

Fetal & Neonatal Lung Development

Clinical Correlates and
Technologies for the Future

Edited by

Alan H. Jobe • Jeffrey A. Whitsett • Steven H. Abman

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Lung disease affects more than 600 million people worldwide. While some of these lung diseases have an obvious developmental component, there is growing appreciation that processes and pathways critical for normal lung development are also important for postnatal tissue homeostasis and are dysregulated in lung disease.

This book provides an authoritative review of fetal and neonatal lung development designed to provide a diverse group of scientists, spanning the basic to clinical research spectrum, with the latest developments on the cellular and molecular mechanisms of normal lung development and injury-repair processes, and how they are dysregulated in disease. The book includes genetics, omics, and systems biology, as well as new imaging techniques that are transforming studies of lung development. The reader will learn where the field of lung development has been, where it is presently, and where it is going to improve outcomes for patients with common and rare lung diseases.

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Lung Growth, Development, and Disease

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Contents

List of contributors vi

Preface ix

-
- 1 **The Genetic Programs Regulating Embryonic Lung Development and Induced Pluripotent Stem Cell Differentiation** 1
Finn Hawkins, Scott A. Rankin, Darrell N. Kotton, and Aaron M. Zorn
 - 2 **Early Development of the Mammalian Lung—Branching Morphogenesis** 22
Kathleen M. Stewart and Edward E. Morrisey
 - 3 **Pulmonary Vascular Development** 34
Timothy D. Le Cras and Marlene Rabinovitch
 - 4 **Transcriptional Mechanisms Regulating Pulmonary Epithelial Maturation: A Systems Biology Approach** 58
Jeffrey A. Whitsett and Yan Xu
 - 5 **Environmental Effects on Lung Morphogenesis and Function: Tobacco Products, Combustion Products, and Other Sources of Pollution** 77
Cindy T. McEvoy and Eliot R. Spindel
 - 6 **Congenital Malformations of the Lung** 94
Susan E. Wert and Kathryn A. Wikenheiser-Brokamp
 - 7 **Lung Structure at Preterm and Term Birth** 126
Jason C. Woods and Johannes C. Schittny
 - 8 **Surfactant During Lung Development** 141
Timothy E. Weaver, Lawrence M. Nogee, and Alan H. Jobe
 - 9 **Initiation of Breathing at Birth** 164
Arjan B. te Pas and Stuart Hooper
 - 10 **Perinatal Modifiers of Lung Structure and Function** 187
Suhas G. Kallapur and Sailesh Kotecha
 - 11 **Chronic Neonatal Lung Injury and Care Strategies to Decrease Injury** 205
Robert P. Jankov and A. Keith Tanswell
 - 12 **Apnea and Control of Breathing** 223
Christopher C. Stryker, Andrew Dylag, and Richard J. Martin
 - 13 **Alveolarization into Adulthood** 238
Manjith Narayanan
 - 14 **Physiologic Assessment of Lung Growth and Development Throughout Infancy and Childhood** 253
Anne-Marie Gibson, Sarath Ranganathan, and Lex W. Doyle
 - 15 **Perinatal Disruptions of Lung Development: Mechanisms and Implications for Chronic Lung Diseases** 269
Michael A. O'Reilly
 - 16 **Lung Growth Through the “Life Course” and Predictors and Determinants of Chronic Respiratory Disorders** 286
Fernando D. Martinez
 - 17 **The Lung Structure Maintenance Program: Sustaining Lung Structure during Adulthood and Implications for COPD Risk** 303
Norbert F. Voelkel and Masahiro Sakagami
-

Index 311

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Preface

These chapters by experts in all aspects of lung growth and development will provide scholarly reviews of the state of the art of lung biology and function. A further goal is to emphasize new technologies and approaches that will transform the understanding of lung development in the near future. These new insights will then inform us about human lung diseases and new treatments. Lung biology initially focused on the amazing structure of the lung following the development of electron microscopy. Subsequent progress resulted primarily from analyses of the cell biology and metabolic processes of the lung. A major success came with the development of surfactant treatments for respiratory distress

syndrome (RDS) in preterm infants. Transgenic technologies then provided tools for exploring lung development, lung injury, and disease models. Currently modern molecular and genetic techniques and systems biology are transforming how lung development can be studied and directly linked to diseases throughout the human life span. The field has come full circle with remarkable advances in imaging of lung cell and septal developmental processes in animal models. Now alveolar numbers and size estimates are possible in patients using advanced magnetic resonance (MR) technologies. The expectations are that new insights will result in new therapies.

The Genetic Programs Regulating Embryonic Lung Development and Induced Pluripotent Stem Cell Differentiation

Finn Hawkins, Scott A. Rankin, Darrell N. Kotton, and Aaron M. Zorn

Abstract

This chapter reviews the current knowledge of the molecular mechanisms controlling embryonic lung development in animal models from the initial specification of a small number of respiratory progenitor cells in the ventral foregut endoderm through the formation of the mature adult lung with regionally specialized epithelial, interstitial, and vascular compartments. In the second half of this chapter we introduce induced pluripotent stem cells (iPSCs) as a compelling new platform to study human lung biology at developmental time-points previously inaccessible to researchers. iPSCs offer the potential to generate functional lung tissue *in vitro* by translating the knowledge gained from studying respiratory system development in different animal models where many of the signaling pathways or airway branching mechanisms are evolutionarily conserved. There are many exciting possible applications of iPSC-derived lung tissue, including the ability to model human lung disease, screen novel drug therapies, and ultimately generate functional, transplantable lung cells or 3-D tissues for those suffering from one of the many forms of end-stage lung disease.

Keywords:

Embryonic lung development, iPSCs, *Nkx2-1*, respiratory progenitors, animal models

Introduction

The mammalian respiratory system is an essential, complex, and highly specialized organ. Each lung is composed of two branching networks, epithelial and vascular, that deliver air and deoxygenated blood to the alveoli, where the primary function of the respiratory system, gas exchange, takes place. The respiratory epithelium contains numerous distinct cell types to accomplish this function. The epithelial tubes, comprised of the trachea, bronchi, and bronchioles, contain secretory, multiciliated, neuroendocrine, and basal cells that humidify and clean the inhaled air while maintaining the airway. The alveolar epithelium contains surfactant-secreting type II pneumocytes (ATII) that maintain patency of the alveoli while type I pneumocytes (ATI) lie in close association with endothelial cells to facilitate efficient gas exchange.

In this chapter we first review the current knowledge of the molecular mechanisms that

control lung development in animal models from the initial specification of a small number of respiratory progenitor cells in the ventral foregut endoderm through the formation of the mature adult lung with regionally specialized epithelial, interstitial, and vascular compartments. In the second half of the chapter we introduce induced pluripotent stem cells (iPSCs) as a compelling new platform to study human lung biology at developmental time-points previously inaccessible to researchers. iPSCs offer the potential to generate functional lung tissue *in vitro* by translating the knowledge gained from studying respiratory system development in different animal models where many of the signaling pathways or airway branching mechanisms are evolutionarily conserved. There are many exciting possible applications of iPSC-derived lung tissue, including the ability to model human lung disease, screen novel drug therapies, and ultimately generate functional, transplantable lung

cells or 3-D tissues for those suffering from one of the many forms of end-stage lung disease.

Development of the Respiratory System

Overview of Lung Development

The billions of epithelial cells lining the respiratory system are derived from just a few hundred progenitor cells in the ventral foregut of the early embryo. Studies in animal models have shown that specification of lung progenitors, growth and morphogenesis of the fetal lung, and epithelial differentiation are regulated by a progressive series of bidirectional inductive interactions between the endoderm and the surrounding mesoderm. These growth factor-mediated interactions not only control epithelial development, but are also critical for the mesoderm-derived tissues, ensuring the coordinated differentiation of the respiratory epithelium with the vascular capillary network to produce the intricate air-blood interface essential for respiration.

The origins of the lung epithelium can be traced back to the initial formation of the endoderm germ layer during gastrulation, starting at embryonic day (E) 6.5 in the mouse (Figure 1-1). Shortly after gastrulation from E7.5–8.5, the endoderm forms a primitive gut tube that is patterned along the anterior–posterior (A–P) axis into broad foregut and hindgut domains by secreted factors from the posterior mesoderm. Between E8.5 and E9.5, signals from the cardiac and splanchnic mesoderm progressively subdivide the foregut epithelium along its A–P and dorsal–ventral (D–V) axes into thyroid, thymus, trachea, lung, esophagus, liver, pancreas, and stomach progenitor cell populations (Figure 1-1) (1,2). At the same time the epithelium signals back to maintain the mesenchyme and promote the proper development of the heart, visceral muscle, and pulmonary vasculature precursors (1,3,4).

The respiratory epithelial progenitors can first be identified by the localized expression of the homeobox gene *Nkx2-1* in a subset of the ventral foregut endoderm at ~E9.0 in the mouse and around 28 days in human gestation (1,5). Morphogenesis of the respiratory system begins between E9.5 and E10.5 when the ventral *Nkx2-1*⁺ cells evaginate forming two primary lung buds and the foregut begins to separate into two tubes:

a ventral trachea and dorsal esophagus. During the pseudoglandular stage of fetal lung development (E12.5–16.5) the primary lung buds grow through a stereotypical process of branching morphogenesis to generate the highly arborized airway tree. This process is controlled by temporally and spatially dynamic signaling interactions between the growing lung bud tips and the surrounding lung mesenchyme. Branching morphogenesis is tightly coordinated with proximal–distal patterning of the lung to generate proximal epithelial progenitors that give rise to the mucociliary cells of the conducting airways and distal epithelial progenitors that give rise to pneumocytes in the peripheral alveoli. In the canalicular and saccular stages of lung development from E16.5 to postnatal day (P) 5, branching morphogenesis ceases, and the epithelium begins to differentiate as the distal lung walls become thinner and the terminal branches expand forming sacs surrounded by vascular endothelium. Finally in the perinatal period the terminal airway sacs are further subdivided and inflate creating mature alveoli lined by squamous ATI cells that facilitate gas exchange and cuboidal ATII cells that secrete surfactant, allowing the lung to inflate.

The mesenchymal–epithelial interactions regulating lung development are mediated by a number of signaling pathways, including Wnt, bone morphogenesis protein (BMP), TGF- β , fibroblast growth factor (FGF), retinoic acid (RA), and Hedgehog (HH), which are used reiteratively with distinct roles at distinct times during lung organogenesis (1–3). Although research has identified key roles for these factors, precisely how combinatorial signaling is regulated and how temporal and spatial specificity of the cellular responses is controlled are areas of active investigation. This information is critical to effectively mimic lung organogenesis *in vitro* with stem cells. Here we review the molecular mechanism of lung development, focusing on epithelial differentiation, and highlight some of the important outstanding questions.

Formation and Early A–P Patterning of the Endoderm

In all vertebrate species, signaling by the TGF β -family ligand Nodal is necessary and sufficient to induce the endoderm and mesoderm germ layers during gastrulation (2). This was first

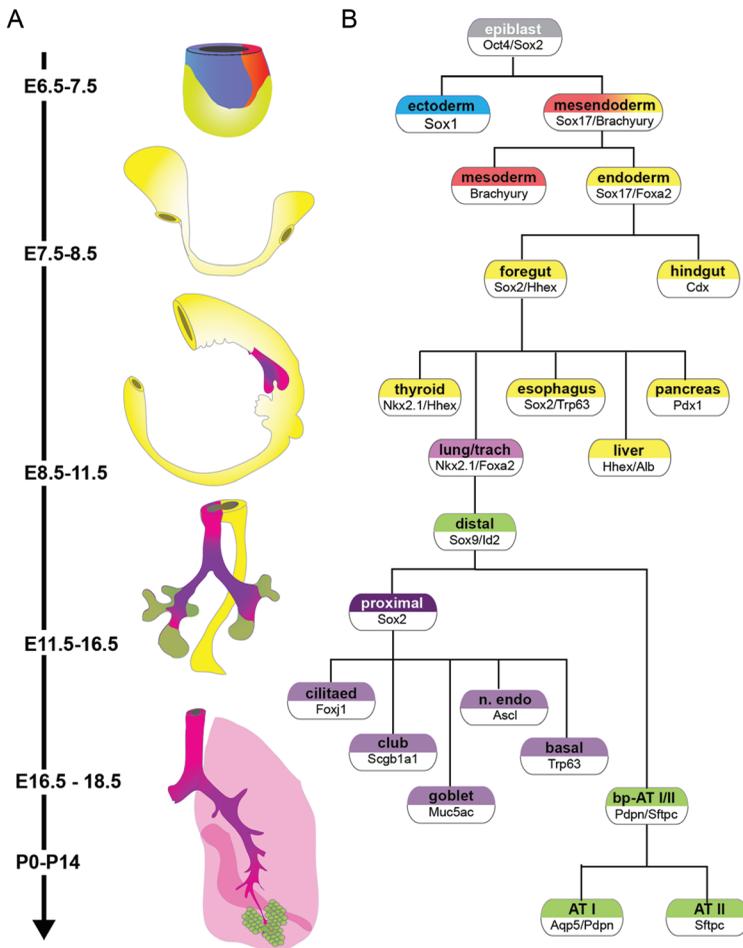


Figure 1-1. Overview of lung development. (A) A time line showing the major steps of lung development in the mouse and (B) a diagram showing the ontogeny of cell lineages (bold; with key marker genes indicated), as the endoderm progressively gives rise to the epithelial cells lining the respiratory system. The endoderm and mesoderm germ layers are segregated from a bipotential mesendoderm progenitor during gastrulation at E6.5–7.5. Between E7.5 and E8.5 the endodermal sheet folds over on the anterior and posterior ends to form a primitive gut tube (anterior left) that is patterned along the A–P axis into foregut and hindgut progenitors. At E8.5–9.5 the foregut is further patterned along the A–P and D–V axes to generate organ-specific lineages, with *Nkx2-1*⁺ respiratory progenitors (magenta) being detected by E9.0. Between E9.5 and E11.5 the single foregut tube is separated into *Sox2*⁺ esophagus and *Nkx2-1*⁺ trachea with primary lung buds emerging. The fetal lung grows by branching morphogenesis in the pseudoglandular stage (E12.5–16.5) during which time the epithelium is patterned along the P–D axis into *Sox9*⁺ progenitors (green) at the distal tips, which give rise to both *Sox2*⁺ proximal airway lineages (purple) as well as distal alveolar epithelium. During the Canalicular (E16.E–17.5) and Saccular (E18–birth) stages of lung development, the proximal epithelia (purple) begins to differentiate into neuroendocrine (n. endo), multiciliated, secretory (club and goblet cell), and basal cells. Alveolarization occurs in the postnatal (P) period, when bipotential alveolar precursors (bp-AT I/II) at the lung periphery differentiate into gas-exchanging ATI cells interspersed with surfactant-expressing ATII cells lining the alveoli (green).

demonstrated when pluripotent cells isolated from pregastrula *Xenopus* embryos were cultured in high concentrations of Activin (a TGF β ligand that activates the same receptors as Nodal), which caused them to adopt an endoderm fate, whereas lower Activin concentrations induced the cells to become mesoderm (2). As we describe later, these classic embryological experiments provided the proof of principle for directing endoderm and

mesoderm differentiation from mouse and human pluripotent stem cells.

In mice, Nodal is expressed in the primitive streak, an embryonic structure through which cells migrate during gastrulation. As cells pass through the primitive streak and are exposed to Nodal ligands, it is thought that they assume a transient bipotential “mesendoderm” state, expressing genes characteristic of both the

endoderm and mesoderm lineages. Cells that emerge from the posterior primitive streak experience lower levels of Nodal and become mesoderm, whereas those that ingress through the anterior end of the primitive streak experience higher Nodal levels and become definitive endoderm (DE) (2). As the DE tissue migrates out of the streak, it intercalates with and displaces the visceral endoderm (VE), an epithelium that gives rise to extraembryonic tissues such as the yolk sac. Nodal-binding to transmembrane receptor complexes results in the phosphorylation and nuclear translocation of the effector protein Smad2, which stimulates the expression of other key transcription factors, including *Sox17* and *Foxa2* in the endoderm, and Goosecoid and Brachyury (T) in the mesoderm (Figure 1-1) (2). *Sox17* is also expressed in the VE, whereas *Foxa2* is also expressed in the axial mesoderm; thus coexpression of *Sox17* and *Foxa2* is a signature of endoderm cells and is commonly used to identify DE cells within stem cell cultures (6,7).

The canonical Wnt/ β -catenin pathway cooperates with Nodal signaling to promote specification and pre patterning of the DE during gastrulation. Wnt-binding to Frizzled-LRP coreceptor complexes results in the stabilization of β -catenin, which translocates to the nucleus, where it interacts with Tcf/Lef DNA-binding proteins to regulate transcription. In zebrafish, *Xenopus*, and mice, β -catenin is essential for gastrulation and helps maintain the high levels of Nodal expression needed for endoderm induction (2). In addition β -catenin/Tcf complexes cooperate with Smad2 to promote the transcription of many mesendoderm genes, including *Sox17* and *Foxa2* (8,9). Initial pre patterning of the endoderm occurs around the same time as endoderm formation with *Foxa2* and *Sox17* being differentially required for anterior and posterior endoderm respectively (2).

After gastrulation, between E7.5 and E9.0 in mice, the sheet of DE cells is transformed into a primitive gut tube that is broadly patterned along the A–P axis with the foregut expressing the transcription factors Hhex and *Sox2*, while the hindgut epithelium expresses the caudal transcription factors Cdx1-4 (Figure 1-1). At this stage in development, regional identity of the endoderm is labile, and if anterior endoderm is experimentally placed in contact with posterior mesoderm, it can be reprogrammed to adopt a hindgut fate (10,11).

The posterior mesoderm secretes RA, Wnt, FGF, and BMP ligands, which together promote intestinal identity in the adjacent endoderm. In contrast the anterior region of the embryo expressed a number of Wnt- and BMP-antagonists including Dkk1, Sfrp, Cerberus, Noggin, and Chordin that protect the anterior endoderm from these posteriorizing signals (2). The available evidence suggests that the foregut epithelium is uniquely capable of becoming lung, liver, or pancreas but the molecular basis of this competence is poorly understood.

Specification of *Nkx2-1*⁺ Respiratory Progenitors

The foregut endoderm is segregated into organ-specific lineages between E8.0 and E9.5 in mice (Figure 1-1) with specification of the respiratory progenitors defined by the downregulation of *Sox2* and the induction of *Nkx2-1* in a subset of the ventral foregut cells at E9.0 (Figure 1-2) (5). Although commonly used as a marker of the respiratory lineage, *Nkx2-1* is also expressed in the brain and presumptive thyroid and thus is not a completely specific indicator of respiratory identity. Recent expression profiling of the early mouse embryo has revealed a combinatorial transcription factor code for different foregut lineages with expression of *Nkx2-1* and the lack of Hhex and Pax8 marking lung, whereas the coexpression of *Nkx2-1*/Hhex/Pax8 marks the thyroid (12,13). *Nkx2-1* directly regulates the expression of many genes in the respiratory epithelium (14), and *Nkx2-1* null mutant mice exhibit severe pulmonary defects including trachea-esophageal fistula, poor branching morphogenesis, and a failure in differentiation of the lung epithelium along with thyroid and brain defects (15,16). The fact that *Nkx2-1* mutants still make lung tissue indicates that other epithelial transcription factors must act in concert with *Nkx2-1* to specify the respiratory lineage. Candidates include a number of Hox-family transcription factors, which display regional expression in the foregut. Consistent with this possibility *Hoxa5* and *Hoxb5* double mutants exhibit hypoplastic lungs and perinatal lethality (17). An important challenge in the future will be to identify how *Nkx2-1*, Hox, and other transcription factors interact to control the complete transcriptome of the respiratory lineage.

Experiments with cultured embryonic mouse tissue suggest that between E8.0 and E9.0

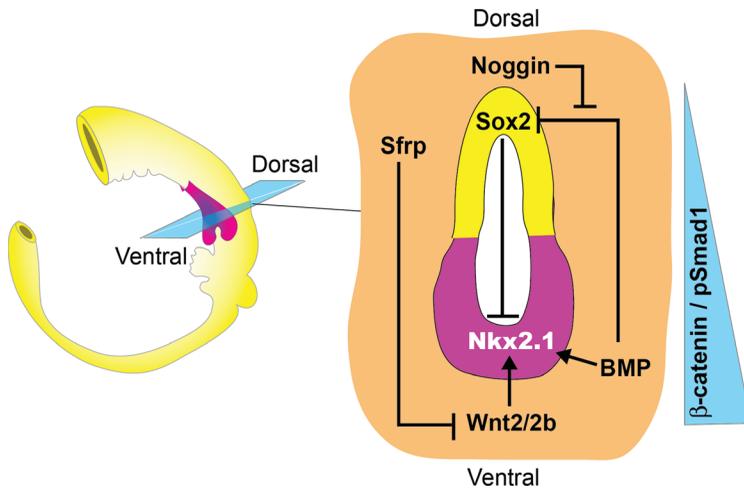


Figure 1-2. D-V foregut patterning and specification of *Nkx2.1*⁺ progenitors. Schematic of an E9.5 mouse gut tube showing a section through the foregut. It shows that *Wnt2/2b* and BMP paracrine signals from the splanchnic mesoderm (orange) generate a gradient of β -catenin and pSmad1 activity along the D-V axis of the foregut epithelium. *Wnt*/ β -catenin induces *Nkx2-1*⁺ respiratory progenitors (pink) in the ventral foregut, whereas BMP/pSmad1 downregulates the ventral expression of the transcription factor *Sox2*, which suppresses *Nkx2-1*. *Wnt* and BMP ligands are counteracted dorsally by the secreted antagonists *Noggin* and *Sfrp*, thus maintaining *Sox2* expression in the dorsal foregut (yellow) that will give rise to the esophagus.

dose-dependent FGF signals from the cardiac mesoderm promote the initial segregation of the ventral foregut into lung, liver, and pancreas lineages. High concentrations of recombinant FGF2 induced expression of *Nkx2-1* and the ATII gene *Sftpc* in isolated foregut endoderm, while moderate FGF doses induced liver (18). Similarly, hyperactivation of FGF receptors (FGFR) in *Xenopus* and chicken embryos expands the *Nkx2-1*-expressing lung domain (19,20). Pharmacological inhibition in *Xenopus* embryos indicates that endogenous FGFR signaling via mitogen-activated protein kinase (MAPK) and Akt pathways is required for lung and liver development *in vivo* and suggests that prolonged FGFR activity is critical for lineage specification, but the molecular mechanisms remain obscure (20,21). This putative role for FGFs in mammalian foregut patterning remains to be genetically validated, as no single or compound *Fgf* mutant described to date exhibits a failure to specify *Nkx2-1*⁺ progenitors, even though a number of FGF ligands play critical roles in fetal lung development (3).

Canonical Wnt signaling is critical for inducing respiratory progenitors. *Wnt2* and *Wnt2b* (*Wnt2/2b*), expressed in the splanchnic mesoderm surrounding the ventral foregut at E8.5–10.5, are redundantly required for lung development (Figure 1-2). The combined mutation of both *Wnt2* and *Wnt2b*, or the deletion of β -catenin from the epithelium at E9.0, resulted in respiratory agenesis and a lack of robust *Nkx2-1* expression, although there may be a very transient low level of *Nkx2-1* even in the absence of β -catenin (22,23). In addition, hyperactivation of β -catenin is sufficient

to dramatically expand the *Nkx2-1* expression domain. *Wnt2/2b* signaling via β -catenin is also necessary and sufficient to specify respiratory epithelium in *Xenopus* (24). Epistasis experiments in frog embryos further suggest that FGF-mediated foregut patterning may promote lung fate in part by regulating *Wnt2/2b* expression in the mesoderm. However, the situation is almost certainly more complicated as compound *Wnt2/2b* mutant mouse embryos have reduced *Fgf10* in the mesenchyme surrounding the nascent lung buds (22). Moreover, analysis of the *Fgf10* and *Fgf9* mutants indicates that FGFs and Wnts regulate each other's expression throughout fetal lung development by complex feedback loops both within the mesenchyme and between the mesenchyme and the epithelium (3).

BMP signaling cooperates with *Wnt2/2b* to promote specification of the respiratory lineage. At E9.0–10, BMP ligands expressed in the cardiac and splanchnic mesenchyme signal to the adjacent ventral foregut epithelium activating the downstream nuclear effectors Smad1/5/8 (Figure 1-2) (25,26). Conditional deletion of BMP receptor genes *Bmpr1a* and *Bmpr1b* (*Bmpr1a/b*) from the endoderm around E9.0, rendering the epithelium unable to respond to BMPs, resulted in reduced *Nkx2-1* and tracheal agenesis (27). Curiously *Bmpr1a/b* deletion also caused ectopic lung buds to emerge from the foregut tube, suggesting that trachea and lung bud progenitors may have different requirements for BMP signaling. Importantly BMPR activity was essential for the ectopic *Nkx2-1* expression induced by the experimental hyperactivation of β -catenin. The available evidence

suggests that BMP signaling acts in part by repressing *Sox2*, thereby allowing *Nkx2-1* expression in the presumptive lung field (27). *Sox2* appears to repress *Nkx2-1* transcription, thus downregulation of *Sox2* by BMP facilitates Wnt-mediated induction of respiratory progenitors (Figure 1-2). Exactly how *Sox2* inhibits *Nkx2-1* expression is unclear as a few days later both of these genes are coexpressed in the developing trachea at E11.5 (28), making it unlikely that *Sox2* directly represses *Nkx2-1* transcription.

Recent evidence from *Xenopus* embryos suggests that the role of BMPs may be more complex with both “prolung” and “antilung” activities by repressing *Sox2* in the epithelium while at the same time restricting the *Wnt2/2b* expression domain in the mesenchyme (24). This dual function may help to coordinate the location of lung-inducing signals within the region of the foregut competent to respond. In the future it will be important to analyze the enhancers and promoters of the *Nkx2-1*, *Sox2*, and *Wnt2/2b* genomic loci in vivo by chromatin immunoprecipitation (ChIP) to determine how direct DNA-binding of β -catenin/Tcf and Smad1 complexes control their transcription.

D–V Patterning of the Foregut into Trachea and Esophagus

Coincident with induction of *Nkx2-1*⁺ respiratory progenitors, differential BMP/Wnt signals also pattern the foregut along the D–V axis such that it separates into distinct tracheal and esophageal tubes. BMP ligands from the ventral mesenchyme are counteracted by Noggin, a BMP-antagonist secreted from the notochord and dorsal foregut (25,26). Similarly the Wnt-antagonists *Sfrp1* and *Sfrp2* are expressed in the dorsal foregut mesoderm surrounding the developing esophagus, where they appear to restrict the activity of ventrally produced *Wnt2/2b* (29). This spatial expression pattern of ligands and antagonists results in a graded level of β -catenin and Smad1 activity along the D–V axis of the foregut tube that is highest in the ventral *Nkx2-1*⁺ presumptive trachea and lowest in the dorsal *Sox2*⁺ presumptive esophagus. The correct balance of BMP/Wnt activity is essential for proper separation of the trachea and esophagus. Reductions in *Wnt2/2b* or BMP activity, such as in *Bmp4*^{-/-} or *Wnt2*^{-/-}; *Wnt2b*^{-/-} compound mutant mice, results in

tracheal atresia (22,25,26), whereas inactivating mutations in Noggin or *Sox2* can result in varying degrees of esophageal atresia and trachea-esophageal fistulas (EA/TEF) in mice and human patients (26,28).

The HH pathway is also essential for early respiratory development and separation of trachea and esophagus. Sonic hedgehog ligand (*Shh*) is expressed throughout the gut tube epithelium, and *Shh*^{-/-} mice mutant embryos display tracheal atresia with hyperplastic lungs (30). One of the main roles of *Shh* signals from the epithelium is to maintain the proliferation and survival of the surrounding mesenchyme, which in turn signals back to the endoderm. The combined mutation of *Gli2* and *Gli3*, downstream transcription factors in the HH pathway, results in a more severe phenotype than the *Shh* mutants, with a complete lack of respiratory system and a single hypoplastic foregut tube (31). Exactly how the Wnt, BMP, and HH pathways modulate cell behaviors to cause the single foregut tube to separate into separate tracheal and esophageal tubes is still poorly understood.

Primary Lung Bud Outgrowth

Initial outgrowth of the primary lung buds around E10.5 in mice requires mesenchymal FGF10 signaling to FGFR2b expressed in the *Nkx1*⁺ foregut epithelium. *Fgf10*^{-/-} and *Fgfr2b*^{-/-} knockout mice exhibit a complete lack of lung buds, although respiratory progenitors are specified as indicated by the rudimentary trachea in the mutant embryos (32,33). RA signaling is also essential for primary lung bud formation. If mice lacking the key RA synthesizing enzyme *Raldh2* are transiently supplemented with RA between E7.5 and 8.5 to overcome early embryonic lethality, the resulting *Raldh2*^{-/-} embryos lack lung buds similar to *Fgf10* and *Fgfr2b* mutants (34,35). Studies have shown that RA orchestrates a complex signaling cascade involving FGF, Wnt, and TGF β pathways to control lung bud growth. On one hand, RA represses expression of the Wnt-antagonist *Dkk1* and thus generates a permissive territory where *Wnt2/2b* can maintain *Nkx2-1* expression (36). On the other hand, RA signaling promotes *Fgf10* expression by suppressing the TGF β /pSmad2 pathway (37).

RA may also indirectly maintain *Fgf10* and *Wnt2/2b* expression by promoting the expression

of mesenchymal transcription factors such as *Tbx4*, *Tbx5*, and *Hoxa5*. These transcription factors are RA targets in several cellular contexts, and depletion of *Tbx4* and/or *Tbx5* results in reduced *Fgf10* and *Wnt2b* in chicken and mouse embryos (19,38). Moreover, the Hox cofactors *Pbx* and *Meis* have recently been shown to cooperatively regulate *Fgf10* transcription (39). It is likely that other signals also cooperate with RA and FGF to regulate lung bud outgrowth. The fact that epithelium-specific deletion of *Bmpr1a/b* results in ectopic lung buds (27) suggests that BMP signaling may constrain inappropriate lung bud outgrowth, but the relationship of this activity to RA and FGF10 remains to be determined.

The observation that *Bmpr1a/b* are required for trachea but repress lung budding (27) suggests that trachea and lungs might have distinct progenitors with different molecular programs maintaining their growth. For example, *Nkx2-1*, *Shh*, and *Bmp4* mutants all have lung tissue but exhibit tracheal dysgenesis (16,25,30), whereas *Fgf10* and *Fgfr2* mutants lack lungs but form a trachea (32,33). Further work is needed to investigate the segregation of trachea and lung lineages.

Proximal-Distal Patterning, Branching Morphogenesis, and Growth of the Fetal Lung

During the pseudoglandular stage of lung development from E12.5 to E16.5 in mice, the fetal lung grows through stereotypical branching morphogenesis where the distal lung tips undergo a reiterative series of bifurcations to produce the highly arborized tree-like structure of the lung (40). Coincident with branching morphogenesis, the immature respiratory epithelium becomes patterned along the proximal–distal (P–D) axis of the lung. Epithelial progenitors in the proximal regions of the fetal lung, which will line the conducting airways, gives rise to neuroendocrine, secretory, ciliated, and basal cells, whereas the distal peripheral airway epithelium gives rise to gas-exchanging ATI cells and surfactant-producing ATII cells (1,3). Studies have identified a discrete junction between these two epithelial compartments termed the bronchoalveolar duct junction, which displays a clear change in cell type and morphology identifiable around E17 (41). This P–D patterning can first be observed at the molecular level, in the fetal mouse lung by E11 when trachea and proximal lung epithelium re-express *Sox2* and the epithelium

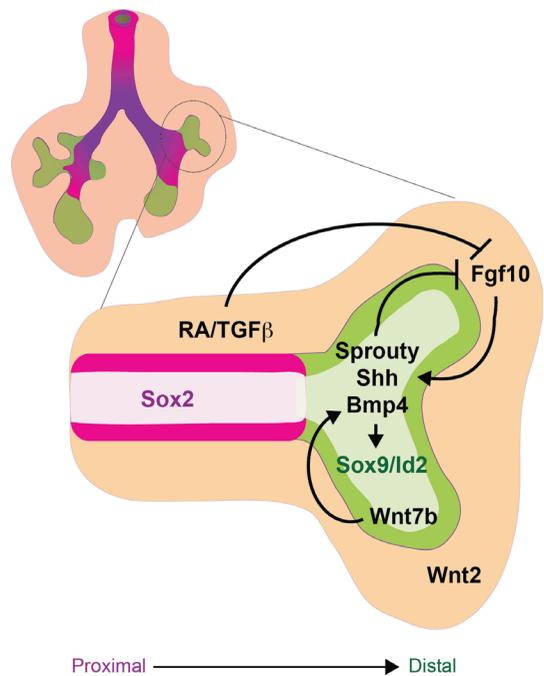


Figure 1-3. P–D patterning and branching morphogenesis of the fetal lung. During the pseudoglandular stage of lung development (E12.5–16.5), the fetal lung is patterning along the proximal–distal axis as it grows by branching morphogenesis. The schematic of an early fetal lung with a magnification of one branch shows that the distal tip expressing *Sox9* and *Id2* (green) is undergoing branching morphogenesis, whereas the proximal airways express *Sox2* (magenta). The mesenchyme (orange) surrounding the distal tip expresses FGF10, which along with BMP and Wnt forms a distal signaling center that maintains proliferation and progenitor status of the *Sox9/Id2*⁺ distal epithelium. FGF10 induces expression of *Sprouty*, *Shh*, and *Bmp4* in the distal epithelium, which along with RA and TGFβ, signal back through a negative feedback loop to restrict the expression and activity of FGF signaling in the stalk region. As a result of proliferation, epithelial cells that are destined to give rise to conducting airway are eventually displaced from the influence of the distal signaling center, causing them to downregulate *Sox9/Id2* and adopt a *Sox2*⁺ proximal fate.

of the branching distal lung tips express the genes *Sox9* and *Id2* (Figure 1-3) (1,3). *Sox2* itself is required for the formation of secretory and ciliated cells in the proximal airway (28,42) while *Sox9* is required for distal alveolar differentiation and normal lung branching (43,44).

P–D patterning, branching morphogenesis, and fetal lung growth are all intimately coordinated by a complex reciprocal cross-talk between the epithelium and mesenchyme involving many of the same signaling factors that regulate respiratory specification and primary lung bud outgrowth namely: FGF, BMP, Wnt, RA, and HH. These interactions generate a signaling center at

the distal lung bud tips, which maintains the proliferation of *Sox9*/*Id2*⁺ distal progenitors, promotes branching morphogenesis, and represses *Sox2*⁺ proximal fate. As the lung tips grow, epithelial cells that give rise to the stalks of the branching airways eventually become displaced from the influence of distal signaling, and as a result they no longer maintain distal identity and adopt a *Sox2*⁺ proximal epithelial fate. Consistent with this model, genetic lineage labeling has shown that *Id2*-expressing progenitor cells in the distal tips of the E11.5–13.5 fetal lungs can self-renew and contribute to both proximal airway and distal alveolar epithelial lineages (45). *Id2*-expressing tip cells subsequently lose this multipotent capacity after this developmental stage when they become restricted solely to distal epithelial fates, consistent with a model of increasing lineage restriction of distal progenitors as developmental stages proceed.

Although many of the molecular details remain to be elucidated, a key component of this distal signaling center is the localized expression of FGF10 in the mesenchyme surrounding the distal tips, which signals to *Fgfr2b* in the epithelium.

Experiments with fetal lung explants and conditional *Fgfr2* and *Fgf10* knockouts, using CRE-drivers that delete during fetal lung growth (to overcome earlier lung agenesis), revealed that FGF10 is a critical mitogen and chemoattractant for the epithelium that drives the branching morphogenesis program (46,47) and maintains distal *Sox9*⁺ cells in a progenitor-like state while repressing proximal *Sox2*⁺ airway fate (48,49). Much of FGF10's activity appears to be mediated by cross-talk with other pathways in the distal signaling center (Figure 1-3). FGF10 promotes the localized expression of *Shh*, *Bmp4*, and its target *Id2* in the distal epithelium of the branching tips (46). The distal tip epithelium also expresses *Wnt7b*, which in turn promotes epithelial *Bmp4* and *Fgfr2* expression (50). Within the epithelium, canonical Wnt/ β -catenin activity (stimulated by epithelial *Wnt7b* and mesenchymal *Wnt2*) together with autocrine BMP signaling maintains proliferation and differentiation of the distal epithelium, while suppressing proximal fate (Figure 1-3) (1,3). The exact mechanisms by which Wnt/ β -catenin and BMP signaling suppresses proximal fate are unclear but it may be similar to the repression of early *Sox2* during D–V foregut patterning.

Reciprocal signaling from the epithelium back to the mesenchyme plays a critical role in maintaining the distal signaling center at the growing lung bud tips. In addition to promoting *Shh* and *Bmp4* expression, FGF10 also activates the expression of the FGF-feedback inhibitor Sprouty in the distal tip epithelium. Sprouty then cell-autonomously restricts FGF signal transduction in the epithelium, whereas *Shh* and *Bmp4* signal back to the mesenchyme to suppress the expression and activity of *Fgf10*. As a result *Fgf10* activity is attenuated in the stalk region proximal to the branching buds. This negative feedback along with additional levels of regulation by *Fgf9*, RA, and TGF β localizes the dynamic expression of mesenchymal *Fgf10* and epithelial *Bmp4* to the growing distal tips during branching morphogenesis (Figure 1-3). These complex tissue interactions also regulate the proliferation and differentiation of the mesenchymal components of the lung. How these complex interactions play out in the directed differentiation of stem cell cultures, where endoderm and mesoderm are often present, remains to be fully explored.

Recent studies have discovered a number of additional layers of regulation in fetal lung patterning and differentiation. For example, genetic studies in mice have identified a role for the Hippo/yes-associated protein (YAP) pathway in defining the border between the *Sox2*⁺ airway progenitors and the distal *Sox9*⁺ presumptive alveolar region (51,52). In addition noncoding microRNAs, which regulate mRNA translation, have been shown to govern the balance between progenitor proliferation and differentiation (53,54), whereas long noncoding RNAs, which are thought to regulate transcription, are reported to modulate early *Nkx2-1* expression (55). Epigenetic modification of chromatin can also impact gene expression in the developing lung. For example histone deacetylases (HDACs) have been shown to promote proliferation and P–D differentiation through the regulation of cell cycle genes and *Bmp4* expression (53,56). The challenge now is to figure out how these different modes of regulation are integrated and to identify strategies to manipulate them in stem cell differentiation protocols. For a more detailed consideration of the complex signaling interactions that modulate P–D patterning, branching morphogenesis, and fetal lung growth, see recent excellent reviews on the subject (1,3).

Epithelial Differentiation

Differentiation and physiological maturation of the respiratory epithelium occurs in a proximal to distal wave beginning at the late fetal stages and continuing into the postnatal period (1). Starting in the proximal airway, *Sox2*⁺ progenitors give rise to neuroendocrine cells (*Ascl1*⁺), secretory Club/Clara cells (*Scgb1a1*⁺) in mice, and Goblet cells (*Muc5ac*⁺) in human, multiciliated cells (*Foxj1*⁺) and basal cells (*Trp63*⁺) located at the base of the pseudostratified epithelium of the trachea and main stem bronchi (Figure 1-1B). The distal epithelium differentiates last as peripheral lung tips undergo septation and expansion into alveoli lined with gas-exchanging squamous ATI cells (*Aqp5*⁺/*Pdpr*⁺) that make up more than 90% of the epithelium, interspersed by surfactant-producing cuboidal ATII cells (*Sftpc*⁺; Figure 1-1B).

The cell-cell signaling events that govern epithelial differentiation are poorly understood, but gene knockouts in the mouse and in vitro tissue culture experiments have identified many transcription factors important for epithelial differentiation. In some cases these factors are broadly required for the differentiation of many, if not all, epithelial cell types from both the proximal and distal lineages, such as *Nkx2-1*, *Gata6*, and *Foxa1/2* (1,3,14), whereas others have restricted function such as *Sox2* and *Sox9* in proximal and distal lineages respectively (28,42–44). However, some transcription factors are critical for specific epithelial cell types such as *Ascl1* for neuroendocrine cells, *Spdef* for goblet cells, *Foxj1* and *Myb* for ciliated cells, and *Trp63* for basal cells; for more details on the diverse role of different transcription factors in respiratory epithelium differentiation, we direct the reader to a number of excellent reviews (1,14,53) and references therein.

One signaling pathway known to regulate lineage segregation in the proximal airway is Notch. First Notch signaling between cells within the epithelium defines whether epithelial cells adopt a neuroendocrine (NE) or nonneuroendocrine (Non-NE) fate (57), via the downstream transcription factors *Hes1* and *Ascl1*. Then a second Notch-mediated event regulates the balance between secretory and ciliated cell fate, such that they are distributed in a salt and pepper manner throughout the airway epithelium (58–60). Precisely how the different temporal effects of Notch activation versus Notch inhibition regulates the

various lung lineages remains an active area of investigation.

During the postnatal period the alveoli mature to form the gas-exchanging units of the lung, where the differentiating epithelium is in intimate contact with the vascular endothelium and mesenchymal fibroblasts that form the alveolar septa, but how these interactions impact the differentiation of ATI and ATII cells is poorly understood. Recent evidence indicates that glucocorticoids, which are commonly used to treat premature babies, promotes ATII cell maturation (1,41). In vitro studies suggest that transcriptional changes in response to glucocorticoids involve alterations in *Nkx2-1* binding and activation of downstream genes (61). Recent single cell transcriptome analysis and lineage tracing studies of the distal lung epithelium during late fetal development have begun to shed new light on a novel bipotential progenitor of the ATI and ATII cells and has identified candidate regulatory proteins that might regulate the segregation of these two lineages (62,63). It appears that these bipotential progenitors express genes characteristic of both ATI and ATII and that lineage restriction involves repression of one of the two genetic programs in late fetal stages, as these bipotential alveolar progenitors are not detected postnatally (62).

Lung Tissue from Pluripotent Stem Cells

Overview

Our understanding of lung development has been significantly advanced through the careful study of animal models, but more work needs to explore the molecular mechanisms driving differentiation of the respiratory epithelium. The goal of this research is to ultimately advance our knowledge of human lung development and disease. A major hurdle to such research is the reality that the developing human lung is generally inaccessible to study. In recent years, the discovery of pluripotent stem cells, including iPSCs, has significantly advanced our ability to model human development and disease in vitro, providing unprecedented access to human cells undergoing developmental cell fate decisions and lung differentiation. Both the discovery of how to generate iPSCs from humans as well as the techniques for differentiating these cells in vitro is founded on

many years of basic science investigations into the mechanisms that regulate pluripotency as well as the signaling pathways that enable *in vitro* differentiation of embryonic stem cells (ESCs) or other pluripotent stem cell populations. In the second part of this chapter we now introduce pluripotent stem cells, including ESCs and iPSCs, before we review the success to date in applying the knowledge gained from studying the molecular mechanisms of respiratory development in animal models to generating lung epithelium from human pluripotent stem cells (hPSCs).

Embryonic Stem Cells

Pluripotent stem cells have the capacity to self-renew indefinitely and form somatic lineages of all three germ layers (ectoderm, mesoderm, and endoderm). Decades prior to the derivation of the first ESC, the concept of a pluripotency emerged from the study of teratocarcinomas. Teratomas (benign) and teratocarcinomas (malignant) are spontaneously occurring tumors that contain derivatives of all three germ layers. Transplantation of a single cell from a teratocarcinoma induced new tumors composed of tissues derived from the different germ layers (64). Teratomas frequently contain areas that resemble early embryos, termed embryoid bodies. This suggested an embryonic nature of the tumor and led to the observation that engrafting pregastrulation mouse embryos at extra-uterine sites of an adult mouse resulted in the formation of a teratocarcinoma (65,66). These observations suggested that a transient population of pluripotent stem cells was transiently present in the epiblast of the developing mouse embryo. Efforts to isolate and propagate these putative pluripotent stem cells from the developing embryo in culture were finally successful in 1981 with the derivation *in vitro* of pluripotent stem cell lines, termed embryonic stem cells (ESCs), from the inner cell mass of mouse blastocyst embryos (67). Generating mouse ESCs is now commonplace. ESCs have the capacity to form teratomas when injected *in vivo* and participate in embryogenesis when injected into mouse blastocysts. Transfer of these chimeric blastocysts into foster mouse mothers has demonstrated that ESCs can contribute to all lineages of the developing organism, including the germline, resulting in the production of viable animals. Of note, ESCs are restricted in their ability to contribute

to the extra-embryonic lineages such as the trophoctoderm. However, when ESCs are injected into a tetraploid blastocyst, the tetraploid host cells can generate the extra-embryonic lineages, while the donor ESCs, remarkably, form the entire fetus proper (68,69). The ability to manipulate the genome of ESCs to target specific genes and subsequently engineer mice (knock-out, knock-in, or knock-down) to interrogate their function has been a powerful development in accelerating our understanding of development and disease (70–72).

Seventeen years after the discovery of mouse ESCs, a method of isolating human ESCs from preimplantation human blastocyst embryos and culturing these cells while maintain their undifferentiated state was first described (73). Similar to mouse ESCs, human ESCs can also differentiate into all three germ layers as evidenced by their ability to form teratomas when injected into mice. Their discovery was met by excitement and controversy. The excitement was fueled by a hope that human ESCs would herald the era of regenerative medicine and the *de novo* generation of human cells, tissues, and organs. Controversy stemmed from the use of discarded human embryos to generate the ESC lines. Although sufficient discussion of the ethical, political, or religious controversies that followed is beyond the scope of this review, from a scientific perspective ESCs have offered invaluable insights into the genetic program of early human development and have paved the way for the discovery of iPSCs.

Induced Pluripotent Stem Cells (iPSCs)

The discovery of somatic cell reprogramming to produce iPSCs resulted from fifty years of research focused on mechanisms that regulate nuclear reprogramming and the engineered induction of pluripotency in amphibians and mammals (74). In 1962, Jim Gurdon demonstrated that replacing the nucleus of a frog egg with that of an adult frog intestinal cell could produce a viable tadpole (75) and subsequently a viable adult frog. This process of nuclear transfer proved that the nuclei of somatic cells contain the necessary genetic information to generate an entire organism and that the epigenetic and gene expression states that govern and restrict adult cellular identity can be reset or reprogrammed to a pluripotent, embryonic state. In 1997, applying this nuclear reprogramming

technique to sheep mammary epithelial cells, Dolly was the first mammal to be cloned (76).

A second field of research that led to the discovery of iPSCs was focused on master transcription factors. Transcription factors (TFs), such as *Nkx2-1* reviewed in the first half of this chapter, are diverse and abundant proteins that regulate gene expression by binding to hundreds or thousands of target sequences in the genome. Master transcription factors control cell-specific genes and can potentially determine cell fate. For example, ectopic expression of a single master transcription factor, MyoD, in fibroblasts results in phenotypic conversion into myoblasts (77).

Combining the knowledge of cellular reprogramming, master transcription factor regulation of fate, and the biology of embryonic stem cells, Takahashi and Yamanaka demonstrated in 2006 that the transient, ectopic overexpression of four transcription factors (Oct4, Klf4, Sox2, and c-Myc) could reset the epigenetic state of mouse somatic cells (e.g., fibroblasts) into a pluripotent state virtually indistinguishable from mouse ESCs (78). iPSCs are similar to mouse ESCs in their global gene expression profiles, pluripotency, germ-line competence, and capacity to form an entire mouse (79–81). A year later, this finding was reproduced by reprogramming using human skin fibroblasts into pluripotent cells that phenotypically resemble human ESCs (82,83).

Generation of human iPSCs from skin biopsies, plucked hair follicles, or peripheral blood samples is now well established (82,84,85). Alternative combinations of transcription factors have been identified, including Nanog, Lin28, ESRRB, NR5A2, that establish the core transcriptional circuitry sufficient to reprogram somatic cells into iPSCs (83,86). iPSCs have several advantages over ESCs; they are genetically identical to the person from whom they are generated, and they overcome the ethical controversy that surrounds and limits the study of ESCs or other cell types that require the use of cells obtained from human embryos. As human iPSCs share the capacity for multilineage differentiation, human ESC studies are now focused on recapitulating key developmental processes in vitro, manipulating gene function to interrogate development and disease to generate organ-specific cell types for both pharmaceutical discovery/toxicity studies and potential cell-based therapies.

Deriving Lung Epithelium De Novo via the “Directed Differentiation” of ESCs/iPSCs

The in vitro differentiation of either ESCs or iPSCs into specific tissue can be guided by adding combinations of growth factors or small molecules to the media at specific times during culture to recapitulate the signaling pathways that regulate in vivo organ development (87), a strategy that requires a detailed understanding of normal organogenesis. This process of recapitulating development in vitro to sequentially pattern pluripotent stem cells toward desired fates is termed “directed differentiation” and has been successfully applied for deriving multiple cell types from ESCs/iPSCs, including neurons, retinal epithelium, cardiomyocytes, hepatocytes, intestinal epithelium, and pancreatic cells (88–94). To date most of the cell types produced from ESCs/iPSCs have an immature phenotype and are not yet ready for clinical applications; however, retinal pigment epithelial cells and photoreceptors derived from iPSCs have a particularly well-characterized functional phenotype and exhibit in vivo functional, vision-restoring potential in preclinical animal testing. Hence, the first clinical trial using these hPSC-derived cells to treat patients suffering from age-related macular degeneration was launched in 2014 (*Nature* doi:10.1038/nature.2014.15915).

Initial attempts at deriving lung epithelium from hPSCs were inefficient, stochastic, used incompletely defined media, or relied on the presence of drug-resistance genes (95–97). The difficulty in part was due to our lack of information regarding normal lung development in vivo. However, since 2011, a number of groups have made significant progress leveraging recent advances in our understanding of respiratory development. The resulting strategies to direct the differentiation of human iPSCs toward a lung epithelial fate use the exogenous addition of growth factors and inhibitors at specific times and concentrations to mimic the progressive cell signaling between the endoderm and mesoderm that specify definitive endoderm, pattern the endoderm, and ultimately induce *Nkx2-1*⁺ respiratory progenitors that differentiate into mature lung epithelium (Figure 1-4). The following sections review these critical stages in the directed differentiation of lung tissue from PSC in vitro.

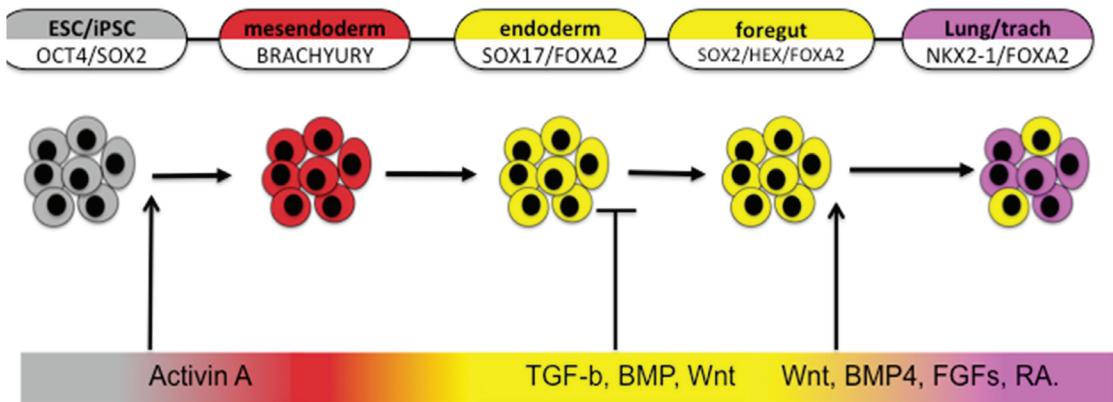


Figure 1-4. A schematic of the directed differentiation of ESCs/iPSCs to lung epithelial progenitors. The key signaling factors and marker genes of the main stages of the in vitro derivation of lung epithelial progenitors are outlined. First ESCs/iPSCs are exposed to high concentrations of Activin A, to mimic Nodal signaling and induced definitive endoderm. PSC exit pluripotency and migrate through a primitive streak-like state (Brachyury+). Definitive endoderm is identified by the expression of a number of genes, including *Foxa2* and *Sox17*. Subsequently, the inhibition of TGF- β , BMP4, and Wnt results in a population reminiscent of anterior foregut (*Foxa2*⁺/*Sox2*⁺). The addition of growth factors, including Wnts, BMP4, FGFs, and RA leads to *Nkx2-1* induction.

In Vitro Definitive Endoderm Induction

In the first part of this chapter we explained that Nodal/TGFB signaling is required to pattern the primitive streak to form endoderm and mesoderm germ layers during gastrulation in all vertebrates. Just as pregastrula *Xenopus* cells will adopt a DE fate when cultured in a high concentration of the exogenously added Nodal signaling inducer, Activin A, so too will hPSCs (6,7). DE induction is the first step for all endoderm-derived directed differentiation protocols. The temporal dynamics of gene expression during DE induction in vitro is similar to that in the mouse embryo (98). hPSC-derived definitive endoderm transiently expresses brachyury, similar to cells migrating through primitive streak in the embryo, and subsequently expresses key endodermal genes (e.g., *Foxa2*, *Sox17*). Studies using mouse embryonic stem cells engineered to carry reporter genes (e.g., green fluorescence protein; GFP) targeted to the loci encoding several primitive streak or DE-associated TFs have demonstrated that similar to the embryo, Activin-induced ESCs/PSCs pass through a transient mesendoderm state prior to DE formation (99,100). Ectoderm and mesodermal fates are suppressed during Activin A induced differentiation of ESCs/iPSCs, and cell surface markers, including C-kit, CXCR4, and EPCAM are useful tools in the in vitro-directed differentiation to help distinguish and quantify definitive endoderm induction (101,102).

Anterior Foregut Endoderm Induction in PSCs

The next major step in directing the in vitro differentiation of DE toward a lung fate is broadly analogous to the gut tube patterning of the E7.0–E9.0 mouse embryo starting with HHEX and *Sox2* expressing foregut progenitors and *Cdx1-4*⁺ hindgut cells. In contrast to the success in generating intestinal, hepatic, and pancreatic progenitors from hPSC-derived DE, more anterior foregut lineages, including lung and thyroid, have only been derived recently. We have already discussed that during normal development the anterior foregut is exposed to a number of Wnt and BMP antagonists, preventing it from adopting a more posterior fate. Capitalizing on this observation, Green et al. demonstrated that stage-specific inhibition of BMP4 and TGF- β , after DE induction from hESC, resulted in a cell population reminiscent of anterior foregut endoderm (AFE) (103). Specifically, the endodermal marker *Foxa2* was maintained, the anterior foregut markers *Sox2*, PAX9, and TBX1 were upregulated, whereas the hindgut marker CDX2 was suppressed. Most important, this AFE-like population was competent to respond to subsequently added, Wnts, BMPs, and FGFs, growth factors resulting in lung and thyroid specification within the cultures (103–106). Further evidence that this population resembles AFE was demonstrated by injecting these cells under the kidney capsule of a mouse

(103). This produced luminal epithelia largely limited to AFE derivatives, including evidence of surfactant protein C+ (*Sftpc*⁺) distal lung epithelial lineages. Using this stage-specific TGF- β /BMP4 inhibition and employing a hESC *Sox2*-GFP reporter line to identify and purify the AFE-like population resulted in the identification of two surface markers, CD56⁺ (NCAM) and CD271⁺ (NGFR), which are proposed to distinguish the *Sox2*⁺ AFE-like population from other cells in heterogeneous in vitro cultures (107). Although a key part of current lung differentiation protocols, the molecular basis for TGF- β and BMP inhibition in rendering DE competent to induce anterior foregut lineages is not yet understood. Indeed, this is still not well understood in normal development of animal models, and it is possible that the experimental advantages of ES/iPSC cultures might help solve this mystery.

Lung Specification from ESC/iPSC-Derived Endoderm

Between E8 and E9.5, Wnt, BMP, and FGF signals segregate the foregut endoderm into organ-specific domains (Figure 1-2), with the prospective thyroid and lung being identified by the expression of *Nkx2-1* at E8.5 and E9, respectively. Although the activity of these signaling pathways is not fully understood, they have proved to be critical in protocols to direct lung differentiation from ESC/iPSC cultures (103–105)

Applying the knowledge of how lung fate is specified in vivo, a number of groups have recently established protocols to derive lung epithelial-like cells from both mouse and human PSCs (104–106,108). Fundamental to these protocols is activin-induced DE, followed by inhibition of TGF- β and/or inhibition of BMP to promote anterior foregut and subsequent exposure to some combination of Wnt activators, BMP4 and FGFs (FGF2, 7, and/or 10). In a relatively short time there has been significant progress in the field with evidence of improving efficiencies of lung lineage specification, more defined protocols, and better characterization of the putative lung progenitors being produced from ESC/iPSC populations (106). Utilizing an ESC line carrying a *Nkx2-1*-GFP knock-in reporter to identify, track, quantify, and purify cells committed to the lung or thyroid lineage has illustrated the kinetics of respiratory or thyroid fate induction

in ESC-/iPSC-derived endoderm, confirming the importance of stage-specific inhibition of TGF- β and BMP4 using SB431542 and Noggin, respectively. Subsequent exposure to the combination of *Wnt3a*, BMP4, FGF2, FGF7, FGF10, EGF, and heparin sulfate yielded 21.3% \pm 2.7% *Nkx2-1*⁺ cells after 15 days of differentiation, resulting in approximately 160 *Nkx2-1*⁺ endodermal cells produced per starting single ESC (104). The *Nkx2-1* knock-in GFP reporter enabled sorting of the cells by fluorescence-activated cell sorting (FACS) and further expansion of this proliferative *Nkx2-1* positive population without requiring the presence of mesenchymal supporting cells. In the presence of FGF2, FGF10, and subsequently a media containing dexamethasone, cyclic AMP, IBMX, and FGF7 (DCI+K) to promote maturation of putative lung epithelial lineages, the expression of both proximal (CC10, *Foxj1*) and distal (*Sftpc*, *Sftpb*, *Pdpm*) lung epithelial markers increased. Rajagopal et al. similarly demonstrated the necessity of TGF- β inhibition after definitive endoderm induction and the expression of endodermal *Nkx2-1* in response to Wnt, BMP4, and FGF2 signaling (105). The authors demonstrated that the BMP signaling necessary for *Nkx2-1* induction was dependent on the canonical BMP, Smad-dependent, pathway as opposed to the MAPK pathway because the application of dorsomorphin (a chemical inhibitor of Smad-dependent BMP signaling) significantly reduced the efficiency of *Nkx2-1* induction, whereas an MEK1/2 inhibitor, PD98059, had little effect. This protocol was translatable to human iPSCs as *Nkx2-1*⁺ endodermal progenitors were successfully derived from iPSCs generated from a patient with cystic fibrosis (105).

Proximal-Distal Patterning of ESC-/iPSC-Derived Lung Lineages

Between E12.5 and E16.5 the lung undergoes continued branching morphogenesis, and the immature lung epithelium is patterned along a proximal (*Nkx2-1*⁺/*Sox2*⁺) versus distal (*Nkx2-1*⁺/*Sox9*⁺) axis (Figure 1-3). The proximally patterned airways will give rise to the conducting airways lined with secretory, ciliated, neuroendocrine, and basal cells. The distal tips will give rise to the alveolar compartments lined by ATI and ATII. As described in the previous section, morphogenesis and P-D patterning is tightly

orchestrated by temporally and spatially restricted reciprocal signaling between the lung epithelium and mesenchyme. The role of mesenchyme in the ESC-/iPSC-directed differentiation of the lung has not yet been explored, and thus reproducing this complex patterning in vitro remains a challenge. Efficient, defined protocols do not yet exist to derive distinct, mature proximal and distal lung epithelial lineages; however, promising recent studies have derived a number of cell types of the lung epithelium. Generating airway-ciliated cells is of particular interest to the study of cystic fibrosis and primary ciliary dyskinesia. Rajagopal et al. generated *Nkx2-1*⁺ endodermal progenitors, as described earlier, using BMP4, Wnt, and FGF2 signaling, followed by exposure to BMP7 and FGF7, while inhibiting Wnt/MAPK/ERK signaling. This resulted in a small population of *Nkx2-1*⁺/*Sox2*⁺ cells and rare cells coexpressing *Nkx2-1*⁺ and *p63*⁺, a signature suggestive of airway basal cell-like differentiation. Further differentiation to more defined cell types of the airway required an in vivo environment, accomplished by injecting these unsorted cells in bulk by subcutaneous injection into immunodeficient mice. The injected ESC-/iPSC-derived cells gave rise to organized, luminal epithelia. Cells within these mouse or human grafted epithelia growing subcutaneously in mice expressed *Nkx2-1* as well as markers of airway basal (*p63*), club (*Clara*) (*CC10*), and goblet (*Muc5AC*) cells. In addition, cells formed cilia and expressed *Foxj1*.

The use of air-liquid interface cultures of ESC/iPSC-derived endoderm has also been employed in attempts to derive monolayered epithelial that might resemble the lung airway. Although lung airway differentiation in the developing embryo proceeds without exposure to air until the time of birth, exposure of monolayered primary airway cell cultures to an apical air-liquid interface provides a strong stimulus for apical-basal polarization as well as ciliation of epithelial cells (109). To attempt to induce these programs in differentiating PSC, Wong et al. used high doses of FGF2 and *Shh* to derive putative anterior foregut progenitors followed by treatment with FGF7, FGF10, and BMP4. FGF18 addition and culture in an air-liquid interface was then employed to produce a monolayered epithelium expressing cystic fibrosis transmembrane

conductance regulator (CFTR). As a proof of concept, translocation of deltaF508 mutant CFTR, the ion channel misfolded in cystic fibrosis (CF), to the plasma membrane was achieved by treating CF iPSC cell-derived CFTR-expressing cells with a CF “corrector” investigational drug. Firth et al. also adopted the sequential, stage-specific approach of inducing definitive endoderm, then anterior foregut endoderm followed by the addition of FGF2, FGF7, FGF10, and BMP4 to specify lung progenitors. Introducing the cell monolayer to an air-liquid interface and inhibiting Notch signaling led to a functional airway epithelium with multiciliated, *FOXJ1*⁺ cells and *CC10*⁺ cells organized into a monolayered epithelium, which displayed barrier function and ion-fluxing epithelial electrophysiology (110).

Distal lung epithelial cells expressing surfactant protein markers have been generated from ESC-/iPSC-derived endodermal precursors in a variety of publications (103,104,106,108). Longmire et al. sorted mouse *Nkx2-1*⁺ endodermal precursors to purity for further expansion in FGF2- and FGF10-supplemented media to generate cells expressing distal transcripts, *Sftpc* and *Sftpb* (104). Huang et al. optimized conditions for derivation of human-ventralized AFE previously reported by this same group (103), resulting in cultures containing about 85% *Foxa2*⁺*Nkx2-1*⁺ cells. These cells were competent for multilineage lung differentiation because further culturing of the bulk population or kidney capsule transplantation into immunodeficient mice gave rise to cells expressing markers of ciliated, club, basal, type I, and type II lung alveolar epithelial cells in vitro or in vivo. In vitro the cells were able to differentiate to *Sftpb*⁺ progeny with less efficient induction of *Sftpc*, by combined Wnt, FGF10, and keratinocyte growth factor exposure followed by dexamethasone and cAMP.

Further work and the use of careful controls will be necessary to characterize how closely iPSC-derived lung-like epithelial lineages resemble their in vivo counterparts. Fortunately, the improving efficiencies of differentiation being reported in these latest publications should make it relatively easy to determine the reproducibility and utility of these protocols for generating distal and proximal lung lineages, allowing investigators to precisely and rigorously test the cellular state of maturation.

Functional Assays of ESC-/iPSC-Derived Putative Lung Lineages

Despite the rapid advances in deriving lung epithelial lineages from hPSCs, the generation of complex 3-D tissue structures composed of mature lung epithelium or even functional organs from these cells remains a high hurdle. Protocols for directed differentiation to lung or other endodermal lineages typically involves the culture of cells as a monolayer. This approach does not allow the study of organ morphogenesis or in the case of the lung, testing the functional capacity of most derived cell types. There is evidence that transitioning to 3-D culture in an extracellular matrix such as matrigel can improve the maturity of hepatic, pancreatic, and more recently, lung-directed differentiations (111–113).

Tissue engineering is rapidly emerging as an approach for generating complex, multicellular structures that might mimic functional lung tissues. Tubes in the shape of upper airways, such as trachea and bronchi, have already been engineered *in vitro* and coated with various cell preparations, such as bone marrow derivatives, prior to surgical grafting into selected patients suffering from regions of tracheal or bronchial atresia (114,115). Beyond the successful generation of functional tubes able to conduct airflow, the engineering of functional alveolar tissue for *in vivo* use remains an unmet challenge. One exciting approach recently published in animal models is the use of “decellularized lungs,” where the perfusion of animal lungs with detergents is used to remove cells, leaving a 3-D scaffold comprised solely of lung extracellular matrix (116,117). These lung scaffolds have then been recellularized with epithelial cell lines (A549 or C10) and endothelial cells (116,117), mesenchymal stem cells (MSCs) (118), whole lung cell suspension digests (116), and even differentiated ESCs/iPSCs in preliminary studies (104). The resulting recellularized lungs can be ventilated and perfused with blood and have had partial function, including capacity for gas exchange, demonstrated through a variety of physiological measurements. Some investigators have even accomplished orthotopic transplantation of these bioartificial lung grafts into pneumonectomized rodents, with partial function *in vivo* demonstrated for a short time period (116,117). Adapting the decellularization–recellularization approach to

construct 3-D lung-like tissues from human ESCs/iPSCs has also recently been demonstrated *ex vivo* (108) and remains an active area of research, although clinical application of this approach remains uncertain and will likely take many decades to develop.

To date most reports on ESC-/iPSC-derived lung cells have focused on attempting to generate lung epithelial lineages. The complex multicellular structure of the lung raises the question of whether all lung lineages, including vascular, interstitial, and immune, can all be derived clonally from a single starting pluripotent stem cell. Developmentally these lineages all appear to derive via the mesodermal germ layer. Fortunately it has been far easier to generate mesoderm from mouse and human ESC/iPSC populations. Hence there is a longer history of investigators successfully generating ESC-/iPSC-derived mesoderm with subsequent formation of a variety of leukocyte, vascular endothelial, smooth muscle, and fibroblastic lineages (119,120). Whether any of these lineages are lung specific remains uncertain, although two recent reports have employed lung disease-specific human iPSCs to generate macrophage and other leukocytic lineages to successfully model pulmonary alveolar proteinosis, a disease that results from defective alveolar macrophage function (121,122). Adapting existing protocols to generate lung-specific phenotypes of these key mesodermal-derived lung vascular and interstitial lineages has not yet been reported and will likely be required to enable *in vitro* study of the full diversity of lung lineages and 3-D tissues engineered from ESCs/iPSCs. Codifferentiation or cocultures of combinations of epithelial and mesenchymal lung progenitors may also enhance the development and maturation of the full diversity of lung lineages by establishing microenvironments and epithelial–mesenchymal signaling niches that regulate normal coordinated development of these tissues.

Summary and the Future

In summary, complex molecular mechanisms coordinate lung development. Decades of careful studies in insects, amphibians, and mammals have uncovered conserved signaling pathways that lead to lung specification and subsequent morphogenesis. High levels of Nodal signaling during gastrulation are required to specify DE. The DE then

forms the gut tube, which is patterned along the A–P axis into broad foregut (*Sox2*⁺, *Hhex*⁺) and hindgut (*Cdx1–4*) domains. Signals from the cardiac and splanchnic mesoderm progressively subdivide the foregut epithelium along its A–P and D–V axes into organ domains. Lung specification is first identified by the expression of *Nkx2-1* in the anterior foregut and is induced by the secretion of Wnts, BMPs, and FGFs from the surrounding mesoderm. Temporally and spatially dynamic signaling interactions between the growing lung bud tips and the surrounding lung mesenchyme results in branching morphogenesis and proximal (*Sox2*⁺) and distal (*Sox9/Id2*⁺) patterning. This complex reciprocal signaling involving FGF10, Wnt/ β -catenin, BMP4, and Tgf β results in a signaling center at the distal lung bud tips, which maintains the proliferation of distal progenitors, promotes branching morphogenesis, and represses *Sox2*⁺ proximal fate. The Hippo/Yap pathway plays a role in defining the proximal–distal boundary, and Notch is known to regulate the further segregation of distinct cell lineages in the proximal airway.

A crucial obstacle to confirming the role of these pathways in human lung development is the inaccessibility of this developmental time point to study. iPSCs offer the potential to study the molecular mechanisms of human development in vitro. Though discovered relatively recently, the generation of iPSCs is now well and widely established. The emerging data from this nascent field confirms that recapitulating the key developmental milestones that regulate lung development in vivo sequentially patterns human iPSCs toward fetal lung epithelium. Further careful work will be aimed at dissecting the subsequent fate decisions involved in deriving distinct cell types of the proximal and distal lung epithelium and in generating cell types that closely resemble their in vivo counterparts. An in vitro platform to study lung development and acquired/genetic lung disease using iPSC-derived lung epithelium has many promising applications from screening novel drug therapies to ultimately generating functional, transplantable lung tissue for patients with end-stage lung disease.

References

- Morrissey EE, Hogan BLM. Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell*. 2010 Jan 19;18(1):8–23.
- Zorn AM, Wells JM. Vertebrate endoderm development and organ formation. *Annu Rev Cell Dev Biol*. 2009;25(1):221–251.
- Ornitz DM, Yin Y. Signaling networks regulating development of the lower respiratory tract. *Cold Spring Harb Perspect Biol*. 2012 May;4(5):a008318–008318.
- Peng T, Tian Y, Boogerd CJ, et al. Coordination of heart and lung co-development by a multipotent cardiopulmonary progenitor. *Nature*. 2013 Aug 29;500(7464):589–592.
- Lazzaro D, Price M, de Felice M, Di Lauro R. The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development*. 1991 Dec;113(4):1093–1104.
- Kubo A, Shinozaki K, Shannon JM, et al. Development of definitive endoderm from embryonic stem cells in culture. *Development*. 2004 Apr;131(7):1651–1662.
- D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol*. 2005 Dec;23(12):1534–1541.
- Engert S, Burtscher I, Liao WP, Dulev S, Schotta G, Lickert H. Wnt/ β -catenin signalling regulates *Sox17* expression and is essential for organizer and endoderm formation in the mouse. *Development. The Company of Biologists Limited*. 2013 Aug;140(15):3128–3138.
- Sinner D, Rankin S, Lee M, Zorn AM. *Sox17* and β -catenin cooperate to regulate the transcription of endodermal genes. *Development*. 2004 Jul;131(13):3069–3080.
- Horb ME, Slack JM. Endoderm specification and differentiation in *Xenopus* embryos. *Dev Biol*. 2001 Aug 15;236(2):330–343.
- Kumar M, Jordan N, Melton D, Grapin-Botton A. Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate. *Dev Biol*. 2003 Jul 1;259(1):109–122.
- Fagman H, Amendola E, Parrillo L, et al. Gene expression profiling at early organogenesis reveals both common and diverse mechanisms in foregut patterning. *Dev Biol*. 2011 Nov 15;359(2):163–175.

- 13 Millien G, Beane J, Lenburg M, et al. Characterization of the mid-foregut transcriptome identifies genes regulated during lung bud induction. *Gene Expr Patterns*. 2008 Jan;8(2):124–139.
- 14 Maeda Y, Davé V, Whitsett JA. Transcriptional control of lung morphogenesis. *Physiol Rev*. 2007 Jan;87(1):219–244.
- 15 Kimura S, Hara Y, Pineau T, et al. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev*. 1996 Jan 1;10(1):60–69.
- 16 Minoo P, Su G, Drum H, Bringas P, Kimura S. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(–/–) mouse embryos. *Dev Biol*. 1999 May 1;209(1):60–71.
- 17 Boucherat O, Montaron S, Bérubé-Simard F-A, et al. Partial functional redundancy between Hoxa5 and Hoxb5 paralog genes during lung morphogenesis. *Am J Physiol Lung Cell Mol Physiol*. 2013 Jun 15;304(12):L817–830.
- 18 Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development*. 2005 Jan;132(1):35–47.
- 19 Sakiyama J-I, Yamagishi A, Kuroiwa A. Tbx4-FGF10 system controls lung bud formation during chicken embryonic development. *Development*. 2003 Apr;130(7):1225–1234.
- 20 Shifley ET, Kenny AP, Rankin SA, Zorn AM. Prolonged FGF signaling is necessary for lung and liver induction in *Xenopus*. *BMC Dev Biol*. 2012;12(1):27.
- 21 Wang JH, Deimling SJ, D’Alessandro NE, Zhao L, Possmayer F, Drysdale TA. Retinoic acid is a key regulatory switch determining the difference between lung and thyroid fates in *Xenopus laevis*. *BMC Dev Biol*. 2011;11(1):75.
- 22 Goss AM, Tian Y, Tsukiyama T, et al. Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev Cell*. 2009 Aug 1;17(2):290–298.
- 23 Harris-Johnson KS, Domyan ET, Vezina CM, Sun X. Beta-catenin promotes respiratory progenitor identity in mouse foregut. *Proc Nat Acad Sci USA*. 2009 Sep 22;106(38):16287–16292.
- 24 Rankin SA, Gallas AL, Neto A, Gómez-Skarmeta JL, Zorn AM. Suppression of BMP4 signaling by the zinc-finger repressors Osr1 and Osr2 is required for Wnt/ β -catenin-mediated lung specification in *Xenopus*. *Development*. 2012 Aug;139(16):3010–3020.
- 25 Li Y, Gordon J, Manley NR, Litingtung Y, Chiang C. BMP4 is required for tracheal formation: a novel mouse model for tracheal agenesis. *Dev Biol*. 2008 Oct 1;322(1):145–155.
- 26 Que J, Choi M, Ziel JW, Klingensmith J, Hogan BLM. Morphogenesis of the trachea and esophagus: current players and new roles for noggin and BMPs. *Differentiation*. 2006 Sep;74(7):422–437.
- 27 Domyan ET, Ferretti E, Throckmorton K, Mishina Y, Nicolis SK, Sun X. Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. *Development*. 2011 Mar 1;138(5):971–981.
- 28 Que J, Luo X, Schwartz RJ, Hogan BLM. Multiple roles for Sox2 in the developing and adult mouse trachea. *Development*. 2009 Jun;136(11):1899–1907.
- 29 Woo J, Miletich I, Kim B-M, Sharpe PT, Shivdasani RA. Barx1-mediated inhibition of Wnt signaling in the mouse thoracic foregut controls tracheo-esophageal septation and epithelial differentiation. *PLoS One*. 2011;6(7):e22493.
- 30 Litingtung Y, Lei L, Westphal H, Chiang C. Sonic hedgehog is essential to foregut development. *Nat Genet*. 1998 Sep;20(1):58–61.
- 31 Motoyama J, Liu J, Mo R, Ding Q, Post M, Hui CC. Essential function of Gli2 and Gli3 in the formation of lung, trachea and oesophagus. *Nat Genet*. 1998 Sep;20(1):54–57.
- 32 Min H, Danilenko DM, Scully SA, et al. *FGF-10* is required for both limb and lung development and exhibits striking functional similarity to *Drosophila branchless*. *Genes Dev*. 1998 Oct 15;12(20):3156–3161.
- 33 Sekine K, Ohuchi H, Fujiwara M, et al. FGF10 is essential for limb and lung formation. *Nat Genet*. 1999 Jan;21(1):138–141.
- 34 Desai TJ, Chen F, Lü J, et al. Distinct roles for retinoic acid receptors alpha and beta in early lung morphogenesis. *Dev Biol*. 2006 Mar 1;291(1):12–24.
- 35 Wang Z, Dollé P, Cardoso WV, Niederreither K. Retinoic acid regulates morphogenesis and patterning of posterior foregut derivatives. *Dev Biol*. 2006 Sep 15;297(2):433–445.
- 36 Chen F, Cao Y, Qian J, Shao F, Niederreither K, Cardoso WV. A retinoic acid-dependent network in the foregut controls formation of the mouse lung primordium. *J Clin Invest*. 2010 Jun;120(6):2040–2048.
- 37 Chen F, Desai TJ, Qian J, Niederreither K, Lü J, Cardoso WV. Inhibition of TGF beta signaling by endogenous retinoic acid is essential for

- primary lung bud induction. *Development*. 2007 Aug;134(16):2969–2979.
- 38 Arora R, Metzger RJ, Papaioannou VE. Multiple roles and interactions of Tbx4 and Tbx5 in development of the respiratory system. Barsh GS, editor. *PLoS Genet*. 2012;8(8):e1002866.
- 39 Li W, Lin C-Y, Shang C, et al. Pbx1 activates FGF10 in the mesenchyme of developing lungs. *Genesis*. 2014 May;52(5):399–407.
- 40 Metzger RJ, Klein OD, Martin GR, Krasnow MA. The branching programme of mouse lung development. *Nature*. 2008 Jun 5;453(7196):745–750.
- 41 Alanis DM, Chang DR, Akiyama H, Krasnow MA, Chen J. Two nested developmental waves demarcate a compartment boundary in the mouse lung. *Nat Commun*. 2014;5:3923.
- 42 Tompkins DH, Besnard V, Lange AW, et al. Sox2 activates cell proliferation and differentiation in the respiratory epithelium. *Am J Respir Cell Mol Biol*. 2011 Jul;45(1):101–110.
- 43 Chang DR, Martinez Alanis D, Miller RK, et al. Lung epithelial branching program antagonizes alveolar differentiation. *Proc Natl Acad Sci USA*. 2013 Nov 5;110(45):18042–18051.
- 44 Rockich BE, Hrycaj SM, Shih HP, et al. Sox9 plays multiple roles in the lung epithelium during branching morphogenesis. *Proc Natl Acad Sci USA*. 2013 Nov 19;110(47):E4456–4464.
- 45 Rawlins EL, Clark CP, Xue Y, Hogan BLM. The Id2+ distal tip lung epithelium contains individual multipotent embryonic progenitor cells. *Development*. 2009 Nov;136(22):3741–3745.
- 46 Hyatt BA, Shangguan X, Shannon JM. FGF-10 induces SP-C and BMP4 and regulates proximal-distal patterning in embryonic tracheal epithelium. *Am J Physiol Lung Cell Mol Physiol*. 2004 Dec;287(6):L1116–1126.
- 47 Weaver M, Dunn NR, Hogan BL. BMP4 and FGF10 play opposing roles during lung bud morphogenesis. *Development*. 2000 Jun;127(12):2695–2704.
- 48 Ablner LL, Mansour SL, Sun X. Conditional gene inactivation reveals roles for FGF10 and FGFR2 in establishing a normal pattern of epithelial branching in the mouse lung. *Dev Dyn*. 2009 Aug;238(8):1999–2013.
- 49 Volckaert T, Campbell A, Dill E, Li C, Minoo P, De Langhe S. Localized FGF10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. *Development*. 2013 Sep;140(18):3731–3742.
- 50 Rajagopal J, Carroll TJ, Guseh JS, et al. Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme. *Development*. 2008 May;135(9):1625–1634.
- 51 Mahoney JE, Mori M, Szymaniak AD, Varelas X, Cardoso WV. The Hippo pathway effector Yap controls patterning and differentiation of airway epithelial progenitors. *Dev Cell*. 2014 Jul 28;30(2):137–150.
- 52 Zhao R, Fallon TR, Saladi SV, et al. Yap tunes airway epithelial size and architecture by regulating the identity, maintenance, and self-renewal of stem cells. *Dev Cell*. 2014 Jul 28;30(2):151–165.
- 53 Herriges M, Morrisey EE. Lung development: orchestrating the generation and regeneration of a complex organ. *Development*. 2014 Feb;141(3):502–513.
- 54 Tian Y, Zhang Y, Hurd L, et al. Regulation of lung endoderm progenitor cell behavior by miR302/367. *Development*. 2011 Apr;138(7):1235–1245.
- 55 Herriges MJ, Swarr DT, Morley MP, et al. Long noncoding RNAs are spatially correlated with transcription factors and regulate lung development. *Genes Dev. Cold Spring Harbor Lab*. 2014 Jun 15;28(12):1363–1379.
- 56 Wang Y, Tian Y, Morley MP, et al. Development and regeneration of Sox2+ endoderm progenitors are regulated by a Hdac1/2-BMP4/Rb1 regulatory pathway. *Dev Cell*. 2013 Feb 25;24(4):345–358.
- 57 Shan L, Aster JC, Sklar J, Sunday ME. Notch-1 regulates pulmonary neuroendocrine cell differentiation in cell lines and in transgenic mice. *Am J Physiol Lung Cell Mol Physiol*. 2007 Feb;292(2):L500–509.
- 58 Guseh JS, Bores SA, Stanger BZ, et al. Notch signaling promotes airway mucous metaplasia and inhibits alveolar development. *Development*. 2009 May;136(10):1751–1759.
- 59 Morimoto M, Nishinakamura R, Saga Y, Kopan R. Different assemblies of Notch receptors coordinate the distribution of the major bronchial Clara, ciliated and neuroendocrine cells. *Development*. 2012 Dec 1;139(23):4365–4373.
- 60 Tsao P-N, Vasconcelos M, Izvolsky KI, Qian J, Lü J, Cardoso WV. Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. *Development*. 2009 Jul;136(13):2297–2307.
- 61 Wade KC, Guttentag SH, Gonzales LW, et al. Gene

- induction during differentiation of human pulmonary type II cells in vitro. *Am J Respir Cell Mol Biol.* 2006 Jun 1;34(6):727–737.
- 62 Desai TJ, Brownfield DG, Krasnow MA. Alveolar progenitor and stem cells in lung development, renewal and cancer. *Nature.* 2014 Feb 5;507:1–16.
- 63 Treutlein B, Brownfield DG, Wu AR, et al. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature.* 2014 May 15;509(7500):371–375.
- 64 Kleinsmith LJ, Pierce GB. Multipotentiality of single embryonal carcinoma cells. *Cancer Res.* 1964 Oct;24:1544–1551.
- 65 Solter D, Skreb N, Damjanov I. Extrauterine growth of mouse egg-cylinders results in malignant teratoma. *Nature.* 1970 Aug 1;227(5257):503–504.
- 66 Stevens LC. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Dev Biol.* 1970 Mar;21(3):364–382.
- 67 Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* 1981 Jul 9;292(5819):154–156.
- 68 Nagy A, Gócsa E, Diaz EM, Prideaux VR, Iványi E, Markkula M, et al. Embryonic stem cells alone are able to support fetal development in the mouse. *Development.* 1990 Nov;110(3):815–821.
- 69 Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA.* 1993 Sep 15;90(18):8424–8428.
- 70 Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell.* 1987 Nov 6;51(3):503–152.
- 71 Bradley A, Hasty P, Davis A, Ramirez-Solis R. Modifying the mouse: design and desire. *Biotechnology (NY).* 1992 May;10(5):534–539.
- 72 Lewandoski M. Conditional control of gene expression in the mouse. *Nat Rev Genet.* 2001 Oct;2(10):743–755.
- 73 Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998 Nov 6;282(5391):1145–1147.
- 74 Yamanaka S. Induced pluripotent stem cells: past, present, and future. *Cell Stem Cell.* 2012 Jun 14;10(6):678–684.
- 75 Gurdon JB. Adult frogs derived from the nuclei of single somatic cells. *Dev Biol.* 1962 Apr;4:256–273.
- 76 Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature.* 1997 Feb 27;385(6619):810–813.
- 77 Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell.* 1987 Dec 24;51(6):987–1000.
- 78 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006 Aug 25;126(4):663–676.
- 79 Zhao X-Y, Li W, Lv Z, et al. iPS cells produce viable mice through tetraploid complementation. *Nature.* 2009 Sep 3;461(7260):86–90.
- 80 Kang L, Wang J, Zhang Y, Kou Z, Gao S. iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. *Cell Stem Cell.* 2009 Aug 7;5(2):135–138.
- 81 Boland MJ, Hazen JL, Nazor KL, et al. Adult mice generated from induced pluripotent stem cells. *Nature.* 2009 Sep 3;461(7260):91–94.
- 82 Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007 Nov 30;131(5):861–872.
- 83 Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007 Dec 21;318(5858):1917–1920.
- 84 Wang Y, Liu J, Tan X, et al. Induced pluripotent stem cells from human hair follicle mesenchymal stem cells. *Stem Cell Rev.* 2013 Aug;9(4):451–460.
- 85 Loh Y-H, Agarwal S, Park I-H, et al. Generation of induced pluripotent stem cells from human blood. *Blood.* 2009 May 28;113(22):5476–5479.
- 86 Ichida JK, Blanchard J, Lam K, et al. A small-molecule inhibitor of TGF-β signaling replaces Sox2 in reprogramming by inducing Nanog. *Cell Stem Cell.* 2009 Nov 6;5(5):491–503.
- 87 Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell.* 2008 Feb 22;132(4):661–680.
- 88 Kriks S, Shim J-W, Piao J, et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature.* 2011 Dec 22;480(7378):547–551.
- 89 Hirami Y, Osakada F, Takahashi K, et al. Generation of retinal cells from mouse and human induced pluripotent stem cells. *Neurosci Lett.* 2009 Jul 24;458(3):126–131.

- 90 Shiba Y, Fernandes S, Zhu W-Z, et al. Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. *Nature*. 2012 Sep 13;489(7415):322–325.
- 91 van Laake LW, Passier R, Monshouwer-Kloots J, et al. Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. *Stem Cell Res*. 2007 Oct;1(1):9–24.
- 92 Nostro MC, Sarangi F, Ogawa S, et al. Stage-specific signaling through TGF β family members and Wnt regulates patterning and pancreatic specification of human pluripotent stem cells. *Development*. 2011 Mar;138(5):861–871.
- 93 Cai J, Zhao Y, Liu Y, et al. Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology*. 2007 May;45(5):1229–1239.
- 94 Spence JR, Mayhew CN, Rankin SA, et al. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature*. 2011 Feb 3;470(7332):105–109.
- 95 Coraux C, Nawrocki-Raby B, Hinnrasky J, et al. Embryonic stem cells generate airway epithelial tissue. *Am J Respir Cell Mol Biol*. 2005 Feb;32(2):87–92.
- 96 Van Haute L, De Block G, Liebaers I, Sermon K, De Rycke M. Generation of lung epithelial-like tissue from human embryonic stem cells. *Respir Res*. 2009;10(1):105.
- 97 Wang D, Haviland DL, Burns AR, Zsigmond E, Wetsel RA. A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA*. 2007 Mar 13;104(11):4449–4454.
- 98 Christodoulou C, Longmire TA, Shen SS, et al. Mouse ES and iPS cells can form similar definitive endoderm despite differences in imprinted genes. *J Clin Invest*. 2011 Jun;121(6):2313–2325.
- 99 Gadue P, Huber TL, Paddison PJ, Keller GM. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. *Proc Natl Acad Sci USA*. 2006 Nov 7;103(45):16806–16811.
- 100 Tada S, Era T, Furusawa C, et al. Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development*. 2005 Oct;132(19):4363–4374.
- 101 Yasunaga M, Tada S, Torikai-Nishikawa S, et al. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nat Biotechnol*. 2005 Dec;23(12):1542–1550.
- 102 Gouon-Evans V, Boussemart L, Gadue P, et al. BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. *Nat Biotechnol*. 2006 Nov;24(11):1402–1411.
- 103 Green MD, Chen A, Nostro M-C, et al. Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. *Nat Biotechnol*. 2011 Mar;29(3):267–272.
- 104 Longmire TA, Ikonomou L, Hawkins F, et al. Efficient derivation of purified lung and thyroid progenitors from embryonic stem cells. *Cell Stem Cell*. 2012 Apr 6;10(4):398–411.
- 105 Mou H, Zhao R, Sherwood R, et al. Generation of multipotent lung and airway progenitors from mouse ESCs and patient-specific cystic fibrosis iPSCs. *Cell Stem Cell*. 2012 Apr 6;10(4):385–397.
- 106 Huang SXL, Islam MN, O'Neill J, et al. Efficient generation of lung and airway epithelial cells from human pluripotent stem cells. *Nature*. 2014 Jan 1;32(1):84–91.
- 107 Brafman DA, Moya N, Allen-Soltero S, Fellner T, Robinson M, McMillen ZL, et al. Analysis of SOX2-expressing cell populations derived from human pluripotent stem cells. *Stem Cell Rep*. 2013 Nov;1(5):464–478.
- 108 Ghaedi M, Calle EA, Mendez JJ, et al. Human iPS cell-derived alveolar epithelium repopulates lung extracellular matrix. *J Clin Invest*. 2013 Nov 1;123(11):4950–4962.
- 109 You Y, Richer EJ, Huang T, Brody SL. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. *Am J Physiol Lung Cell Mol Physiol*. 2002 Dec;283(6):L1315–1321.
- 110 Firth AL, Dargitz CT, Qualls SJ, et al. Generation of multiciliated cells in functional airway epithelia from human induced pluripotent stem cells. *Proc Natl Acad Sci USA*. 2014 Apr 29;111(17):E1723–1730.
- 111 Ogawa S, Surapisitchat J, Virtanen C, et al. Three-dimensional culture and cAMP signaling promote the maturation of human pluripotent stem cell-derived hepatocytes. *Development*. 2013 Jul 16;140(15):3285–3296.
- 112 Saito H, Takeuchi M, Chida K, Miyajima A. Generation of glucose-responsive functional islets with a three-dimensional

- structure from mouse fetal pancreatic cells and iPS cells in vitro. *PLoS One*. 2011;6(12):e28209.
- 113 Gotoh S, Ito I, Nagasaki T, et al. Generation of alveolar epithelial spheroids via isolated progenitor cells from human pluripotent stem cells. *Stem Cell Rep*. 2014 Sep;3(3):394–403.
- 114 Baiguera S, Jungebluth P, Burns A, et al. Tissue engineered human tracheas for in vivo implantation. *Biomaterials*. 2010 Dec;31(34):8931–8938.
- 115 Macchiarini P, Jungebluth P, Go T, et al. Clinical transplantation of a tissue-engineered airway. *Lancet*. 2008 Dec 13;372(9655):2023–2030.
- 116 Ott HC, Clippinger B, Conrad C, et al. Regeneration and orthotopic transplantation of a bioartificial lung. *Nat Med*. 2010 Aug;16(8):927–933.
- 117 Petersen TH, Calle EA, Zhao L, et al. Tissue-engineered lungs for in vivo implantation. *Science*. 2010 Jul 30;329(5991):538–541.
- 118 Daly AB, Wallis JM, Borg ZD, et al. Initial binding and recellularization of decellularized mouse lung scaffolds with bone marrow-derived mesenchymal stromal cells. *Tissue Eng. Part A*. 2012 Jan;18(1–2):1–16.
- 119 Inoue-Yokoo T, Tani K, Sugiyama D. Mesodermal and hematopoietic differentiation from ES and iPS cells. *Stem Cell Rev*. 2013 Aug;9(4):422–434.
- 120 Era T. Mesoderm cell development from ES cells. *Methods Mol Biol*. 2010;636:87–103.
- 121 Suzuki T, Mayhew C, Salles A, et al. Use of induced pluripotent stem cells to recapitulate pulmonary alveolar proteinosis pathogenesis. *Am J Respir Crit Care Med*. 2014 Jan 15;189(2):183–193.
- 122 Lachmann N, Happle C, Ackermann M, Lüttge D, Wetzke M, Merkert S, et al. Gene correction of human induced pluripotent stem cells repairs the cellular phenotype in pulmonary alveolar proteinosis. *Am J Respir Crit Care Med*. 2014 Jan 15;189(2):167–182.

Early Development of the Mammalian Lung-Branching Morphogenesis

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Abstract

The lung is an essential organ for mammalian health throughout life and represents a critical interface with the external environment in the regulation of gas exchange. To achieve these goals the mammalian lung has evolved into a highly complex system of branching epithelial and vascular structures that connects to a vast network of alveolar gas-exchanging units. The generation of this complex organ involves multiple steps and encompasses events that span prenatal and postnatal life. This chapter focuses on the mechanisms that regulate early lung development and branching morphogenesis and the various signaling molecules, extracellular matrix proteins, and genetic changes that influence this process. These research areas are critical for understanding the various causes of respiratory disease, which are a major contributor to morbidity and mortality at all stages of life.

Keywords:

Lung biology, branching morphogenesis, development, Wnt signaling, pulmonary biology, respiratory system

Introduction

The formation of the respiratory system represents an evolutionary process critical for terrestrial life. The lungs are the primary organ that performs gas exchange between the external environment and the cardiovascular system, and their basic role in respiration is highly conserved among vertebrates as divergent as amphibians and mammals. The mature mammalian lung is a highly complex structure comprised of myriad endodermal- and mesodermal-derived cell lineages that undergo a complex developmental process to form a series of branched tubules (the trachea, bronchi, and bronchioles) that conduct air from the pharynx to a gas-exchange area at terminal, bud-like structures, termed alveoli. The distal alveoli are surrounded by a dense capillary network that facilitates the exchange of oxygen and carbon dioxide between the alveolar air spaces and the pulmonary vasculature.

Normal lung development is determined by a functional integration of genetic changes and physical and chemical changes, including intraluminal fluid pressure and oxygen tension. Genetic factors include (1) transcription factors that directly modulate gene expression; (2) growth

factors and cytokines as well as their related intracellular signaling components that mediate cell proliferation, differentiation, migration and cell-cell interactions; and (3) extracellular matrix components that provide important environmental cues that promote proper lung cell behavior. Lung development has been the object of extensive studies in recent years, resulting in the generation of new insights into the origins of the various cell lineages in the lung as well as the molecular pathways that regulate the development of these lineages. In turn, this has led to insights into important lung diseases including asthma and chronic obstructive pulmonary disease (COPD), as well as the lung's response to acute injury. One of the most remarkable aspects of lung development is that although the organ arises from a simple patch of anterior foregut endoderm ensheathed with lateral mesoderm early in development, it develops through an elegant morphological process into a highly structured and complex organ. One of these processes, called branching morphogenesis, is essential for generating the basic arborized three-dimensional structure of the airway tree, resulting in the extensive surface area required for gas exchange in the adult. The arborization of the airways serves

multiple functions that include allowing for the entering air to be warmed, moistened, and filtered for particulates. In addition, the highly branched network forms a large terminal gas exchange surface in a spatially efficient manner.

This chapter will summarize the current understanding of the major molecular pathways required for branching morphogenesis during lung development. Most of what is described herein refers to mouse lung development because of the genetic data available. Lung vascular development and later events such as sacculization and alveoli formation are not discussed in this chapter but have been well reviewed previously and are included in Chapter 3 (1–3).

Overview of Lung Development and Branching Morphogenesis

Histologically, mouse lung development has been divided into four chronological stages defined by changes in the structure of the airway tubes and morphological modifications of epithelial cells. This staging method for mice has been reviewed elsewhere (4) and is briefly summarized as (1) the embryonic stage (E9.0–E12.5), which includes the specification of the respiratory endoderm followed by the earliest stages of branching morphogenesis; (2) the pseudoglandular stage (E12.5–16.5), when the respiratory tree branches extensively to form the arborized networks of airways that are patterned in a proximal–distal manner; (3) the canalicular stage (E16.5–17.5), where the parallel vascular network begins to come into close apposition to the branched airways; (4) the saccular stage (E17.5 to approximately postnatal day 5 [P5]), when the terminal sacs develop and there is an increase in vascular complexity in the surrounding terminal sacs and the first evidence of alveolar epithelial and mesenchymal differentiation occurs; and (5) the alveolar stage (P5–30), when the terminal sacs develop into mature alveolar units, including the development of primary and secondary septa. This chapter will primarily focus on the morphological and genetic changes that occur in the first half of lung development, including the first two stages—embryonic and pseudoglandular (E9.0–E16.5).

In the mouse, the respiratory system, including the trachea and lungs, arises from the primitive anterior ventral foregut at approximately embryonic day 9–9.5 in the mouse (E9.0–9.5). The foregut endoderm is multipotent and can generate several

organs, including the respiratory system, esophagus, thyroid, and liver, and each of these organ primordia is specified in a unique spatial region along the anterior–posterior axis. Specification of the early respiratory endoderm progenitors in the anterior foregut is controlled by the action of many signaling pathways, including Wnt, Retinoic acid (RA), and Fgf (5). These pathways signal from the surrounding mesoderm to activate the early respiratory endoderm program in the anterior foregut. The ultimate result of these signaling pathways is activation of expression of the transcription factor *Nkx2-1* specifically on the ventral side of the foregut endoderm and restriction of *Sox2* and *Trp63* expression to the dorsal side (6,7). Within 24 hours of this specification event, the ventral *Nkx2-1*⁺ endoderm undergoes a morphogenetic process that leads to a pinching of the foregut endodermal tube to give rise to two new tubes, the dorsal esophagus that leads to the stomach and the ventral trachea, which ultimately leads to the lungs. The trachea and bronchial stalks extend to form the main bronchi, a process completed by approximately E10.5 in the mouse. At this stage the two primitive lungs are fairly symmetrical buds that bulge outward into the pleuroperitoneal cavity.

Growth and patterning of the airways is accompanied by the elongation and repetitive branching of the two lung buds, a process known as branching morphogenesis. This generates an arborized structure of airways with thousands of terminal branches. Examination of the primitive lungs shortly after the two initial buds form show that at this early stage, the lung is relatively simple, essentially two epithelial sacs, surrounded by a fairly undifferentiated mesenchyme, which together encompasses a lumen that is continuous with the trachea. This lumen is maintained throughout development of the lung and as such presents a different model of branching morphogenesis from other branched organs such as the salivary gland (8) and mammary gland (9). Importantly, the subsequent branching process in the mouse is also different from humans in that the bronchial buds give rise to one left and four right lung lobes, which are well established by E12. During this stage of development, the trachea completes its separation from the esophagus. Moreover, the separation of the trachea from the esophagus and the formation of the branching lung appear to be distinct developmental processes, as studies have demonstrated that lung

bud formation still occurs even in the absence of tracheal development (6).

As the lung epithelium continues to grow and branch during the pseudoglandular stage, it becomes patterned along the proximal–distal axis. The proximal to distal patterning of the lung epithelium is directed and maintained by molecular signaling between the mesenchyme and epithelium through a number of signaling pathways, including Fgf, Wnt, and BMP. Lung branching in mice has been shown to be highly reproducible and suggests that the process is genetically hardwired (10). Branching morphogenesis modes have been classified into three types: domain branching, planar bifurcation, and orthogonal bifurcation. These three types of branch formation modalities are initiated at differing times throughout lung development. Domain branch formation occurs earliest in development and is characterized by daughter branches budding from the main bronchial tubule. Domain branching establishes the underlying skeleton of the arborized respiratory tree that develops into the main bronchioles of the lung. At the bud tips, the growing terminal bud expands, flattens, and undergoes bifurcation, either within the plane of the parent branch (planar bifurcation) or perpendicular to the plane of the parent branch (orthogonal bifurcation) (10).

Following the pseudoglandular stage, around E16.5 in the mouse, branching morphogenesis slows and reaches completion. During the canalicular (E16.5–17.5) and saccular stages (E18.5–P5), terminal branches will narrow and then form clusters of epithelial sacs that will later develop into alveoli, which are the primary sites of gas exchange in the lung. In addition the proximal–distal patterning of the epithelium and mesenchyme results in differentiation of more than 40 different cell types that constitute the mature, functional lung. Importantly, throughout all stages of endodermal development, the lung mesoderm or mesenchyme develops and interacts with the lung endoderm to promote branching and cellular differentiation (11).

Transcription Factors in Branching Morphogenesis

Lung development and branching morphogenesis are mediated by dynamic changes in gene

expression, which are controlled in part by the expression and activity of lineage-specific transcription factors. Multiple transcription factor families are expressed in the lung including members of the Nkx, Forkhead box (Fox), Myc, and Gata families. Interestingly, many of the transcription factors required for development of the early foregut are reutilized later in lung morphogenesis (12). Although some of these transcription factors are highly cell type specific, others are expressed in multiple tissue compartments in a distribution pattern that changes during development. We will briefly highlight the key transcription factor proteins or family of proteins that are currently known to play a critical role in branching morphogenesis.

Fox Family Proteins

The Fox family of proteins plays an important role in the regulation of cell differentiation, organogenesis, and gene expression in many organs, including the lung. Foxa1 and Foxa2 are expressed in the foregut endoderm before lung formation and are expressed in an overlapping pattern with Nkx2-1 in respiratory epithelial cells during lung morphogenesis and in the mature lung (13,14). Deletion of both Foxa1 and Foxa2 in mouse models inhibited cell proliferation, epithelial cell differentiation, and branching (15). Increased expression of Foxa2 in respiratory epithelial cells perturbed branching and impaired cell differentiation (14). Members of the Foxp family, including Foxp1, Foxp2, and Foxp4, are expressed in the developing and postnatal lung epithelium. Foxp1/2 act in a redundant manner to regulate early lung epithelial development, and a combined loss of these two transcription factors leads to disruption in the early branching program (16). Foxp1/4 act in a redundant manner to regulate airway secretory cell differentiation by repressing the goblet cell differentiation program (17). Moreover, Foxp1/4 are essential for regeneration of secretory cells in the adult lung after naphthalene-based injury (17). In the mesenchyme, Foxf1 plays a critical role in embryonic development, and severe lung malformations were observed in *Foxf1*^{+/-} mice (18,19). Mutations in Foxf1 underlie an important congenital lung disease called alveolar capillary dysplasia (20–22).

HOX Family Proteins

The HOX genes are a family of transcription factors that act to specify regional identity along the anterior–posterior body axis by regulating specific downstream sets of effectors, which in turn direct morphogenetic events (23). A subset of HOX genes are expressed in the developing lung, including *Hoxa5* and *Hoxb5*, which have been shown to be important for lung branching morphogenesis. *Hoxa5* null mice exhibit primary lung defects leading to respiratory distress and to partially penetrant lethality at birth (24, 25). *Hoxb5* null embryos show decreased branching (26), which is thought to be due to downregulation in tenascin-C, an extracellular matrix component required for branching morphogenesis (27, 28).

N-myc

N-myc is a proto-oncogene that is expressed during the differentiation of several cell lineages during mammalian embryogenesis and, when overexpressed, participates in neoplastic transformation in cancers. N-Myc null mice die around e10.5 and fail to exhibit lung branching morphogenesis (29–31). Further studies using a hypomorphic mutation survive until birth but later die due to a defect in lung branching morphogenesis as observed by decreased alveolar surface area and less branched airways (32). Lung epithelial specific loss of N-myc leads to decreased epithelial proliferation and differentiation with a concomitant increase in apoptosis (33). In vitro models have suggested that there may be important interactions between N-myc and growth factors during lung morphogenesis, but further research is needed to understand these interactions (31,34).

Nkx2-1

Nkx2-1, also known as thyroid transcription factor-1 (TTF-1), is the earliest known marker of the specification of foregut endoderm into the pulmonary and thyroid cell lineages, appearing before formation of the definitive lung (35). Deletion of Nkx2-1 in the mouse causes malformations of a variety of organs and is required for branching morphogenesis in the lung, leading to a loss of peripheral lung structures (36). Moreover,

mutations in the human *Nkx2-1* gene have been associated with respiratory failure in human infants (37). Nkx2-1 interacts with a multitude of regulatory proteins and transcription factors that regulate surfactant homeostasis, vasculogenesis, host defense, fluid homeostasis, and inflammation before birth (38). A large regulatory network of genes and transcriptional programs is regulated by Nkx2-1, and many of these are essential for proper lung morphogenesis (12).

Epigenetic Influences on Lung Morphogenesis

Histone Deacetylases

Emerging data from studies exploring the role of histone deacetylases (HDACs) shows that these chromatin remodeling factors play an important role in regulating early lung development. HDAC1 and HDAC2 are found in the same macromolecular complexes including NuRD and Sin3a. Combined loss of HDAC1/2 in the developing lung endoderm leads to a complete loss of the Sox2⁺ proximal endoderm progenitor compartment (39). This results in the expansion of the Sox9⁺ distal progenitor compartment and a loss of branching morphogenesis. The role of other HDACs in lung development awaits further investigation.

Polycomb Complex

The polycomb repressive complex is a large multiprotein complex that is involved in gene repression. In the lung the polycomb repressive complex 2 (PRC2) component Ezh2 is widely expressed during development (40). Loss of Ezh2 specifically in the developing lung endoderm leads to a dramatic expansion of the Trp63⁺ basal cell lineage (40). Trp63⁺ basal cells are an important stem cell lineage known to regenerate the pseudostratified airway epithelium of the adult large airways (41). In mice, Trp63⁺ basal cells are not normally found underlying the airways during lung development and are normally only observed in large numbers in the postnatal trachea and main stem bronchi. The expansion of Trp63⁺ basal cells after loss of Ezh2 in the developing lung endoderm suggests that one role of the PRC2 complex during development is to restrict the basal cell lineage.

miRNAs

MicroRNAs (miRNAs) are small, noncoding RNAs that bind to the coding region of target mRNAs to suppress translation and degrade the mRNA. Emerging studies have identified miRNAs that are temporally and spatially regulated within developing organs, including the lung (42–44). Several clusters of miRNAs have been shown to play an important role in balancing lung endoderm progenitor proliferation and differentiation. The miR17–92 cluster is essential for lung development, with loss of its expression leading to lung hypoplasia (45). Conversely, increased expression of miR17–92 leads to increased endoderm proliferation and decreased differentiation (46). The miR302–367 cluster is also important for promoting lung endoderm proliferation and differentiation. Loss of miR302–367 activity leads to decreased proliferation and enhanced epithelial differentiation, whereas increased expression leads to a block in differentiation coupled with increased proliferation (47). Recently miR-221 and miR-130a have been shown to regulate both airway branching and lung microvascular development (48). In these *in vitro* studies, increased miR-221 or decreased miR-130a levels in lung cultures both resulted in reduced airway branching. Identifying the specific miR targets that affect branching morphogenesis is an area of intense research currently.

Long Noncoding RNAs

Long noncoding RNAs (lncRNAs) have recently been shown to play important roles in lung development. Several hundred lncRNAs have been identified in the mouse lung, and some of these have been shown to regulate important molecular processes during lung morphogenesis. One lncRNA called Nkx2-1-associated noncoding intergenic RNA or NNCI is located several kilobases downstream of Nkx2-1 and has been shown to regulate the expression of Nkx2-1 (49). Importantly, there are lncRNAs located near many transcription factors important for lung development besides Nkx2-1, including Gata6, Foxa2, and Foxf1 (49). Conversely, lncRNAs that are located in gene deserts also play an important role. One of these, called LL34, appears to regulate RA signaling in lung epithelial cells (49). Given the large number of lncRNAs expressed in the developing lung, there are likely many more of these poorly

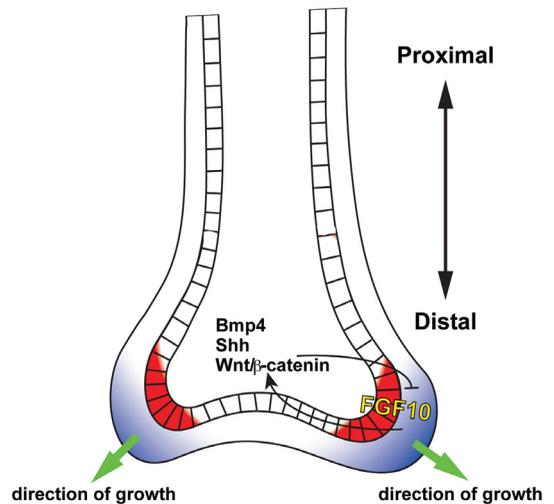


Figure 2-1. Reciprocal interactions between the developing lung endoderm and mesenchyme promote growth and branching of the airways. The developing lung airways are patterned in a proximal–distal manner with mesenchymal factors such as Fgf10 promoting growth and branching at the distal tip. Fgf10 and other mesenchymal factors act in concert with endodermally expressed signaling pathways such as BMP, Shh, and Wnt/ β -catenin, which are all required for proper branching and growth of the developing airways.

understood transcripts that regulate important processes during lung development.

Signaling Pathways in Branching Morphogenesis

As introduced earlier, branching morphogenesis of the lung requires precise reciprocal signaling from the epithelial and mesenchymal compartments to regulate cell proliferation and differentiation. This results in regional lung-specific gene expression and proper patterning of the lineages within the developing lung. Signaling factors expressed in the distal lung mesenchyme such as Fgf10 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 distal lung epithelium (50–52). These findings emphasize the importance of autocrine and paracrine factors produced within the lung mesenchyme, which are necessary and sufficient for lung branching morphogenesis (Figure 2-1). Here we will briefly summarize the key signaling pathways that are known to regulate branching morphogenesis in the developing lung.

Fgf Signaling

Fibroblast growth factors (Fgfs) and their receptors (Fgfrs) regulate a wide range of biological functions, including cellular proliferation, migration, and differentiation. Despite the numerous Fgfs expressed in the lung, Fgf10 in the mesenchyme and its receptor, Fgfr2b in the epithelium, have emerged as the primary Fgf ligand/receptor interaction required for branching morphogenesis in the lung (53). Fgf10 mutant lungs recapitulate the Fgfr2b mutants, with both mutants displaying complete lung agenesis distal to the trachea (54–56). Analysis of Fgf10 hypomorphs revealed fewer and shorter branches with loss of Fgf10 and complete loss of the accessory lobe. In addition, conditional loss of Fgf10 in the lung mesenchyme resulted in secondary branching defects (57). Together these results demonstrate a requirement for Fgf10 signaling in the initial budding of the lung from the trachea and strongly suggest a role for Fgf10 in later branching processes. Fgf9 is another important signaling ligand in lung development, as Fgf9 mutants showed decreased lung branching and changes in epithelial cell morphology (58).

Wnt Signaling

Nineteen Wnts are expressed in vertebrates, and the expression patterns of 5 Wnts have been characterized in the lung: Wnt2, Wnt2b, Wnt7b, Wnt5a, and Wnt11. Wnt2 and Wnt2b are expressed exclusively in the lung mesenchyme, whereas Wnt7b is expressed exclusively in the developing lung endoderm. Wnt5a is expressed in both the developing mesenchyme and endoderm of the lung in a temporal specific fashion (59,60). Loss of Wnt2 and Wnt2b, Wnt7b, and Wnt5a, as well as loss of β -catenin (a downstream effector of Wnt signaling), all cause various defects in lung development, establishing an important role for this pathway in lung development (61). The Wnt signaling pathway is activated when a Wnt ligand binds the Wnt coreceptors, including frizzleds and Lrp5/6. There have been at least five frizzled genes reported in the mouse lung (62). Once the ligand–receptor interaction has occurred, the canonical, or β -catenin-dependent pathway and the noncanonical, or β -catenin-independent pathways are activated. Canonical Wnt signaling frequently effects cell

proliferation or cell fate specification; in contrast, the noncanonical Wnt pathways control components of cell migration and polarity that have effects on tissue morphogenesis.

Wnt2 and Wnt2b are required in a combinatorial fashion for lung endoderm specification. Loss of Wnt2/2b leads to complete respiratory organ agenesis, including loss of both trachea and lung development and loss of Nkx2-1 expression (63). Loss of both Wnt2 and Wnt7b causes loss of branching morphogenesis in the lungs after their initial formation (64). Moreover, Wnt2/7b double knockouts have significantly reduced smooth muscle development. Additional work is required to establish whether the defect in branching morphogenesis with loss of Wnt2/Wnt7b is due to the loss of smooth muscle or due to unreported defects in the epithelium (64). Wnt5a mutant animals have a very distinct defect in lung development characterized by lung hyperplasia, increased proliferation, and overbranching of the distal airways, without changes in cell specification (60). Overexpression of Wnt5a in the lung epithelium causes decreased branching, shortened outgrowth of new buds, and complete loss of the accessory lobe (60).

The Wnt receptor Fzd2 plays a key role in regulating epithelial cell behavior and tube morphology necessary for formation of new branch points during airway morphogenesis. Fzd2 is essential for regulating changes in epithelial cell shape and cell lengthening along the apical–basal axis, which is critical for formation of new domain branch points and maintaining proper airway tube shape in the developing lung (65). Loss of Fzd2 leads to decreased apical expression of phospho-myosin light chain 2 indicative of decreased Rho signaling, which is required for thickening of the lung epithelium prior to new branch formation (65).

Hedgehog Signaling

The canonical Hedgehog signaling pathway involves binding of the hedgehog ligand to one of the patched (Ptc) twelve-pass membrane protein receptors. Upon binding of hedgehog ligand to Ptc, inhibition of smoothed by Ptc is reversed, and Smo activates the Hedgehog pathway. Sonic hedgehog (Shh) signaling is required for branching morphogenesis and serves as a paracrine signal that regulates smooth muscle

differentiation in the lung mesenchyme (66,67). *Shh*^{-/-} mice have tracheoesophageal fistula and simple cyst-like lung sacs that fail to branch, although they exhibit some cell type specific differentiation (66, 68).

BMP Pathway

Evidence from normal and targeted misexpression studies in mice further suggests that BMP4 a TGF- β family peptide, plays a role in embryonic lung morphogenesis (69). Misexpression of BMP4 in *Sftpc*⁺ distal lung epithelium during development results in lungs that are smaller than normal with grossly distended terminal buds and large air-filled sacs at birth (69). Developmental loss of the BMP4 receptor BMPRIa leads to defects in distal lung epithelial proliferation and survival, and some of these defects are mimicked by loss of BMP4 in the developing lung epithelium (70).

Influence of Extracellular Matrix on Branching Morphogenesis

The ECM (extracellular matrix) is defined as the diverse collection of proteins and sugars that surrounds cells in all solid tissues. This tissue compartment provides structural support by maintaining an insoluble scaffold, and this in turn helps to define the characteristic shape and dimensions of organs and complex tissues. The lung ECM is primarily composed of collagen IV, laminins, nidogen, and proteoglycans (71,72). Although the ECM has historically been perceived as performing a primarily structural and hence biomechanical role, the ability of the ECM to provide the contextual information responsible for controlling both individual and collective cellular behavior has been recognized in recent years. Basement membrane components also play a dynamic role as a barrier and reservoir of growth factors, which in turn regulate epithelial and mesenchymal cell proliferation and differentiation. Absence or inhibition of the interaction of epithelial cells with the basement membrane has a direct consequence in the failure of normal lung development (73,74). Moreover, proper expression and function of extracellular matrix (ECM) molecules are necessary for branching to occur normally. However, little is known about the role of epithelial cell surface molecules that mediate epithelial-matrix interactions during this process.

Collagens

Collagens are a large family of structural proteins that are widespread throughout the body and are important for a broad range of functions, including tissue scaffolding, cell adhesion, cell migration, cancer, angiogenesis, tissue morphogenesis, and tissue repair. Previous work has shown that treatment with collagenase or inhibitors of procollagen synthesis disrupts lung epithelial branching (75–77). Furthermore, Collagen IV, the predominant collagen in the developing lung, is frequently found to be downregulated in mouse models where lung branching morphogenesis is perturbed (78). Together these data suggest a role for collagens in promoting and maintaining branching morphogenesis, and the direct mechanisms behind this influence are currently an active area of investigation.

Laminins

Laminins (LNs) are a family of extracellular matrix glycoproteins involved in cell adhesion, migration, proliferation, and differentiation during tissue development. LNs are composed of three chains, one central (α) and two lateral (β and γ) that are linked by disulfide bonds to form a cross-shaped molecule (79). To date five α , three β , and three γ chain isoforms have been described, which suggests that their combination can lead to approximately 30 variants of LN (80–85). Mouse embryos with a mutated LN α 5 chain isoform die by E17.5, and their lungs show poor lobe septation and bronchiolar branching, suggesting that this LN α chain is indispensable for lung branching morphogenesis (86–88). Antilaminin antibodies were also shown to inhibit branching morphogenesis of lung explants (89). The mechanisms by which the branching epithelium interacts with the extracellular matrix to bring about this morphogenetic event are still unknown. Several integrin cell surface receptors that bind laminins may mediate the interaction between the matrix molecules and the epithelium to promote lung branching morphogenesis (90)

Integrins

Integrins are heterodimeric cell surface receptors composed of a single α and β peptide subunit

(91). The extracellular domains of integrins interact with the ECM or other cell surface molecules, and some cytoplasmic domains have been shown to interact with the cytoskeleton (92,93). Within an integrin subfamily, a single β subunit is able to form heterodimers with several α integrins (91). The extracellular domain of the α integrin confers the binding specificity for the heterodimer, and the particular biological response to binding is determined by the α subunit cytoplasmic domain (94–96). Upon integrin binding of components of the ECM, signals are transduced that control diverse cell behaviors such as cell adhesion and migration (97). Loss of integrin alpha 3, which primarily associates with laminins, results in reduced branching with the large bronchi extending to the periphery of the lung (78). Loss of integrin beta 1, which primarily associates with collagens, results in branching defects and a loss of proper formation of a single-layered epithelium in the distal branch points of the airway tree (98,99). These results implicate a role for integrin receptors in

basement membrane organization and lung-branching morphogenesis.

Summary and the Future

In this chapter we have given a brief overview of the various genetic factors, signaling molecules, and extracellular matrix components that contribute to branching morphogenesis in the lung. Despite the increased understanding of the molecular processes that regulate branching morphogenesis and early lung development, the physical processes that drive lung branching morphogenesis are just beginning to be elucidated (100). New advancements in imaging techniques, cell lineage specific reporter tools, and development of computational models for branching morphogenesis will allow researchers to explore the dynamic nature of this process in far greater detail than in previous studies (101,102). Together these new avenues of research will help unravel the systems biology of branching morphogenesis at the both the molecular and mechanical levels.

References

- Bourbon J, Boucherat O, Chailley-Heu B, Delacourt C. Control mechanisms of lung alveolar development and their disorders in bronchopulmonary dysplasia. *Pediatric Research*. 2005 May;57(5 Pt 2):38R–46R.
- Pauling MH, Vu TH. Mechanisms and regulation of lung vascular development. *Current Topics in Developmental Biology*. 2004;64:73–99.
- Williams MC. Alveolar type I cells: molecular phenotype and development. *Annual Review of Physiology*. 2003;65:669–695.
- Ten Have-Opbroek AA. Lung development in the mouse embryo. *Experimental Lung Research*. 1991 Mar-Apr;17(2):111–130.
- Morrissey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. *Developmental Cell*. 2010 Jan 19;18(1):8–23.
- Domyan ET, Ferretti E, Throckmorton K, Mishina Y, Nicolis SK, Sun X. Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. *Development*. 2011 Mar;138(5):971–981.
- Kaufman MH. *The atlas of mouse development*. London England: Harcourt Brace & Company; 1992.
- Varner VD, Nelson CM. Cellular and physical mechanisms of branching morphogenesis. *Development*. 2014 Jul;141(14):2750–2759.
- Watson CJ, Khaled WT. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development*. 2008 Mar;135(6):995–1003.
- Metzger RJ, Klein OD, Martin GR, Krasnow MA. The branching programme of mouse lung development. *Nature*. 2008 Jun 5;453(7196):745–750.
- Mund SI, Stampanoni M, Schittny JC. Developmental alveolarization of the mouse lung. *Developmental Dynamics*. 2008 Aug;237(8):2108–2116.
- Maeda Y, Dave V, Whitsett JA. Transcriptional control of lung morphogenesis. *Physiological Reviews*. 2007 Jan;87(1):219–44.
- Besnard V, Wert SE, Hull WM, Whitsett JA. Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. *Gene Expression Patterns*. 2004 Dec;5(2):193–208.
- Zhou L, Lim L, Costa RH, Whitsett JA. Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. *The Journal of Histochemistry and Cytochemistry*. 1996 Oct;44(10):1183–1193.

- 15 Wan H, Dingle S, Xu Y, Besnard V, Kaestner KH, Ang SL, et al. Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis. *The Journal of Biological Chemistry*. 2005 Apr 8;280(14):13809–13816.
- 16 Shu W, Lu MM, Zhang Y, Tucker PW, Zhou D, Morrisey EE. Foxp2 and Foxp1 cooperatively regulate lung and esophagus development. *Development*. 2007 May;134(10):1991–2000.
- 17 Li S, Wang Y, Zhang Y, Lu MM, DeMayo FJ, Dekker JD, et al. Foxp1/4 control epithelial cell fate during lung development and regeneration through regulation of anterior gradient 2. *Development*. 2012 Jul;139(14):2500–2509.
- 18 Kalinichenko VV, Lim L, Stolz DB, Shin B, Rausa FM, Clark J, et al. Defects in pulmonary vasculature and perinatal lung hemorrhage in mice heterozygous null for the Forkhead Box f1 transcription factor. *Developmental Biology*. 2001 Jul 15;235(2):489–506.
- 19 Mahlapuu M, Ormestad M, Enerback S, Carlsson P. The forkhead transcription factor Foxf1 is required for differentiation of extra-embryonic and lateral plate mesoderm. *Development*. 2001 Jan;128(2):155–166.
- 20 Sen P, Yang Y, Navarro C, Silva I, Szafranski P, Kolodziejaska KE, et al. Novel FOXF1 mutations in sporadic and familial cases of alveolar capillary dysplasia with misaligned pulmonary veins imply a role for its DNA binding domain. *Human Mutation*. 2013 Jun;34(6):801–811.
- 21 Miranda J, Rocha G, Soares P, Morgado H, Baptista MJ, Azevedo I, et al. A novel mutation in FOXF1 gene associated with alveolar capillary dysplasia with misalignment of pulmonary veins, intestinal malrotation and annular pancreas. *Neonatology*. 2013;103(4):241–245.
- 22 Sen P, Gerychova R, Janku P, Jezova M, Valaskova I, Navarro C, et al. A familial case of alveolar capillary dysplasia with misalignment of pulmonary veins supports paternal imprinting of FOXF1 in human. *European Journal of Human Genetics*. 2013 Apr;21(4):474–477.
- 23 Krumlauf R. Hox genes in vertebrate development. *Cell*. 1994 Jul 29;78(2):191–201.
- 24 Aubin J, Lemieux M, Tremblay M, Berard J, Jeannotte L. Early postnatal lethality in Hoxa-5 mutant mice is attributable to respiratory tract defects. *Developmental Biology*. 1997 Dec 15;192(2):432–445.
- 25 Herriges JC, Yi L, Hines EA, Harvey JF, Xu G, Gray PA, et al. Genome-scale study of transcription factor expression in the branching mouse lung. *Developmental Dynamics*. 2012 Sep;241(9):1432–1453.
- 26 Boucherat O, Montaron S, Berube-Simard FA, Aubin J, Philippidou P, Wellik DM, et al. Partial functional redundancy between Hoxa5 and Hoxb5 paralog genes during lung morphogenesis. *American Journal of Physiology–Lung Cellular and Molecular Physiology*. 2013 Jun 15;304(12):L817–830.
- 27 Volpe MV, Ramadurai SM, Pham LD, Nielsen HC. Hoxb-5 down regulation alters Tenascin-C, FGF10 and Hoxb gene expression patterns in pseudoglandular period fetal mouse lung. *Frontiers in Bioscience*. 2007;12:860–873.
- 28 Roth-Kleiner M, Hirsch E, Schittny JC. Fetal lungs of tenascin-C-deficient mice grow well, but branch poorly in organ culture. *American Journal of Respiratory Cell and Molecular Biology*. 2004 Mar;30(3):360–366.
- 29 Charron J, Malynn BA, Fisher P, Stewart V, Jeannotte L, Goff SP, et al. Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene. *Genes & Development*. 1992 Dec;6(12A):2248–2257.
- 30 Stanton BR, Perkins AS, Tessarollo L, Sassoon DA, Parada LF. Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes & Development*. 1992 Dec;6(12A):2235–2247.
- 31 Sawai S, Shimono A, Wakamatsu Y, Palmes C, Hanaoka K, Kondoh H. Defects of embryonic organogenesis resulting from targeted disruption of the N-myc gene in the mouse. *Development*. 1993 Apr;117(4):1445–1455.
- 32 Moens CB, Auerbach AB, Conlon RA, Joyner AL, Rossant J. A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. *Genes & Development*. 1992 May;6(5):691–704.
- 33 Okubo T, Knoepfler PS, Eisenman RN, Hogan BL. Nmyc plays an essential role during lung development as a dosage-sensitive regulator of progenitor cell proliferation and differentiation. *Development*. 2005 Mar;132(6):1363–74.
- 34 Serra R, Pelton RW, Moses HL. TGF beta 1 inhibits branching morphogenesis and N-myc expression in lung bud organ cultures. *Development*. 1994 Aug;120(8):2153–2161.
- 35 Lazzaro D, Price M, de Felice M, Di Lauro R. The transcription factor TTF-1 is expressed at the onset of thyroid and lung

- morphogenesis and in restricted regions of the foetal brain. *Development*. 1991 Dec;113(4):1093–1104.
36. Kimura S. Thyroid-specific enhancer-binding protein Role in thyroid function and organogenesis. *Trends in Endocrinology and Metabolism*. 1996 Sep;7(7):247–252.
37. Devriendt K, Vanhole C, Matthijs G, de Zegher F. Deletion of thyroid transcription factor-1 gene in an infant with neonatal thyroid dysfunction and respiratory failure. *The New England Journal of Medicine*. 1998 Apr 30;338(18):1317–1318.
38. DeFelice M, Silberschmidt D, DiLauro R, Xu Y, Wert SE, Weaver TE, et al. TTF-1 phosphorylation is required for peripheral lung morphogenesis, perinatal survival, and tissue-specific gene expression. *The Journal of Biological Chemistry*. 2003 Sep 12;278(37):35574–35583.
39. LeBoeuf M, Terrell A, Trivedi S, Sinha S, Epstein JA, Olson EN, et al. Hdac1 and Hdac2 act redundantly to control p63 and p53 functions in epidermal progenitor cells. *Developmental Cell*. 2010 Dec 14;19(6):807–818.
40. Snitow ME, Li S, Morley MP, Rathi K, Lu MM, Kadzik RS, et al. Ezh2 represses the basal cell lineage during lung endoderm development. *Development*. 2015 Jan 1;142(1):108–117.
41. Hogan BL, Barkauskas CE, Chapman HA, Epstein JA, Jain R, Hsia CC, et al. Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. *Cell Stem Cell*. 2014 Aug 7;15(2):123–138.
42. Dong J, Jiang G, Asmann YW, Tomaszek S, Jen J, Kislinger T, et al. MicroRNA networks in mouse lung organogenesis. *PLoS One*. 2010;5(5):e10854.
43. Lim L, Kalinichenko VV, Whitsett JA, Costa RH. Fusion of lung lobes and vessels in mouse embryos heterozygous for the forkhead box f1 targeted allele. *American Journal of Physiology–Lung Cellular and Molecular Physiology*. 2002 May;282(5):L1012–1022.
44. Williams AE, Moschos SA, Perry MM, Barnes PJ, Lindsay MA. Maternally imprinted microRNAs are differentially expressed during mouse and human lung development. *Developmental Dynamics*. 2007 Feb;236(2):572–580.
45. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkland SJ, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell*. 2008 Mar 7;132(5):875–886.
46. Lu Y, Thomson JM, Wong HY, Hammond SM, Hogan BL. Transgenic over-expression of the microRNA miR-17–92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. *Developmental Biology*. 2007 Oct 15;310(2):442–453.
47. Tian Y, Zhang Y, Hurd L, Hannenhalli S, Liu F, Lu MM, et al. Regulation of lung endoderm progenitor cell behavior by miR302/367. *Development*. 2011 Apr;138(7):1235–1245.
48. Mujahid S, Nielsen HC, Volpe MV. MiR-221 and miR-130a regulate lung airway and vascular development. *PLoS One*. 2013;8(2):e55911.
49. Herriges MJ, Swarr DT, Morley MP, Rathi KS, Peng T, Stewart KM, et al. Long noncoding RNAs are spatially correlated with transcription factors and regulate lung development. *Genes & Development*. 2014 Jun 15;28(12):1363–1379.
50. Alescio T, Cassini A. Induction in vitro of tracheal buds by pulmonary mesenchyme grafted on tracheal epithelium. *The Journal of Experimental Zoology*. 1962 Jul;150:83–94.
51. Shannon JM. Induction of alveolar type II cell differentiation in fetal tracheal epithelium by grafted distal lung mesenchyme. *Developmental Biology*. 1994 Dec;166(2):600–614.
52. Taderera JV. Control of lung differentiation in vitro. *Developmental Biology*. 1967 Nov;16(5):489–512.
53. Warburton D, Bellusci S, De Langhe S, Del Moral PM, Fleury V, Mailleux A, et al. Molecular mechanisms of early lung specification and branching morphogenesis. *Pediatric Research*. 2005 May;57(5 Pt 2):26R–37R.
54. Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, et al. Fgf10 is essential for limb and lung formation. *Nature Genetics*. 1999 Jan;21(1):138–141.
55. De Moerloose L, Spencer-Dene B, Revest JM, Hajihosseini M, Rosewell I, Dickson C. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development*. 2000 Feb;127(3):483–492.
56. Arman E, Haffner-Krausz R, Gorivodsky M, Lonai P. Fgfr2 is required for limb outgrowth and lung-branching morphogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 1999 Oct 12;96(21):11895–11899.
57. Abler LL, Mansour SL, Sun X. Conditional gene inactivation reveals roles for Fgf10 and Fgfr2 in establishing a normal

- pattern of epithelial branching in the mouse lung. *Developmental Dynamics*. 2009 Aug;238(8):1999–2013.
- 58 Colvin JS, White AC, Pratt SJ, Ornitz DM. Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme. *Development*. 2001 Jun;128(11):2095–2106.
- 59 Li C, Hu L, Xiao J, Chen H, Li JT, Bellusci S, et al. Wnt5a regulates Shh and Fgf10 signaling during lung development. *Developmental Biology*. 2005 Nov 1;287(1):86–97.
- 60 Li C, Xiao J, Hormi K, Borok Z, Minoo P. Wnt5a participates in distal lung morphogenesis. *Developmental Biology*. 2002 Aug 1;248(1):68–81.
- 61 Goss AM, Tian Y, Cheng L, Yang J, Zhou D, Cohen ED, et al. Wnt2 signaling is necessary and sufficient to activate the airway smooth muscle program in the lung by regulating myocardin/Mrtf-B and Fgf10 expression. *Developmental Biology*. 2011 Aug 15;356(2):541–552.
- 62 Finch PW, He X, Kelley MJ, Uren A, Schaudies RP, Popescu NC, et al. Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. *Proceedings of the National Academy of Sciences of the United States of America*. 1997 Jun 24;94(13):6770–6775.
- 63 Goss AM, Tian Y, Tsukiyama T, Cohen ED, Zhou D, Lu MM, et al. Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. *Developmental Cell*. 2009 Aug;17(2):290–298.
- 64 Miller MF, Cohen ED, Baggs JE, Lu MM, Hogenesch JB, Morrisey EE. Wnt ligands signal in a cooperative manner to promote foregut organogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2012 Sep 18;109(38):15348–15353.
- 65 Kadzik RS, Cohen ED, Morley MP, Stewart KM, Lu MM, Morrisey EE. Wnt ligand/ Frizzled 2 receptor signaling regulates tube shape and branch-point formation in the lung through control of epithelial cell shape. *Proceedings of the National Academy of Sciences of the United States of America*. 2014 Aug 26;111(34):12444–12449.
- 66 Pepicelli CV, Lewis PM, McMahon AP. Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Current Biology*. 1998 Sep 24;8(19):1083–1086.
- 67 Weaver M, Batts L, Hogan BL. Tissue interactions pattern the mesenchyme of the embryonic mouse lung. *Developmental Biology*. 2003 Jun 1;258(1):169–184.
- 68 Litingtung Y, Lei L, Westphal H, Chiang C. Sonic hedgehog is essential to foregut development. *Nature Genetics*. 1998 Sep;20(1):58–61.
- 69 Bellusci S, Henderson R, Winnier G, Oikawa T, Hogan BL. Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development*. 1996 Jun;122(6):1693–1702.
- 70 Eblaghie MC, Reedy M, Oliver T, Mishina Y, Hogan BL. Evidence that autocrine signaling through Bmpr1a regulates the proliferation, survival and morphogenetic behavior of distal lung epithelial cells. *Developmental Biology*. 2006 Mar 1;291(1):67–82.
- 71 Thibeault DW, Mabry SM, Ekekezie, II, Zhang X, Truog WE. Collagen scaffolding during development and its deformation with chronic lung disease. *Pediatrics*. 2003 Apr;111(4 Pt 1):766–776.
- 72 Wasowicz M, Biczysko W, Marszalek A, Yokoyama S, Nakayama I. Ultrastructural studies on selected elements of the extracellular matrix in the developing rat lung alveolus. *Folia Histochemica et Cytobiologica*. 1998;36(1):3–13.
- 73 Hilfer SR. Morphogenesis of the lung: control of embryonic and fetal branching. *Annual Review of Physiology*. 1996;58:93–113.
- 74 Minoo P, King RJ. Epithelial-mesenchymal interactions in lung development. *Annual Review of Physiology*. 1994;56:13–45.
- 75 Grobstein C, Cohen J. Collagenase: effect on the morphogenesis of embryonic salivary epithelium in vitro. *Science*. 1965 Oct 29;150(3696):626–628.
- 76 Wessells NK, Cohen JH. Effects of collagenase on developing epithelia in vitro: lung, ureteric bud, and pancreas. *Developmental Biology*. 1968 Sep;18(3):294–309.
- 77 Spooner BS, Faubion JM. Collagen involvement in branching morphogenesis of embryonic lung and salivary gland. *Developmental Biology*. 1980 Jun 1;77(1):84–102.
- 78 Kreidberg JA, Donovan MJ, Goldstein SL, Rennke H, Shepherd K, Jones RC, et al. Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development*. 1996 Nov;122(11):3537–3547.
- 79 Timpl R, Brown JC. The laminins. *Matrix Biology*. 1994 Aug;14(4):275–281.
- 80 Ehrig K, Leivo I, Engvall E. Merosin and laminin. Molecular relationship and role in nerve-muscle development. *Annals of the New York Academy of Sciences*. 1990;580:276–280.

- 81 Galliano MF, Aberdam D, Aguzzi A, Ortonne JP, Meneguzzi G. Cloning and complete primary structure of the mouse laminin alpha 3 chain. Distinct expression pattern of the laminin alpha 3A and alpha 3B chain isoforms. *The Journal of Biological Chemistry*. 1995 Sep 15;270(37):21820–21826.
- 82 Koch M, Olson PF, Albus A, Jin W, Hunter DD, Brunken WJ, et al. Characterization and expression of the laminin gamma3 chain: a novel, non-basement membrane-associated, laminin chain. *The Journal of Cell Biology*. 1999 May 3;145(3):605–618.
- 83 Pierce RA, Griffin GL, Mudd MS, Moxley MA, Longmore WJ, Sanes JR, et al. Expression of laminin alpha3, alpha4, and alpha5 chains by alveolar epithelial cells and fibroblasts. *American Journal of Respiratory Cell and Molecular Biology*. 1998 Aug;19(2):237–244.
- 84 Sunada Y, Bernier SM, Kozak CA, Yamada Y, Campbell KP. Deficiency of merosin in dystrophic dy mice and genetic linkage of laminin M chain gene to dy locus. *The Journal of Biological Chemistry*. 1994 May 13;269(19):13729–13732.
- 85 Vuolteenaho R, Nissinen M, Sainio K, Byers M, Eddy R, Hirvonen H, et al. Human laminin M chain (merosin): complete primary structure, chromosomal assignment, and expression of the M and A chain in human fetal tissues. *The Journal of Cell Biology*. 1994 Feb;124(3):381–394.
- 86 Miner JH, Cunningham J, Sanes JR. Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. *The Journal of Cell Biology*. 1998 Dec 14;143(6):1713–1723.
- 87 Miner JH, Lewis RM, Sanes JR. Molecular cloning of a novel laminin chain, alpha 5, and widespread expression in adult mouse tissues. *The Journal of Biological Chemistry*. 1995 Dec 1;270(48):28523–28526.
- 88 Nguyen NM, Miner JH, Pierce RA, Senior RM. Laminin alpha 5 is required for lobar septation and visceral pleural basement membrane formation in the developing mouse lung. *Developmental Biology*. 2002 Jun 15;246(2):231–244.
- 89 Schuger L, O'Shea S, Rheinheimer J, Varani J. Laminin in lung development: effects of anti-laminin antibody in murine lung morphogenesis. *Developmental Biology*. 1990 Jan;137(1):26–32.
- 90 Wu JE, Santoro SA. Differential expression of integrin alpha subunits supports distinct roles during lung branching morphogenesis. *Developmental Dynamics*. 1996 Jun;206(2):169–181.
- 91 Hynes RO. Specificity of cell adhesion in development: the cadherin superfamily. *Current Opinion in Genetics & Development*. 1992 Aug;2(4):621–624.
- 92 Chen YP, O'Toole TE, Leong L, Liu BQ, Diaz-Gonzalez F, Ginsberg MH. Beta 3 integrin-mediated fibrin clot retraction by nucleated cells: differing behavior of alpha IIb beta 3 and alpha v beta 3. *Blood*. 1995 Oct 1;86(7):2606–2615.
- 93 Otey CA, Vasquez GB, Burrige K, Erickson BW. Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain. *The Journal of Biological Chemistry*. 1993 Oct 5;268(28):21193–21197.
- 94 Chan BM, Kassner PD, Schiro JA, Byers HR, Kupper TS, Hemler ME. Distinct cellular functions mediated by different VLA integrin alpha subunit cytoplasmic domains. *Cell*. 1992 Mar 20;68(6):1051–1060.
- 95 Kassner PD, Hemler ME. Interchangeable alpha chain cytoplasmic domains play a positive role in control of cell adhesion mediated by VLA-4, a beta 1 integrin. *The Journal of Experimental Medicine*. 1993 Aug 1;178(2):649–660.
- 96 Kawaguchi S, Hemler ME. Role of the alpha subunit cytoplasmic domain in regulation of adhesive activity mediated by the integrin VLA-2. *The Journal of Biological Chemistry*. 1993 Aug 5;268(22):16279–16285.
- 97 Clark EA, Brugge JS. Integrins and signal transduction pathways: the road taken. *Science*. 1995 Apr 14;268(5208):233–239.
- 98 Chen J, Krasnow MA. Integrin Beta 1 suppresses multilayering of a simple epithelium. *PLoS One*. 2012;7(12):e52886.
- 99 Plosa EJ, Young LR, Gulleman PM, Polosukhin VV, Zaynagetdinov R, Benjamin JT, et al. Epithelial beta1 integrin is required for lung branching morphogenesis and alveolarization. *Development*. 2014 Dec;141(24):4751–4762.
- 100 Nelson CM, Gleghorn JP. Sculpting organs: mechanical regulation of tissue development. *Annual Review of Biomedical Engineering*. 2012;14:129–154.
- 101 Chi X, Michos O, Shakya R, Riccio P, Enomoto H, Licht JD, et al. Ret-dependent cell rearrangements in the Wolffian duct epithelium initiate ureteric bud morphogenesis. *Development Cell*. 2009 Aug;17(2):199–209.
- 102 Schnatwinkel C, Niswander L. Multiparametric image analysis of lung-branching morphogenesis. *Developmental Dynamics*. 2013 Jun;242(6):622–637.

Pulmonary Vascular Development

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Abstract

The lung is the most vascularized organ in the body due to its primary function to perform exchange of respiratory gases for the entire body. This chapter reviews the structure, developmental origins, timing, and patterning of the arterial, capillary, venous, and lymphatic systems in the lung as well as the main pulmonary arteries and veins that connect the heart to the lung. Both the pre- and postnatal stages (phases) of pulmonary vascular morphogenesis are covered. The relative importance of vasculogenesis versus angiogenesis in the initial formation of pulmonary vessels is discussed as well as other potential mechanisms. Current knowledge of cellular and molecular pathways and mechanisms that orchestrate and regulate vascular morphogenesis are discussed, along with the relative contributions of endothelial cells, smooth muscle cells, pericytes, and fibroblasts. The coordinate regulation of the pulmonary vascular system with airway development is critical and is also reviewed. Responses of the pulmonary circulation to injury and the potential for regression and regeneration of the lung vasculature as well as unanswered questions and future directions in pulmonary vascular development are also presented.

Keywords:

Vasculogenesis, angiogenesis, arteriogenesis, lymphangiogenesis, pulmonary vascular development, lung development, lymphatic development, lung vasculature, vascular structure, vascular function

Pulmonary Vascular Structure and Function

Because the primary function of the lungs is to perform exchange of respiratory gases for the entire body, the pulmonary vascular system is the most extensive of any organ due to the large surface area that is needed. Pulmonary circulation is formed by blood ejected from the right side of the heart being delivered to the alveolar capillaries through an extensive system of conducting arteries and arterioles (Figure 3-1). A similarly elaborate venous system returns the oxygenated blood to the left side of the heart. The pulmonary lymphatic system collects and returns interstitial fluid to the circulation and in addition contains an extensive system of lymph nodes. Last, to support the multiple large airways and their branches, there is also a systemic bronchial circulation. This system appears rather later in development (after the ninth week of gestation). An extensive network of vasa vasorum exists in the adventitia of the pulmonary arteries.

The vessels of the pulmonary vasculature are generated during fetal lung development through branching processes. Further development and

remodeling occurs after birth during the postnatal phase of lung morphogenesis and also as the lungs grow throughout childhood to match and support the increase in size of the body. This chapter will focus on the patterning and development of the arterial, capillary, venous, and lymphatic systems in the lung as well as the main pulmonary arteries and veins that connect the heart to the lung.

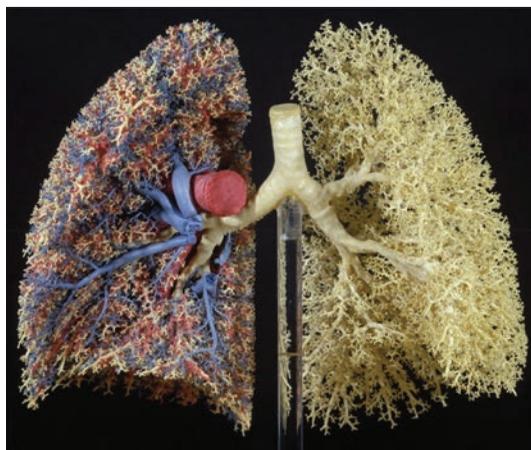


Figure 3-1. Casts of the airway (yellow/white), arterial (red) and venous (blue) systems in the human lung. Courtesy of Prof. Ewald Weibel, Institute of Anatomy, University of Berne.

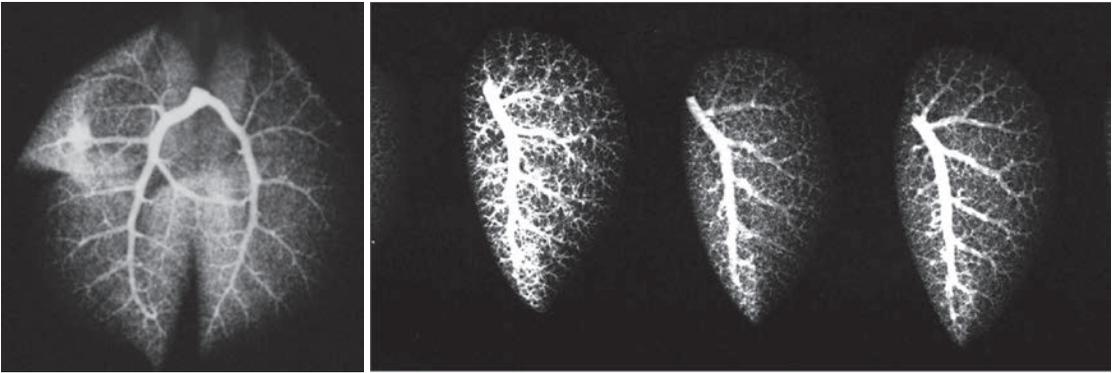


Figure 3-2. Arteriogram of the whole adult mouse lung (left panel). Arteriograms of left lungs from three adult mice (right panel). Barium mixed with gelatin were instilled into the main pulmonary artery and the lungs inflation fixed. X-ray images were then obtained. Provided by T. D. Le Cras.

Current knowledge of cellular and molecular pathways that orchestrate and regulate vascular morphogenesis will be discussed. Coordinate regulation of the pulmonary vascular system with airway development will also be introduced. Detailed information on the stages and regulation of airway morphogenesis is presented in Chapter 2 and will not be repeated here. For a long period of time the main period of pulmonary vascular development was believed to occur principally during the third canalicular stage of lung morphogenesis. As will be discussed later in this chapter, with transgenic mice that utilize endothelial specific markers (1) vascular morphogenesis actually starts much earlier in the embryonic phase as the lung buds develop from the foregut endoderm.

Before discussing pulmonary vascular development, it is important to understand the structure of the mature vascular system that is generated. The pulmonary arterial system in the adult lung is highly extensive and extremely complex and for the most part mirrors the airway system (Figure 3-1). The venous and lymphatic systems are similarly complex but have different patterning and locations within the lung. While the airways conduct air in and out through the same system, the vascular system conducts blood in and out through different systems with the arterial system bringing deoxygenated blood from the right side of the heart to the alveolar capillary surface and the venous system returning oxygenated blood to the left. Like the airways, the vascular systems are highly stereotyped and have similar branching patterns from individual to

individual, indicating that regulation of the patterning of these systems is robust and reproducible (Figure 3-2). The development of the pulmonary vascular system is closely coordinated with the airways. Genes and molecular pathways that play a role in airway epithelial to mesenchymal signaling are discussed later in this chapter as each of the vascular systems is reviewed. The geometry of the airway and vascular systems are tree-like in structure (Figures 3-1 and 3-2) and are proposed to be fractal in nature, so their generation is likely governed by simple recursive rules, probably involves simple iterative processes, and possibly involves the interaction of only a few pathways and genes/proteins (2).

In addition to the complex three-dimensional branching structure of the pulmonary circulation, the caliber and vessel wall structure varies considerably depending on the size, location, and to which system the vessel belongs. The tapering caliber and branching pattern of the arterial system in the adult mouse lung is shown in Figure 3-2. The diameter of the pulmonary arteries varies considerably from proximal to distal arteries. The cellular and molecular structure of the vessel wall as well as the vessel patterning must be carefully regulated so that hemodynamic forces are accommodated, especially because large changes in hemodynamics occur with exercise. In addition to regulation of branching patterns of the vascular and airway systems and the structural changes in the walls of these systems, the developing lung must fit within the constraints of the chest and be connected to the heart and upper respiratory tract, respectively.

Development of the Pulmonary Arteries

Structure, Origins, Timing, and Patterning

It is widely accepted that the airways act as a template for vascular morphogenesis in the lung, especially as the developing arteries align with the conducting airways as they undergo branching morphogenesis. The timing and naming of the stages of lung morphogenesis relied heavily on the histologic appearance of the developing airways. Hislop (3) developed a matching scheme outlining the timing and stages of pulmonary vascular development relative to airway morphogenesis (Figure 3-3). The arteries that run alongside the conducting airways are referred to as conventional arteries and extend out to the respiratory bronchioles and alveolar ducts (3). Supernumerary arteries arise from the conventional arteries and lead in a direction away from the conducting airways to supply the alveolar regions (Figure 3-4). The main (extra) pulmonary arteries and veins that connect the heart to the pulmonary circulation were originally thought to develop by angiogenesis, sprouting off the heart and growing into the developing lungs (4). However, there is also evidence that they may form by vasculogenesis, as had already been reported for the aorta (5). Further discussion of this occurs later in the chapter.

Two major processes are believed to account for the development of pulmonary vessels. One is

vasculogenesis, the de novo formation of vessels by differentiation of mesoderm progenitor cells into endothelial cells. Expression of specific genes, including *VEGFR2* (Flk-1), *CD31* (PECAM), and *Sox-17* (see later), defines these cells as well as their location and the structures they form. Studies suggest that early/primitive endothelial cells come together to form primitive tubes or sinusoids. The second process by which vessels are formed is called angiogenesis. In this process new vessels are formed by branching or sprouting from a preexisting vessel. Angiogenesis still occurs in adult organs (although vasculogenesis is not believed to occur) and is one of the key processes that permits tumors to grow. However, it is uncertain if vasculogenesis occurs outside the periods of organogenesis. The relative contribution of these two processes to pulmonary vascular development and the timing has been the cause of some debate. Early attempts to answer these questions in the mouse lung were approached using a variety of injection techniques to generate vascular casts and angiograms as well as histology (4). DeMello and colleagues (4) suggested that vasculogenesis occurs in the peripheral lung to form blood lakes and that angiogenesis is the primary process in the proximal lung that forms the main arteries and veins. They also reported that branching of the airways and vessels is relatively synchronized, although branching of the vasculature is slightly behind the airways. Using their injection techniques, they did not detect fusion between the developing central and peripheral

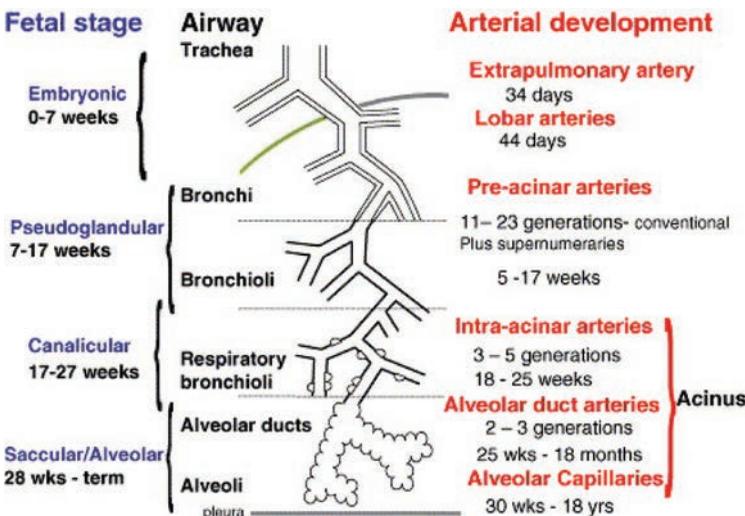


Figure 3-3. Stages and timing of airway and arterial development in humans. Reprinted with permission from A. Hislop, *Paediatric Respiratory Reviews*, 2005;6(1):35-43.



Figure 3-4. Casts of the airway (yellow/white) & arterial (red) systems in the human lung shows close coordination between airway and arterial structure. Arrows indicate supernumerary pulmonary arteries coming off the main conventional arteries and supplying the alveolar regions. Courtesy of Prof. Ewald Weibel, Institute of Anatomy, University of Berne.

vascular systems until E13–14 in the mouse, and only by E17 was an extensive network present. The concept of peripheral vasculogenesis generating the distal vessels and central angiogenesis the main vessels developed from this report (6).

The identification of genes and molecular markers of early endothelial cells enabled transgenic reporter mice to be developed to facilitate the characterization of pulmonary vascular development. The VEGF receptor 2 (VEGFR2) also known as fetal liver kinase1 (Flk) is an early marker of endothelial cells and is required for endothelial cell differentiation and vasculogenesis. Schachtner and colleagues (1) used transgenic mice with the β -galactosidase gene (LacZ) knocked into the Flk gene locus to identify newly differentiating endothelial cells in the embryonic mouse lung. Their analysis started at E10.5, soon after the lung buds appear, and then continued

the analysis through pre- and postnatal development and into the adult lung. Their study demonstrated that vascular development occurs at the earliest stages of lung development, in the embryonic phase as the lung buds first develop. A primitive vascular plexus or “halo” of differentiating endothelial cells formed around each airway bud as it emerged into the underlying splanchnic mesenchyme. In addition to the primitive vascular plexus forming around the airway buds, they also showed that the main pulmonary artery was already forming at E10.5 and had at least a partial lumen. This was much earlier than previously thought, and the developing pulmonary artery was already contiguous with the aortic sac of the heart at E10.5. Because differentiating endothelial cells forming the pulmonary artery could already be seen prior to the formation of a complete lumen, Schachtner and colleagues (1) further suggested that vasculogenesis is the principal mechanism by which proximal arteries are formed, rather than angiogenesis, similar to the aorta that also forms initially by vasculogenesis.

The study by Schachtner et al. (1) highlighted three important concepts in pulmonary vascular development: First, vasculogenesis is the initial process giving rise to the main pulmonary artery that connects the heart to the lung. Second, vascular development in the lung occurs early as the lung bud emerges and so is already well underway by the canalicular stage of lung morphogenesis. Third, pulmonary vascular development continues through all stages of lung morphogenesis. However, the relative contribution of vasculogenesis versus angiogenesis to pulmonary vascular development is still not conclusively resolved, and a third mechanism of new vessel formation has also been proposed: Intussusceptive angiogenesis is a process by which new vessels are generated from existing vessels through the formation of tissue pillars that divide or split the vessel (7). The role of this process in pulmonary vascular development is unclear.

As lung morphogenesis proceeds from E10.5–13.5 in the mouse and the lung buds continue to branch into the splanchnic mesenchyme, extensive endothelial plexuses form around each epithelial bud, including apparent lumens that contain red blood cells (1) (Figure 3-5). Extension of the vascular plexus continues from the embryonic phase through the end of the pseudoglandular phase. Further analysis of Flk-LacZ mice

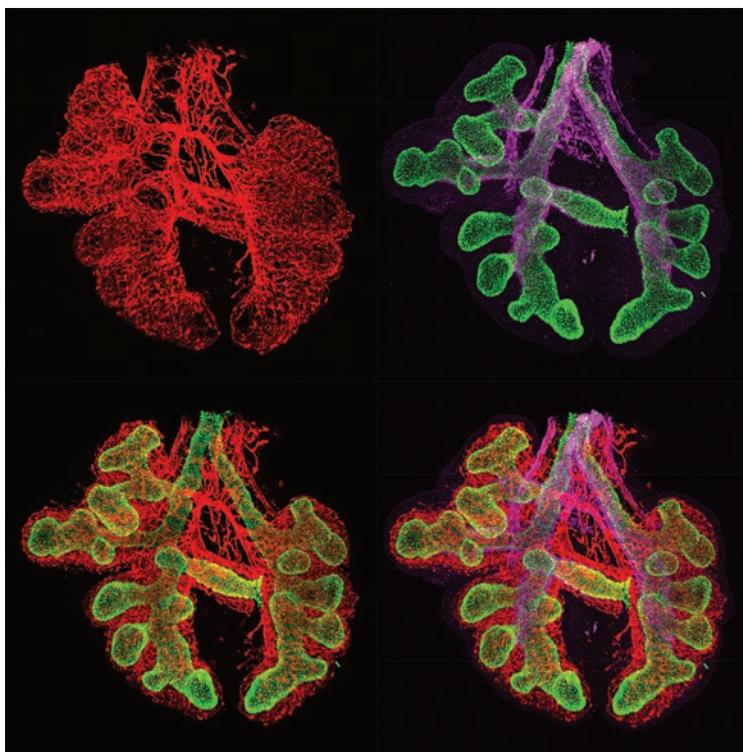


Figure 3-5. Immunofluorescent staining of fetal mouse lung embryonic day 12.5 (E12.5) showing epithelial cells in green (TTF-1/NKX2.1), endothelial cells in red (endomucin), and smooth muscle cells in purple (smooth muscle α -actin). Provided by J. M. Shannon.

showed that the distal vascular plexus expands and refines so that by E12.5 and E13.5, an extensive system of channels has formed a few cell layers away from the epithelial tubes (1).

Immunostaining for Sox-17, one of the Sox family of transcription factors, also suggests that the process of vasculogenesis plays an important role in the distal lung mesenchyme. Sox-17 marks the differentiation of primitive cells in the mesenchyme into endothelial cells at E12.5 that have not yet formed a lumen (Figure 3-6). In addition, Sox17 expression is also in endothelial cells that have formed primitive tubes and contain red blood cells. In Figure 3-6, showing further immunostaining for Sox17, endothelial cells align with and form vessels with lumens around the developing airways. From E12.5–15.5 the vascular network becomes increasingly more complex and organized with a circulation already present, including veins. By the end of the pseudoglandular phase, all the preacinar arteries and veins have formed (3). The saccular and alveolar phases of lung morphogenesis are characterized by extensive changes in the terminal airways to give rise to alveolar ducts and alveoli, the gas exchange structures of the lung. In parallel there is extensive development of a capillary bed in the distal lung

and around the alveoli. Once again this was beautifully shown in the Flk-LacZ mice (1). These later stages are also characterized by thinning of the mesenchyme and epithelial layers to create the thin interface between distal airways and the capillary system.

After formation of a primary vascular plexus, the patterning of the arteries, veins, and lymphatics is generally thought to occur in a proximal to distal fashion. One of the best examples of this is the recruitment of smooth muscle around the arteries (and airways) first proximally but then extending more distally as lung development proceeds. These developing vessels also remodel, laying down a vital extracellular matrix in very coordinated layers and with an exact composition. This secondary stage in vascular morphogenesis is vital, as if it is not done correctly, vascular leak and/or hemorrhage can result. Depending on the location and diameter of the artery, the variable thickness, and composition of the arterial wall, and there may be one or more layers of smooth muscle cells (8). Another important cell type in the vessel wall is the fibroblast. Fibroblasts are derived from mesenchymal cells and can be abundant in the outer walls of vessels, especially proximal arteries, where they form the so-called

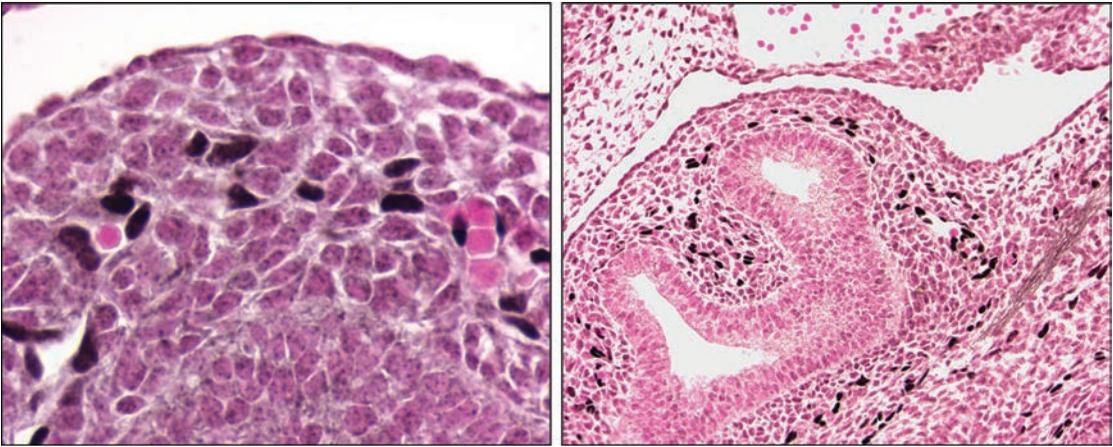


Figure 3-6. Immunostaining shows differentiation of mesenchymal cells into Sox-17 positive endothelial cells (black) in the mouse lung at embryonic day 12.5 (E12.5). Left panel shows a high-power picture of Sox-17 positive cells (black nuclei) where no patent vessel appears to be present, as well as Sox-17 positive cells, where small vessels contain red blood cells. Right panel shows a lower-power image of E12.5 mouse lung with the location of Sox-17 positive cells (black) in the mesenchyme around the developing airway in the center. Provided by J. A. Whitsett, S. Wert, and T. D. Le Cras.

adventitial layer along with extracellular matrix. The relative number of cells and thickness of the smooth muscle (medial layer; and fibroblasts (adventitial layer) varies considerably between proximal and distal vessels. At the level of the capillaries, most studies suggest that pericytes are the primary support cells.

Recently a study by Peng et al. (5) examined the origins and development of the pulmonary arteries and veins that connect the lung to the heart (Figure 3-7). They examined development of these major vessels in a lung agenesis model created by deletion of β -catenin from the anterior foregut (AFG) endoderm. They showed that the pulmonary arteries and veins still develop in the absence of lung development and intersect in the region where the lung normally forms. In β -catenin knockout mice, the main pulmonary arteries and veins persisted through embryonic development, although they did not branch or develop further. What is especially important about this study is that Peng and colleagues used lineage tracing and conditional gene deletion experiments to identify a common population of cardiopulmonary progenitor (CPP) cells that give rise to the mesenchymal cells of the cardiac inflow tract and the lung, including proximal endothelial cells, vascular smooth muscle cells, and pericyte-like cells as well as airway smooth muscle (Figure 3-7). These findings are consistent with suggestions by Hall et al. (9) that airway and vascular smooth muscle cells

have a common origin. Peng et al. (5) further suggested that endothelial cells in the distal capillaries (in the alveolar regions) are not derived from CPP cells, but from a different population of mesenchymal progenitor cells that express VE-cadherin. When it comes to alveolar development, there is ample evidence that it is driven by the architecture of the capillaries as reviewed in (10).

Cellular and Molecular Mechanisms

The developing airways are thought to play a major role in the regulation of pulmonary vascular morphogenesis. As mentioned earlier, pulmonary arteries closely follow the conducting airways, and the lung epithelium is a source of important paracrine factors, including sonic hedgehog and vascular endothelial growth factor (VEGF; see following), whose cognate receptors are located in the mesenchyme. In the following sections we will discuss factors that are known to regulate development of the pulmonary arterial system. Many more genes/factors have been identified as regulating vascular development in other organs or animal models that do not participate in lung development. In addition, mice in which many of the genes for these vascular factors have been inactivated die prior to or during the early stages of lung morphogenesis, and so the specific role that these factors play in pulmonary vascular development remains unclear.

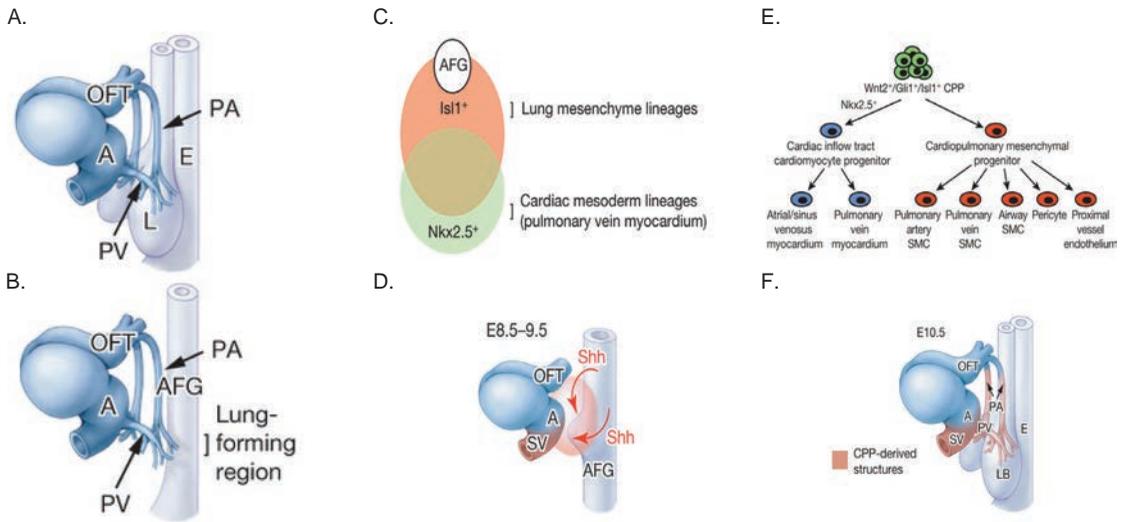


Figure 3-7. Panel A: The schematic shows the large pulmonary arteries (PA) and pulmonary veins (PV) in relation to the developing esophagus (E), outflow tract (OFT), and atrium (AT) of the heart and the lung (L) at E10.5 in the mouse. Panel B: The PA and PV still develop in *Shh^{Cre} Ctnnb1^{flox/flox}* mutants, despite the absence of lung specification. Panel C: Schematic depicts lung and cardiac mesodermal lineages, overlap, and molecular markers (Isl1 and Nkx2.5, respectively) in relation to the anterior foregut (AFG). Panel D: Schematic shows how sonic hedgehog (Shh) produced by the AFG endoderm stimulates cardiopulmonary mesoderm progenitor cells (orange zone) to form the pulmonary artery (PA) and pulmonary veins (PV). Panel E: The schematic shows how the different lung mesenchymal cell populations develop from cardiopulmonary mesoderm progenitor (CPP) cells. Panel F: Schematic shows the vascular structures formed from cardiopulmonary progenitor (CPP) cells. LB, lung bud; E esophagus; A, atrium; OFT, outflow tract; SV, sinus venosus. Reprinted with permission from Peng et al. *Nature*. 2013;7464(5):589–592.

Sonic Hedgehog and Patched: Sonic hedgehog (Shh) has a critical role in early patterning of the lung and branching morphogenesis. Shh is expressed at high levels in the distal tips of the epithelium. The receptor for Shh is patched (ptc), and it is expressed at high levels in the lung mesenchyme. Overexpression of Shh in the distal epithelium increased proliferation of mesenchymal and epithelial cells and resulted in an abundance of mesenchyme (11). These and other studies (12) show that Shh plays an important role in regulating mesenchymal and epithelial cell populations and patterning of the lung. However, whether Shh directly induces angioblasts and or endothelial cell differentiation or patterning of the vascular network in the lung mesenchyme is unclear. In the study by Peng et al. (5), an important role for Shh was identified in development of the main pulmonary arteries and veins that connect the heart to the lung. The vascular plexus was disorganized and also failed to connect to the heart in *Shh*^{-/-} mice. Shh is expressed in the anterior foregut (AFG) adjacent to the cardiac inflow tract and at the time that pulmonary vascular development is first initiated. Gli is a downstream effector of hedgehog signaling and is

expressed in CPP cells along with Wnt2 and Isl1. Inactivation of smoothened (Smo), which is important for Shh signaling in CPP cells, also disrupted pulmonary vascular plexus formation. Hence, this important study (5) suggests that Shh from the AFG signals to CPP cells to promote formation of the vascular plexus and also connections of the pulmonary arteries and veins to the outflow and inflow tracts of the heart.

Fibroblast Growth Factors: Fibroblast growth factors (FGFs) are important for induction of the primitive mesoderm in the early embryo. In *Xenopus* embryos, which lack FGF receptor-1 activity, vasculogenesis is inhibited, and endothelial cells are not induced (13). In the quail embryo, FGF-2 induces the formation of angioblasts, vascular patterning, and expression of the VEGFR2 receptor, Flk (14,15). FGF-2 may play an important role in the induction of angioblasts, which are subsequently responsive to VEGF. FGF-2 is expressed in epithelial and mesenchymal cells of lungs from midtrimester human fetuses (16). Although FGF-1, -2, -7, -9, -10, and -18 are expressed in the developing lung [reviewed in (17)], the role of these FGFs, and especially FGF2, in regulating angioblast induction in the

splanchnic mesenchyme and pulmonary vascular development remains unclear. Cardiac mesoderm may be important for patterning the foregut endoderm from which the lung buds arise, potentially through FGF-1 and -2 (18). Whether cardiac mesoderm induces angioblasts in the splanchnic mesoderm via FGF2, and so initiates pulmonary vascular morphogenesis, is unclear.

Vascular Endothelial Growth Factors:

Vascular endothelial growth factors (VEGF) are a family of factors that contribute to vessel development and function in a number of organs (19, 20). VEGF-A stimulates vasculogenesis and angiogenesis through its stimulatory effects on the differentiation and growth of endothelial cells. VEGF gene ablation in mice causes severe cardiovascular abnormalities and is embryonic lethal. Even heterozygous mice die at E10.5, with abnormal spatial organization of vessels and impaired differentiation of endothelial cells. Hence, even a moderate deficiency in VEGF may be detrimental to lung development and function. VEGF acts through two receptors, VEGFR1 (flt-1) and VEGFR2 (Flk or KDR in humans). The VEGFR2 is an early marker of angioblasts and is vital for both promitotic and pro-survival effects of VEGF on endothelial cells. VEGF plays an important role in the differentiation of angioblasts into endothelial cells (21). There are several isoforms of VEGF that are generated by alternative splicing from a single gene (22). Differential expression of these isoforms reflects distinct functions during vascular development (23). The larger isoforms, including VEGF 206, 188 and 164, bind to heparin proteoglycans and extracellular matrix and are not as freely diffusible as the smaller VEGF 120 isoform. VEGF 164 has intermediate properties as it can diffuse as well as bind to heparin proteoglycans (20). During the early pseudoglandular stage of lung morphogenesis in mice, VEGF expression is diffuse and present in both mesenchyme and epithelial compartments (24). As development proceeds, VEGF expression becomes increasingly restricted to the epithelium, and by E13-15 VEGF expression is most intense in the tips or leading edges of the branching airways, where it stimulates vascular morphogenesis in the surrounding mesenchyme (24).

In the sacular phase (E18 onward) and postnatally, VEGF expression becomes restricted to the distal lung epithelium and specifically alveolar type II cells (23,24). Analysis of the different

isoforms of VEGF-A show that expression levels of VEGF 164 and 120 are relatively constant throughout pre- and postnatal phases of lung morphogenesis. VEGF 188, however, has low expression early and then increases from E13 through birth, remaining high after birth even into adulthood (23). The relative importance of the larger VEGF isoforms has been explored in mice in which VEGF 188 and 164 were deleted leaving only VEGF 120 (20,25). VEGF 120 deficient mice developed to term, but died at birth with abnormalities in pulmonary vascular development (23,25). The role of VEGF after birth was studied by Gerber and colleagues (26) by conditionally deleting the VEGF-A gene and treating newborn mice with a soluble VEGF receptor. In both cases, the mice failed to thrive, and morphogenesis in many organs, including the lung, was disrupted. Inhibition of VEGF receptor signaling in newborn rats also disrupted postnatal vascular and alveolar formation, underscoring the interdependency between vascular and airway development, at least in the final postnatal phase of lung development (27). It is apparent that strict control of VEGF levels in the lung is crucial for normal vascular development and function. Although loss-of-VEGF function is highly detrimental, prenatal overexpression of VEGF-A₁₆₄ also disrupted lung morphogenesis (28,29), and postnatally elevated VEGF-A₁₆₄ levels caused pulmonary vascular leak and alveolar hemorrhage (30). VEGF-D is also expressed in the developing lung and the distinctive patterns of VEGF-A and -D expression have been suggested to represent the unique functions that these different forms of VEGF play in lung development (31). VEGF-D and -C bind to VEGFR-3 and play an important role in lymphatic development.

Epidermal Growth Factor Receptor and

Ligands: The epidermal growth factor (EGF) receptor (EGFR) and its ligands, transforming growth factor- α (TGF- α), EGF, heparin-binding EGF, and amphiregulin, contribute to epithelial development, maturation, and repair. There are conflicting reports on the roles of EGFR ligands in vascular development. In the hamster cheek pouch assay, TGF- α and EGF were found to be potent proangiogenic factors (32). However, in the avian embryo, TGF- α and EGF reduced the hemangiopoietic potential and endothelial cell migration from the splanchnopleural mesoderm (33). In the lung, overexpression of TGF- α and

activation of EGFR signaling disrupt airway and vascular morphogenesis prenatally and postnatally (34–36). Lung development in TGF- α null mice appears normal, and interestingly, the mice are protected from the harmful effects of agents like bleomycin that induce pulmonary fibrosis (37). Knockout of the EGF and EGFR genes in mice is fetal lethal early in development, so their role in pulmonary vascular development remains elusive.

Angiopoietins and Tie Receptors: Tie1 and Tie2 (Tek) belong to a family of tyrosine kinase receptors that are specifically expressed by endothelial cells, including early in vascular development (38). The ligands for Tie receptors are the angiopoietins (Ang) and can have similar structures but also different and opposing effects on vascular development. Although angiopoietins can stimulate angiogenesis *in vitro*, most *in vivo* studies indicate that the angiopoietins and the Tie receptor system play more of a role in the secondary stages of vessel formation, that is, vessel assembly, remodeling, and maturation. The role of the angiopoietins and Tie receptors in lung development is unclear because gene deletion causes embryonic lethality at E10.5–12.5, during the early stages of lung development. Expression of Ang1 is altered in pulmonary diseases, including upregulation in patients with pulmonary hypertension (PH) and may play a role in the disease pathogenesis because increases in Ang1 in an animal model caused PH (39,40). Ang 1 is downregulated in infants with congenital diaphragmatic hernia (CDH), consistent with the pulmonary hypoplasia in these patients (41). However the role of the Ang/Tie system in pulmonary vascular morphogenesis remains uncertain.

Notch System: The Notch system regulates cell fate decisions in all three germ layers and plays important roles in neural, retinal, limb, heart, and vascular morphogenesis, as well as hematopoiesis (42). Four notch receptors have been identified, and ligands include Jagged 1–2 and Delta-like 1, 3, and 4. Deletion studies show that although the Notch system and its downstream signaling molecules are not necessary for vasculogenesis, they are essential for correct remodeling of the early vascular plexus. In mice loss of Notch 1 and 4, or Jagged 1 results in severely disorganized vasculature and is lethal at E9.5–10.5 (43, 44). Studies in zebrafish have shown that the Notch system is also involved in arterial–venous specification (45, 46). In early development the Notch receptors and ligands are expressed throughout the

developing vascular system, but later expression becomes restricted to the arterial system. In zebrafish a hierarchical system has been determined in which Shh regulates VEGF-A and VEGF-A regulates Notch. Loss of gridlock, which is downstream of Notch signaling, caused loss of the arterial markers such as ephrinB2 and increased expression of the ephrin receptor EphB4, a venous marker. In mammals, arterial specification may be regulated by VEGF-A acting through Notch and ephrins and Hey1 and 2 that are downstream of Notch (47). Deletion of Hey 1 and 2 as well as Notch 1 in mice blocked vascular remodeling and caused loss of arterial markers, including ephrin B2 (48). Notch 3 plays an important role in arterial differentiation and maturation of vascular smooth muscle cells (49). Notch 1, 2, and 3 as well as Jagged-1 and -2 are expressed in the developing lung (50). Notch-1 and Jagged-1 and -2 are expressed in both the epithelial and mesenchymal compartments at E14. Although inhibition of Notch-1 expression in fetal lung explant cultures, but not -2 or -3, increased epithelial branching, effects on vascular morphogenesis were not reported. In the lung Notch-2 is regulated by one of the Foxhead box (Fox) transcription factors, Foxf1 (51). The role of Notch signaling in pulmonary vascular morphogenesis remains poorly understood.

Transforming Growth Factor- β , endoglin, Activin Receptor Kinase, and Bone Morphogenic Proteins (BMPs): The transforming growth factor- β (TGF- β) superfamily includes the three isoforms of TGF- β , activins, inhibins, growth and differentiation factors as well as the bone morphogenic proteins (BMPs). Heteromeric complexes of two types of serine/threonine transmembrane kinase receptors bind TGF- β superfamily members and activate downstream Smad proteins. TGF- β plays a vital role in vasculogenesis and angiogenesis, as well as differentiation and maturation of new vessels (52, 53). Activated TGF- β is released by endothelial cells on contact with pericytes. TGF- β inhibits endothelial proliferation and stimulates recruitment and differentiation of mural cells, such as pericytes and smooth muscle cells. TGF- β also stimulates deposition of extracellular matrix that is critical for the structure of larger vessels. Endoglin is an auxiliary receptor that interacts with type II receptors for TGF- β and activin. Loss of endoglin in mice causes a similar phenotype to loss of

TGF- β , suggesting that endoglin is necessary for TGF- β signaling during vascular development (54). Endoglin expression is seen very early in endothelial cells throughout the developing embryo and is strongly expressed in lung mesenchyme (55). Expression of endoglin and Alk1 are similar, and Alk-1, one of the seven type I TGF- β receptors, is expressed by endothelial cells and is critical for the effects of TGF- β on vascular development (56). Alk-1 null mice die in midgestation from vascular defects characterized by excessive fusion of the capillary plexus into cavernous vessels, hyperdilation of larger vessels, and defective recruitment and differentiation of smooth muscle cells (56,57). The role of TGF- β in vascular development may be more complex than was initially appreciated. In proximal tubular cells from the kidney, TGF- β regulates expression of VEGF-A, and angiostatic factors, thrombospondin-1 (TSP-1), and the soluble VEGF receptor, sFlt-1 (58).

BMPs play important roles in vascular development, and BMP receptors are expressed by endothelial and smooth muscle cells. BMP-2/4, BMP-5/7, and Smad1 and-5 deficient mice have enlarged vessels and reduced numbers of smooth muscle cells (59). BMP6 is expressed by endothelial cells and smooth muscle cells and stimulates endothelial cell migration and tube formation through induction of Id1 a helix-loop-helix transcription factor that can repress expression of thrombospondin-1 (TSP-1) that is a potent inhibitor of angiogenesis (60). In the lung most studies have focused on BMP4, as it plays an important role in epithelial-mesenchymal interactions [reviewed in (17)]. Early expression of BMP4 is in the splanchnic mesoderm near the location where the lung buds arise from the foregut endoderm, and then later BMP4 expression is in the proximal mesenchyme and distal epithelial tips. However, whether BMP4 directly regulates pulmonary vascular morphogenesis is unclear. Mutations in the BMPRII receptor gene have been linked to pulmonary vascular diseases such as familial pulmonary arterial hypertension (FPAH), principally seen in adults (61). Interactions between BMPs and the Notch system have been reported, in that BMPs blocked the inhibitory effects of Notch signaling on endothelial cell migration (62). BMPs signal in pulmonary arterial endothelial cells and smooth muscle cells by activating downstream effectors of the Wnt

signaling pathway. In endothelial cells (63), BMPs activate β -catenin and PPAR γ to form a complex that regulates downstream genes in endothelial cells such as apelin that are important in angiogenesis (64). In smooth muscle cells, BMPs transiently activate β -catenin to regulate downstream genes that influence migration (65).

Wnts: Wnts are secreted growth factors that regulate important cell processes, including proliferation, polarity, differentiation, and angiogenesis (66). The Wnt family comprise 18 or more ligands that signal through 10 known frizzled receptors (Fz). Wnt-ligand binding activates multiple intracellular pathways including the β -catenin/LEF-TCF pathway. Wnt-5a, -7a, and -10b are expressed by endothelial cells in vitro, and Wnt-5a by vascular smooth muscle cells (67). Wnt-2 is important for placental vascular development, as fetal-derived placental capillaries were reduced in Wnt-2 $-/-$ mice, resulting in high perinatal mortality and growth restriction of surviving pups (68). The role of Wnts in pulmonary vascular morphogenesis is not well understood. However, Wnt7b is expressed at high levels in the epithelium of distal airways, and inactivation of the Wnt7b gene in mice resulted in perinatal death likely from the lung hypoplasia and pulmonary hemorrhage seen in these animals (69). Examination of lung histology showed that the smooth muscle of the large vessels was hypertrophic and apoptotic, which likely accounted for the vessel rupture and pulmonary hemorrhage. Wnt7b is required for expression of extracellular matrix protein tenascin-C (TN-C) that in turn can regulate pdgfr- α and pdgfr- β expression and proliferation of smooth muscle precursor cells (70).

Platelet Derived Growth Factors: PDGF-A and -B are involved in vessel remodeling and maturation; however, whether PDGF-A regulates angiogenesis is unclear (71, 72). PDGF-B and its receptor PDGF- β can be simultaneously expressed by endothelial cells, suggesting an autocrine role in angiogenesis. Endothelial cells in large vessels express PDGF-B but not the PDGF- β receptor, suggesting a paracrine role, but capillary endothelial cells respond to PDGF-B by increasing capillary density. Interestingly, one study showed that VEGF-A induces VEGFR2-positive embryonic stem cells to differentiate into endothelial cells, whereas PDGF-BB caused VEGFR2-positive embryonic stem cells to differentiate into pericytes and vascular smooth muscle

cells (73). This study addresses an important concept, namely that different growth factors can stimulate the differentiation of potentially a common pool of vascular progenitor cells into different lineages that will form endothelial cells and mural support cells like smooth muscle cells, fibroblasts, and pericytes. Hence, PDGFs are likely important in recruitment and differentiation of mural cells that are critical for vascular structure and function. Whether PDGFs play a similar role in stimulating initial development of vessels in the lungs is unclear, although there are studies examining the role of PDGFs in pulmonary vascular diseases, such as PAH (74).

Insulin-like Growth Factors: The insulin-like growth factor (IGF) system is a complex system that includes two ligands (IGF-1 and IGF-2), two receptors (IGFR1 and IGFR2), at least six IGF binding proteins (IGFBP-1 to-6) and proteases that regulate IGF activity (75). IGF signaling regulates cell proliferation and differentiation and whether cells undergo mitosis or apoptosis. IGF signaling can regulate embryonic development as well as postnatal growth, body size, and longevity. IGFs play an important role in development of a number of organs (75). IGF-1 stimulates proliferation of endothelial cells in vitro, and IGF-1/II can stimulate angiogenesis in the retina. Expression of IGF-I and IGF-II and IGFR-1 receptor have been reported in airway epithelial cells and smooth muscle cells adjacent to the airways, as well as endothelial cells lining the primary vascular plexus of 4- to 12-week-old human lung tissue (76). Treatment of human fetal lung explants with an IGFR-1 blocking antibody increased apoptosis in mesenchymal cells, particularly endothelial cells, and disrupted epithelial branching. Treatment of fetal rat lung explants with the IGFR-1 antibody caused loss of blood islands in the mesenchyme. Hence, there is evidence to support a role for IGFs, acting through IGFR-1, in pulmonary vascular morphogenesis.

Ephrins: Ephrins and Eph receptors are widely expressed throughout the developing embryo and play important roles in vascular development (77). In the ephrin/Eph receptor system, both ligand and receptor must be membrane bound, so that ephrin ligands can cluster and activate receptor signaling. As a result ephrin/Eph signaling interactions are mostly restricted to neighboring cells and important in local cell-to-cell interactions rather than long-range signaling.

Ephrin-B1 and -B2 can promote capillary-like assembly of cultured endothelial cells and angiogenic sprouting. Genetic ablation of ephrin-B2 in embryonic mice resulted in failure of the primary capillary network to remodel and so disrupted vascular development (78). The ephrin/Eph system likely has a role in arterial versus venous specification because ephrin-B2 is expressed in embryonic arteries but not veins, whereas its receptor, EphB4, is expressed in veins but not arteries (79). However, ephrin-B1 is expressed in arterial and venous endothelial cells and EphB3 in veins and some arteries. Ephrin-B2 expression persists in adult arteries and also extends into microvessels, raising into question the concept that capillaries have neither arterial nor venous identity (80). Ephrin-B2 and EphB2 are expressed by mesenchymal cells adjacent to endothelial cells, suggesting that ephrins might play a role in endothelial-mesenchymal interactions (79). Ephrin-B2 is also expressed in the smooth muscle of arteries as well as endothelial cells, but not in venous smooth muscle and indicates a major difference between these smooth muscle beds (80). Expression of ephrin-B2 in arterial smooth muscle cells after endothelial cells suggests that a signal from arterial endothelial cells may be inducing expression of ephrin-B2 in vascular smooth muscle cells. Ephrin-A1, -B1, and -B2 and EphB4 are expressed in capillary endothelial cells from adult mouse lungs, but the role of ephrin/Eph in pulmonary vascular morphogenesis remains unclear.

Angiostatic Factors: A number of naturally produced factors that can inhibit angiogenesis have been identified (81), including angiostatin, endostatin, TSP-1, pigment epithelial derived factor (PEDF), soluble Flt-1, endothelial-monocyte activating polypeptide (EMAPII), and fibulin-5. Angiostatin and endostatin are not produced as angiostatic factors de novo, but are proteolytic fragments of plasminogen and type XVIII collagen. In contrast, TSP-1, and PEDF are produced directly from distinct genes, and hence their angiostatic activity appears to be one of their primary functions. Although there is evidence that these factors regulate vascular morphogenesis in organs, most attention has been focused on the role of these factors in pathologic processes, such as tumor vascularization and their potential use as antitumor agents (82). Therefore while angiostatic factors are expressed in the lung and altered

in disease states, such as lung cancer, pulmonary fibrosis, acute respiratory distress syndrome (ARDS), and emphysema, the role of these factors in pulmonary vascular morphogenesis is not well understood. While lung development appears normal in TSP-1 null mice, there is massive inflammatory cell influx into the lungs after birth, suggesting a role for TSP-1 in lung homeostasis (83), which may be due to the role of TSP-1 in regulating TGF- β (84). PEDF may exert its angiostatic effects by downregulating VEGF expression, although its role in pulmonary vascular morphogenesis is unclear. Alternative splicing of the VEGFR1 or Flt-1 gene generates a soluble form of the receptor, sFlt-1, which is a potent natural antagonist of VEGF. sFlt is expressed in the lung, although its role in normal pulmonary vascular development is also unclear. However, one study showed that excess sFlt-1 in amniotic fluid during late gestation reduced alveolarization and pulmonary vascular growth in infant rats, suggesting that it may play a functional role (85). EMAP II is a cytokine-like molecule that has potent angiostatic activity, inducing cultured endothelial cells to undergo apoptosis. Expression of EMAPII was high during the pseudoglandular stage of lung morphogenesis, but decreased as pulmonary vascular morphogenesis progressed (86). EMAPII may be a negative regulator of pulmonary vascular growth based on fetal lung allograft studies. Hence, there may be interactions between angiostatic factors and proangiogenic factors, which may represent regulatory or homeostatic feedback loops. The presence of such feedback loops would not be surprising given the need to carefully regulate vascular morphogenesis in proportion to airway development.

MicroRNAs: MicroRNAs can regulate networks of genes by binding to multiple RNAs, generally to inactivate and degrade them, thereby transforming cellular phenotype. Micro RNAs are important in angiogenesis and vascular development of the lung, miR-221 being antiangiogenic and miR-130 being proangiogenic (87).

Transcription Factors: A number of transcription factors regulate endothelial cell specification and differentiation into arterial, venous, and lymphatic lineages. Some examples are discussed in the following paragraphs, including the hypoxia-inducible factors (HIF), Hox genes, Fox genes, Sox genes, and members of the GATA family. The reader is directed to reviews for more

information (88–90). Our understanding of the role of these factors in vascular development comes from studies in zebrafish and other models and a few studies in the developing lung.

Hypoxia-inducible transcription factors (HIFs) have been extensively studied because of their role in hypoxia responses. HIF-1 α plays an important role in oxygen homeostasis and regulates VEGF (91,92). Deletion of the HIF-2 α gene (also known as EPAS1) was recently shown to cause respiratory distress and death at birth in mice (93). HIF-2 α null mice had reduced VEGF levels, but structurally the lungs of HIF-2 α null mice did not appear grossly abnormal, except for an increased abundance of immature pneumocytes and some subtle deficits in lung vascularization later in gestation.

Hox transcription factors are mammalian homologues of homeobox genes that regulate body patterning and organogenesis in *Drosophila*. While deletion of some Hox genes disrupt lung morphogenesis, the role of these transcription factors in pulmonary vascular morphogenesis is not well understood (94). For example, Hoxb-5 regulates airway patterning, possibly by its effects on tenascin-C and FGF10 expression, and so may indirectly affect vascular development (95). Paired-related homeobox gene (Prx1) has been implicated more directly in pulmonary vascular development. Prx1 is expressed in endothelial cells and the wall of muscularized vessels early in lung morphogenesis and then later in the medial and adventitial layers (96). Ihida-Stansbury and colleagues suggested that Prx1 plays a role in promoting patterning and differentiation of vessels in the developing lung based on in vitro studies in which Prx1 transfection into fetal lung mesenchymal cells induced an endothelial-like phenotype and the ability to form vascular networks in matrigel (96). Furthermore, Prx1 $^{-/-}$ mice were cyanotic, die soon after birth, and had decreased expression of TN-C, which may have contributed to the hypoplastic lung phenotype and lack of pulmonary vessels in these animals.

Fork-head box (Fox) transcription factors are expressed in mesoderm, including the splanchnic mesoderm, and are important in mesenchymal induction of the lung epithelium. Haploinsufficiency of Foxf1 causes abnormal development of the lung, as well as the gall bladder, esophagus, and trachea. Lung abnormalities in Foxf1 $^{+/-}$ mice included hemorrhage, fusion of lung lobes, and

defects in sacculle and microvascular development (97). Reduced expression of Notch2 and Hes-1, its downstream target, may contribute to the abnormalities caused by Foxf1 haploinsufficiency (51). FoxC1 and FoxC2 have virtually identical DNA-binding domains. Mice that were double mutants for FoxC1 and C2 had arteriovenous malformations, and lymphatic endothelial cells failed to sprout off from the veins likely due to reduced VEGF-C (98). While expression of the venous marker COUP-TFII was normal, double-mutant mice lacked expression of arterial markers, suggesting that FoxC transcription factors play an important role in arterial cell fate specification. However, the role of FoxC1 and C2 in the lung is unclear.

SRY-related high-mobility group box (Sox) transcription factors comprise a family of 20 different members. Studies suggest that the Sox F group, which includes Sox 7, 17, and 18, regulate arteriovenous specification and venous lymphatic specification. Loss of one Sox F member may be compensated for by the other F group members (90). Sox18 has raised a lot of interest, as it can regulate Prox1 and therefore lymphatic development. Sox17 is expressed in mesenchymal progenitors of endothelial cells in the developing lung and is solely found in endothelial cells in the adult lung (99). Conditional deletion of Sox17 in mesoderm resulted in abnormal pulmonary vascular development, including enlarged arteries, vein varices, and decreased microvasculature with alveolar hypoplasia.

There are six GATA transcription factors, of which GATA-1, -2, and -3 play important roles in hematopoietic cells. Studies suggest that GATA-2 regulates differentiation of endothelial cells (90) and key endothelial genes, including VEGFR2 (Flk-1), endomucin, and VEcadherin (100). Knockdown of GATA2 in dermal endothelial cells reduced expression of these genes and increased nonendothelial genes “including” smooth muscle actin and SNAIL, suggesting that failure to progress into an endothelial cell fate leads to direction into other mesenchymal cell fates like smooth muscle.

Extracellular Matrix Molecules: Extracellular matrix (ECM) molecules provide important structural integrity to mature vessels to support their function and a scaffold and cell adhesion that supports the growth and development of vessels. Growth factors such as VEGF, especially the longer isoforms, bind to ECM, although the exact role of

this function is not entirely clear. ECM proteins include collagen, laminin, fibronectin, elastin, and tenascin. Collagen and lamin are produced early in lung development and increase as development proceeds. In contrast, fibronectin expression increases in the pseudoglandular stage when preacinar arteries are forming and then decreases during the canalicular phase (101). Elastin levels also increase in the pseudoglandular stage and continue to rise in the saccular and alveolar stages, when it plays an important role in alveolar and vascular development and structure (102). In transgenic mice, even haploinsufficiency of elastin results in pulmonary hypertension later in life (103).

Integrins: Integrins are composed of α and β subunits that form transmembrane heterodimer complexes. Integrins function as cell surface receptors that interact with other proteins, including ECM. The β 1 integrin family includes receptors that recognize collagen, laminin, and fibronectin. The β 3 integrins include receptors for fibronectin, von Willebrand factor, and thrombospondin. Integrin expression by endothelial cells is complex [see review by Albelda (104)]. The five integrins that contain the α v subunit are widely expressed in most tissues in mice. However, mice that lack the α v subunit, and are therefore deficient in all five integrins, develop normally, suggesting that lung development and cellular differentiation can occur in the absence of these integrins (105).

Development of the Pulmonary Capillary System

Structure, Origins, Timing, and Patterning

The capillary system in the distal lung is extensive and closely integrated with the alveoli to form the large gas exchange surface of the lung. Development of this capillary network expands considerably in the saccular and alveolar stages of lung development after the formation of the preacinar and acinar arteries and arterioles is complete (Figure 3-3).

Cellular and Molecular Mechanisms

Pathways that selectively regulate pulmonary capillary development are poorly understood; however, VEGF and other proangiogenic factors likely play a role. Inhibition of VEGFR2 in the neonatal lung reduced vascular morphogenesis and disrupted alveolar formation during the postnatal

phase of lung development that is normally characterized by expansion of the capillary network (27). The transcription factor *Foxf1* has also been implicated in pulmonary capillary development because conditional inactivation of the *Foxf1* gene in endothelial cells in mice caused a phenotype similar to alveolar-capillary dysplasia in humans (106).

Development of the pulmonary veins

Structure, Origins, Timing, and Patterning

The pulmonary venous system returns blood from the vast alveolar capillary system to the heart. Veins in the peripheral lung are generally located in the tissue regions between the arteries and airways (Figure 3-8) but run close to the major airway and artery in the proximal lung, especially where they emerge from the lung tissue. Early development of the pulmonary venous system is poorly understood. Hall and colleagues (9) used immunohistochemistry and serial reconstruction techniques to characterize venous formation in fetal and neonatal human lungs. Their data suggested that the pulmonary veins initially form in the splanchnic mesenchyme by vasculogenesis. Later expansion of the venous system was more consistent with angiogenesis taking over as the process by which venous development advanced, similar to arterial development. Although airways are thought to play an important role in patterning the arterial system Hall et al. (9) suggested that this was less likely to be the case with the venous system, given that veins develop in the

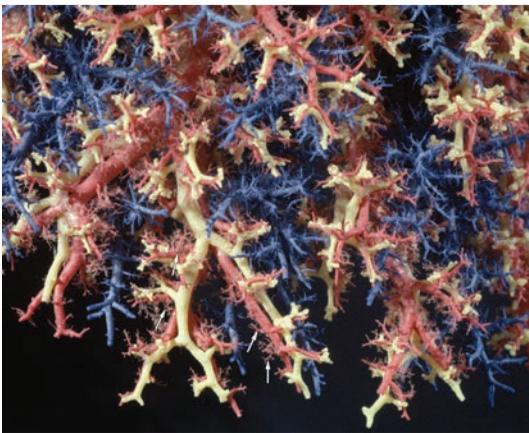


Figure 3-8. Casts show the venous (blue) system in the human lung relative to the airway (yellow/white) and arterial (red) systems. Courtesy of Prof. Ewald Weibel, Institute of Anatomy, University of Berne.

mesenchyme away from the airways and arteries. However, the mechanisms regulating patterning of the venous system remain unclear. By 34 d gestation continuity between the aortic sac, pulmonary artery, capillary plexus, pulmonary veins, and left atrium is seen in the human fetus (9).

Hall et al. also (9) examined the origin of the venous smooth muscle and suggested that venous smooth muscle cells are derived from mesenchymal cell precursors. They also reported that the pulmonary veins acquired smooth muscle somewhat later than pulmonary arteries and that expression of mature smooth muscle proteins was also delayed relative to arterial smooth muscle.

Cellular and Molecular Mechanisms

In the early vertebrate embryo, angioblasts that are the precursors to endothelial cells appear to be committed to either arterial or venous lineages prior to the formation of a functional circulation. However, the pathways that regulate this commitment are not well understood. In the study by Peng et al. (5), deletion of *Smo* from *Isl1* expressing CPP cells caused pulmonary vein atresia as well as severe inflow tract defects, suggesting that CPP cells are important for the development of the pulmonary veins as well as arteries and heart.

Notch signaling, as mentioned earlier, is important for arterial development downstream of VEGF. Ephrin-B2 is expressed in arteries and EphB4 in veins, although this does not appear to be important for the initial specification of arteries versus veins (78). The transcription factor COUP-TFII is expressed in venous endothelial cells in the developing circulation and may regulate venous cell fate by repressing Notch signaling. COUP-TFII is expressed in the mesenchyme of the developing mouse and human lung and regulated by retinoic acid signaling, although its exact role in pulmonary vascular development has not been clearly defined. COUP-TFII is also important in diaphragm development as COUP-TFII null mice develop congenital diaphragmatic hernia (CDH) and associated lung hypoplasia (107).

Development of Pulmonary Lymphatics

Structure, Origins, Timing, and Patterning

The lymphatic system drains lymph fluid from the lung tissues and returns it to the circulation.

It contains lymph nodes that play important roles in the immune system. Lymphatic vessels are lined by endothelial cells and vary in size from thin-walled capillaries to larger vessels. Larger lymphatic vessels frequently contain valves to prevent backflow of lymph. Lymphatic vessels form around airways and arteries, especially the larger caliber ones (108). In the human fetal lung, lymphatics develop in the hilar region in the second month of gestation and then expand over the next month to form an extensive lymphatic network around the bronchi, pulmonary artery, and pulmonary veins as far as the pleura. Valves form in the lymphatics starting with the hilar region and then extending into more distal regions as development proceeds.

The origin of lymphatic vessels has been unclear until recently because of the lack of specific markers for lymphatic endothelial cells versus endothelial cells in the arteries or veins, often referred to as “blood endothelial cells.” Primitive lymphatic sacs were originally thought to develop by budding of endothelial cells off the veins and then further extended to form a peripheral lymphatic network by angiogenesis-like sprouting from these lymphatic sacs. This model implies that the venous system is formed, or at least partially formed, prior to the development of the lymphatic system. An alternative model is that in which lymphatic sacs form via a vasculogenesis-type process in the mesenchyme, independent of veins, and then make venous connections later. Determining which of these models is correct awaited the development of better molecular tools and marker genes. In a study by Wigle and Oliver (109), expression and deletion of the homeobox transcription factor *Prox1* provided strong evidence to support the venous development model of lymphatic endothelial cells budding off veins. *Prox1* is expressed in a subpopulation of endothelial cells that form the lymphatic system. This is described later.

Cellular and Molecular Mechanisms

VEGF receptor 3 (VEGFR3 also known as *Flt4*) is expressed by endothelial cells in developing veins and lymphatics but becomes restricted to lymphatics later in postnatal life. Further insight into the role of VEGFR3 in lymphatics has been hampered by the lack of a viable VEGFR3 null mouse.

Overexpression of VEGF-C, a VEGFR3 ligand, in transgenic mice caused lymphatic hyperplasia (110) and more recently respiratory distress (RDS), chylothorax, and pulmonary lymphangiectasia when expression was directed to the perinatal period (E15.5–P14) (111). VEGF-D also activates VEGFR3, and increases in VEGF-D increase the growth of lymphatic vessels, but not blood vessels consistent with VEGF-D activating only VEGFR3 and not VEGFR-2. VEGF-D is expressed in the developing mouse lung from low levels in the pseudoglandular stage, increasing in the lung mesenchyme (especially *Cadherin-11* positive cells) through the early neonatal period, then declining thereafter and reaching very low levels in the adult lung (31). However the role of VEGF-D is unclear as VEGF-D null mice develop a structurally and functionally normal lymphatic system (112). VEGF-A is typically thought of with respect to arterial development; however, Mallory et al. overexpressed VEGF-A in the lungs of transgenic mice during the perinatal period and observed increased vessels that were identified as lymphatics in the distal lung (113). mRNA levels of VEGF-C/D were unaltered in this study, suggesting that the levels of VEGF-A can influence the balance between arterial and lymphatic development in the lung.

The homeobox transcription factor *Prox1* is often thought to act as a “master” regulator of lymphangiogenesis. Wigle and Oliver (109) examined *Prox1* null mice in which the gene was inactivated by insertion of the β -galactosidase gene. *Prox1* marks a specific subpopulation of endothelial cells in the veins that form the lymphatic sacs that bud off. Using *Prox1*^{+/-} mice, they showed β -galactosidase staining of lymphatic endothelial cells in E14.5 lungs that was already an extensive endothelial plexus around the developing airways, including the trachea and bronchi (Figure 3-9). Analysis of *Prox1*^{-/-} embryos showed that the lymphatic vessels were absent from the trachea and airways, indicating an important role for *Prox1* in lymphangiogenesis and not arteries and veins.

FoxC2 is another transcription factor that acts as a novel regulator of lymphatic development and remodeling (114). *FoxC2* regulates later steps in lymphatic development, including establishing a collecting lymphatic vessel identity by regulating expression of other genes involved in lymphangiogenesis, including PDGF- β , Delta-like 4, and

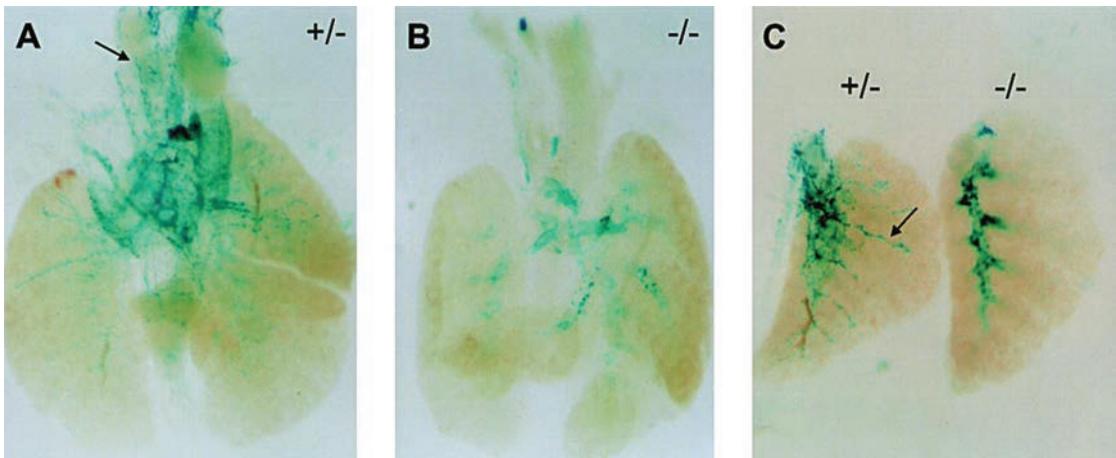


Figure 3-9. Lymphatic development of the E14.5 lungs in Prox^{+/-} (A) and Prox^{-/-} (B) embryos. Lymphatic vessels are shown by LacZ staining around the esophagus, trachea, and bronchi. In Prox^{-/-} embryos these lymphatic vessels were absent as well as the lymphatic vessels in the lung (indicated by the arrow in Prox^{+/-} lung) (B & C). Reprinted with permission from Wigle & Oliver, *Cell*, 1999;98:769–778.

Ang-2. The lymphatics of FoxC2 null mice had increased pericyte recruitment, as well as agenesis of lymphatic valves. FoxC1 null mice died prenatally and perinatally with major cardiovascular, genitourinary, skeletal, and ocular defects. As mentioned earlier, in addition to arteriovenous malformations, lymphatic endothelial cells sprout from the veins in mice that were double mutants for FoxC1 and C2 (FoxC1^{+/-}; FoxC2^{-/-}), likely due to reduced VEGF-C (98). FoxC2 may have roles in both early and later lymphangiogenesis although whether FoxC2 fulfills similar functions in the lung is unclear.

In Alk1-depleted mice, lymphatics were enlarged in a number of organs suggesting that ALK-1 signals regulate lymphatic vessel formation and size (115). The effects of ALK-1 were mediated by BMP-9, which decreased the number of cultured lymphatic endothelial cells. Bmp9-deficient mouse embryos also had enlarged dermal lymphatic vessels, and BMP-9 downregulated the expression of the Prox1, which is critical for lymphatic endothelial cells.

Nuclear factor of activated T cells (NFATc1) is a transcription factor that is important for T-cell differentiation, cardiac valve development, and osteogenesis. NFATc1 also plays a role in lymphatic development. NFATc1 colocalizes with Prox1 and VEGFR3 to endothelial cells that sprout off the cardinal vein and form lymphatic sacs (116). While endothelial cells sprout off the cardinal vein in NFATc1 null mice, they do not coalesce

properly into lymphatic sacs. This and other data from using calcineurin inhibitors that block NFATc1 activation indicate an important role for NFATc1 in lymphatic patterning. In VEGF-A overexpressing transgenic mice, loss of the calcineurin regulatory subunit blocked the increase in lymphangiogenesis.

Spontaneous mutations and genetic deletion of Sox18 in mice caused embryonic lethality with defective lymphatic development characterized by a lack of Prox1 lymphatic cells (72). Sox18 regulates Prox1, and overexpression of Sox18 in endothelial cells increases Prox1 and Podoplanin, key lymphatic endothelial cell markers. Sox18 appears to play a very important role in lymphatic development, and this may be due to its being an upstream regulator of Prox1. GATA2 is another transcription factor that may play a role in lymphangiogenesis as conditional loss-of-function in mouse embryos resulted in abnormal lymphatic development and mixed lymphatic-blood vessel structures.

Unanswered Questions and Future Directions

There remain many unanswered questions in pulmonary vascular development. There is still uncertainty about the relative importance of vasculogenesis versus angiogenesis in the initial formation of pulmonary vessels. It appears that both processes play a role, but the question of what

if any role angiogenesis plays in the initial formation of pulmonary vessels remains unclear. Recent studies identifying CPP cells that give rise to major pulmonary vessels is exciting but also raises again the question of whether there is growth of major vessels that then invade the lung by angiogenesis versus development in situ of these major vessels by vasculogenesis. Whether there is a role for airway smooth muscle cells in endothelial specification and vascular plexus development is unclear. The exact timing/sequence and composition of factors that induce arterial versus capillary versus venous versus lymphatic endothelial cells is unclear. Fractal rules suggest a few factors. The regulation of vessel diameter and how the structural composition of developing vessels is regulated is also poorly understood. Whether early vascular endothelial cells are plastic and can be transformed into a different fate, either another endothelial cell type or another mesenchymal fate, and what is the time window involved is not understood. Answers to these questions may not only illuminate our understanding of lung development but may also aid in discovery of the causes of pulmonary vascular diseases and vessel remodeling later in life.

The precise spatial-temporal regulation of arterial, venous, capillary, and lymphatic development are poorly understood. The spatial regulation of arterial versus venous development is not understood. High concentrations of VEGF and other airway epithelial-derived growth factors promote arterial development near the airways based on their stimulatory effects on endothelial specification and proliferation. However, whether lower levels of these growth factors lends mesenchymal cells to develop along a venous fate is still unclear. It is also possible that higher concentrations of factors derived from the mesenchyme itself might promote venous cell fate. Development of capillaries is also poorly understood but presumably regulated by distal epithelial cells and/or other cells in the alveolar regions. In lymphangiogenesis some pathways and regulators are clearly important, but the regulation of size and patterning of lymphatics remains poorly understood.

Better targeting of pulmonary vessels in experimental models is needed, especially conditional and temporal targeting that can be regulated. Targeting of different endothelial cell populations should be insightful in the future. The application

of single-cell transcriptional analysis during development (117) and the verification and elucidation of regulatory factors and pathways already identified in other organs and nonlung models needs to be studied in the lung.

Response to Injury

Despite many unanswered questions, there is much known about the response of the vasculature to injury and the capacity for regression of disease and lung vascular regeneration. Mechanical ventilation with cyclic stretch impairs lung vascular growth in premature lambs (118) and newborn mice (119) (Figure 3-10). The mechanism is related to upregulation of elastase and TGF- β activation, resulting in enhanced synthesis but impaired assembly of elastin and loss of endothelial cells as a result of apoptosis (120). Hyperoxia is a stimulus often shown to promote impaired alveolar and vascular development (121), and unlike hypoxia in newborn rats, the abnormalities do not appear to reverse (122). Interestingly α 1 antitrypsin prevented vascular changes and abnormal lung compliance associated with hyperoxia in newborn rats (123). Inflammation induced structural abnormalities of the lung and vasculature in rats (124). A model of perinatal high flow due to a left to right shunt induced adverse remodeling in the vasculature of the ovine lung in association with endothelial dysfunction (125). In this model carnitine appeared to improve mitochondrial metabolism and endothelial function (126). In children with congenital heart defects, high pulmonary blood flow leads to abnormal muscularization of distal vessels, medial hypertrophy of muscular arteries, and loss of precapillary arteries that precede occlusive remodeling (plexogenic arteriopathy) (127). Children older than two years show reduced plasticity of these vascular changes and impaired potential for regression after repair of the congenital heart defect. Different vascular changes are seen with defects that cause reduced flow to the lungs (128) or high left-sided pressures (129). With reduced flow the vessels are small in size with reduced muscularity, whereas with high pressure they tend to be increased in number with increased muscularity. With total anomalous pulmonary venous return, prenatal occlusive changes of neointimal formation can be seen in the veins (130).

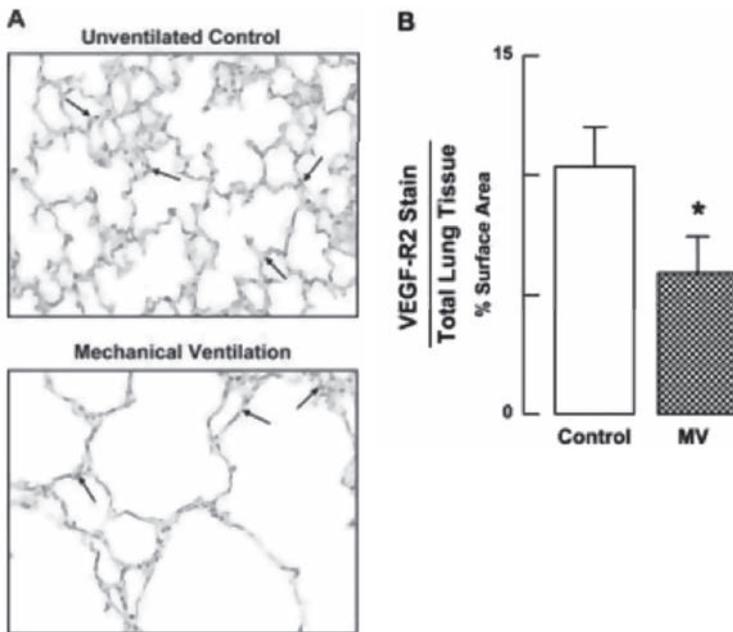


Figure 3-10. Mechanical ventilation (MV) with air for 24 h reduces lung abundance of VEGF-R2 protein. (A) Immunohistochemistry (IHC) staining of zinc-fixed lung tissue section showing reduced VEGF-R2 protein (arrows, brown stain) in distal lung after MV with air for 24 h compared with the unventilated control lung. (B) Summary data (means and SD) show a ~50% decrease in VEGF-R2 stain area relative to tissue area, measured by quantitative image analysis of 20 randomly selected fields (x400) in two tissue slides per animal. *Significant difference compared with Control group, $P < 0.05$. $n = 4$ per group. Reprinted with permission from Mokres et al., *Am. J. Physiol. Lung, Cellular Mol. Physiol.* 2010;298:L23–L35.

Potential for Regression and Regeneration of Normal Lung Vasculature

There has been much recent interest in the role of progenitor cell therapy, particularly with mesenchymal progenitor cells in the regeneration of both lung blood vessels and airways [reviewed in (131)]. In particular both cord blood angiogenic progenitor cells (132) and circulating endothelial progenitor cells (133) have shown efficacy in experimental models. Because these cells are believed to work via a paracrine mechanism, work

specifically related to the vasculature has focused attention on exosomes and how they may be used to regenerate lung and blood vessels and prevent or reverse experimental pulmonary hypertension (134). The pneumonectomy model is one of the best to study the potential for lung regeneration because of the important role of the regenerating vasculature (135) with evidence that macrophages contribute in a pivotal manner (136). In fact, this has been an ideal model to study the role of angiogenesis in alveolar regeneration (137) and the physical factors that influence the process (138).

References

- Schachtner SK, Wang Y, Scott Baldwin H. Qualitative and quantitative analysis of embryonic pulmonary vessel formation. *American Journal of Respiratory Cell and Molecular Biology*. 2000;22(2):157–165.
- Glenny RW. Emergence of matched airway and vascular trees from fractal rules. *Journal of Applied Physiology*. 2011;110(4):1119–1129.
- Hislop A. Developmental biology of the pulmonary circulation. *Paediatric Respiratory Reviews*. 2005;6(1):35–43.
- deMello DE, Sawyer D, Galvin N, Reid LM. Early fetal development of lung vasculature. *American Journal of Respiratory Cell and Molecular Biology*. 1997;16(5):568–581.
- Peng T, Tian Y, Boogerd CJ, Lu MM, Kadzik RS, Stewart KM, et al. Coordination of heart and lung co-development by a multipotent cardiopulmonary progenitor. *Nature*. 2013;500(7464):589–592.
- Galambos C, deMello DE. Molecular mechanisms of pulmonary vascular development. *Pediatric and Developmental Pathology*. 2007;10(1):1–17.
- Djonov V, Schmid M, Tschanz SA, Burri PH. Intussusceptive angiogenesis: its role in embryonic vascular network formation. *Circulation Research*. 2000;86(3):286–292.
- Greif DM, Kumar M, Lighthouse JK, Hum J, An A, Ding L, et al. Radial construction of an arterial wall.

- Developmental Cell*. 2012; 23(3):482–493.
- 9 Hall SM, Hislop AA, Haworth SG. Origin, differentiation, and maturation of human pulmonary veins. *American Journal of Respiratory Cell and Molecular Biology*. 2002; 26(3):333–340.
 - 10 Thebaud B, Abman SH. Bronchopulmonary dysplasia: where have all the vessels gone? Roles of angiogenic growth factors in chronic lung disease. *American Journal of Respiratory and Critical Care Medicine*. 2007;175 (10):978–985.
 - 11 Bellusci S, Furuta Y, Rush MG, Henderson R, Winnier G, Hogan BL. Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development*. 1997;124(1):53–63.
 - 12 Miller LA, Wert SE, Clark JC, Xu Y, Perl AK, Whitsett JA. Role of Sonic hedgehog in patterning of tracheal-bronchial cartilage and the peripheral lung. *Developmental Dynamics*. 2004;231(1):57–71.
 - 13 Amaya E, Musci TJ, Kirschner MW. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell*. 1991;66(2): 257–270.
 - 14 Flamme I, Breier G, Risau W. Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. *Developmental Biology*. 1995;169(2):699–712.
 - 15 Cox CM, Poole TJ. Angioblast differentiation is influenced by the local environment: FGF-2 induces angioblasts and patterns vessel formation in the quail embryo. *Developmental Dynamics*. 2000;218(2):371–382.
 - 16 Gonzalez AM, Hill DJ, Logan A, Maher PA, Baird A. Distribution of fibroblast growth factor (FGF)-2 and FGF receptor-1 messenger RNA expression and protein presence in the mid-trimester human fetus. *Pediatric Research*. 1996;39(3):375–385.
 - 17 Shannon JM, Hyatt BA. Epithelial-mesenchymal interactions in the developing lung. *Annual Review of Physiology*. 2004;66:625–645.
 - 18 Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development*. 2005;132(1):35–47.
 - 19 Zachary I. Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor. *American Journal of Physiology Cell Physiology*. 2001;280(6):C1375–1386.
 - 20 Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nature Medicine*. 2003;9(6):669–676.
 - 21 Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 1995;376(6535):62–66.
 - 22 Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *The Journal of Biological Chemistry*. 1991;266 (18):11947–11954.
 - 23 Ng YS, Rohan R, Sunday ME, deMello DE, D'Amore PA. Differential expression of VEGF isoforms in mouse during development and in the adult. *Developmental Dynamics*. 2001;220(2): 112–121.
 - 24 Gebb SA, Shannon JM. Tissue interactions mediate early events in pulmonary vasculogenesis. *Developmental Dynamics*. 2000;217(2):159–69.
 - 25 Galambos C, Ng YS, Ali A, Noguchi A, Lovejoy S, D'Amore PA, et al. Defective pulmonary development in the absence of heparin-binding vascular endothelial growth factor isoforms. *American Journal of Respiratory Cell and Molecular Biology*. 2002;27 (2):194–203.
 - 26 Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, et al. VEGF is required for growth and survival in neonatal mice. *Development*. 1999;126(6): 1149–1159.
 - 27 Le Cras TD, Markham NE, Tudor RM, Voelkel NF, Abman SH. Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2002;283(3): L555–562.
 - 28 Zeng X, Wert SE, Federici R, Peters KG, Whitsett JA. VEGF enhances pulmonary vasculogenesis and disrupts lung morphogenesis in vivo. *Developmental Dynamics*. 1998;211(3):215–227.
 - 29 Akeson AL, Greenberg JM, Cameron JE, Thompson FY, Brooks SK, Wiginton D, et al. Temporal and spatial regulation of VEGF-A controls vascular patterning in the embryonic lung. *Developmental Biology*. 2003;264(2):443–455.
 - 30 Le Cras TD, Spitzmiller RE, Albertine KH, Greenberg JM, Whitsett JA, Akeson AL. VEGF causes pulmonary hemorrhage, hemosiderosis, and air space enlargement in neonatal mice. *American Journal of Physiology Lung Cellular and Molecular*

- Physiology*. 2004;287(1): L134–142.
- 31 Greenberg JM, Thompson FY, Brooks SK, Shannon JM, McCormick-Shannon K, Cameron JE, et al. Mesenchymal expression of vascular endothelial growth factors D and A defines vascular patterning in developing lung. *Developmental Dynamics*. 2002;224(2):144–153.
- 32 Schreiber AB, Winkler ME, Derynck R. Transforming growth factor- α : a more potent angiogenic mediator than epidermal growth factor. *Science*. 1986;232(4755): 1250–1253.
- 33 Pardanaud L, Dieterlen-Lievre F. Manipulation of the angiopoietic/hemangiopoietic commitment in the avian embryo. *Development*. 1999;126(4):617–627.
- 34 Korfhagen TR, Swantz RJ, Wert SE, McCarty JM, Kerlakian CB, Glasser SW, et al. Respiratory epithelial cell expression of human transforming growth factor- α induces lung fibrosis in transgenic mice. *The Journal of Clinical Investigation*. 1994; 93(4):1691–1699.
- 35 Hardie WD, Bruno MD, Huelsman KM, Iwamoto HS, Carrigan PE, Leikauf GD, et al. Postnatal lung function and morphology in transgenic mice expressing transforming growth factor- α . *The American Journal of Pathology*. 1997;151(4):1075–1083.
- 36 Le Cras TD, Hardie WD, Fagan K, Whitsett JA, Korfhagen TR. Disrupted pulmonary vascular development and pulmonary hypertension in transgenic mice overexpressing transforming growth factor- α . *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2003; 285(5):L1046–1054.
- 37 Madtes DK, Elston AL, Hackman RC, Dunn AR, Clark JG. Transforming growth factor- α deficiency reduces pulmonary fibrosis in transgenic mice. *American Journal of Respiratory Cell and Molecular Biology*. 1999;20 (5):924–934.
- 38 Thurston G, Gale NW. Vascular endothelial growth factor and other signaling pathways in developmental and pathologic angiogenesis. *International Journal of Hematology*. 2004;80(1):7–20.
- 39 Du L, Sullivan CC, Chu D, Cho AJ, Kido M, Wolf PL, et al. Signaling molecules in nonfamilial pulmonary hypertension. *The New England Journal of Medicine*. 2003;348(6):500–509.
- 40 Sullivan CC, Du L, Chu D, Cho AJ, Kido M, Wolf PL, et al. Induction of pulmonary hypertension by an angiopoietin 1/TIE2/serotonin pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(21):12331–12336.
- 41 Chinoy MR. Pulmonary hypoplasia and congenital diaphragmatic hernia: advances in the pathogenetics and regulation of lung development. *The Journal of Surgical Research*. 2002;106 (1):209–223.
- 42 Weinmaster G. The ins and outs of notch signaling. *Molecular and Cellular Neurosciences*. 1997;9(2):91–102.
- 43 Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, et al. Notch signaling is essential for vascular morphogenesis in mice. *Genes & Development*. 2000;14(11):1343–1352.
- 44 Xue Y, Gao X, Lindsell CE, Norton CR, Chang B, Hicks C, et al. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Human Molecular Genetics*. 1999;8(5):723–730.
- 45 Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, et al. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development*. 2001;128(19):3675–3683.
- 46 Zhong TP, Childs S, Leu JP, Fishman MC. Gridlock signalling pathway fashions the first embryonic artery. *Nature*. 2001;414(6860):216–220.
- 47 Visconti RP, Richardson CD, Sato TN. Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99(12):8219–8224.
- 48 Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes & Development*. 2004;18(8):901–911.
- 49 Domenga V, Fardoux P, Lacombe P, Monet M, Maciazek J, Krebs LT, et al. Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes & Development*. 2004;18 (22):2730–2735.
- 50 Kong Y, Glickman J, Subramaniam M, Shahsafaei A, Allamneni KP, Aster JC, et al. Functional diversity of notch family genes in fetal lung development. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2004;286(5): L1075–1083.
- 51 Kalinichenko VV, Gusarova GA, Kim IM, Shin B, Yoder HM, Clark J, et al. Foxf1 haploinsufficiency reduces Notch-2 signaling during

- mouse lung development. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2004;286(3):L521–530.
- 52 Darland DC, D'Amore PA. TGF beta is required for the formation of capillary-like structures in three-dimensional cocultures of 10T1/2 and endothelial cells. *Angiogenesis*. 2001;4(1):11–20.
- 53 Roberts AB, Sporn MB. Regulation of endothelial cell growth, architecture, and matrix synthesis by TGF-beta. *The American Review of Respiratory Disease*. 1989;140(4):1126–1128.
- 54 Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, et al. Defective angiogenesis in mice lacking endoglin. *Science*. 1999;284(5419):1534–1537.
- 55 Jonker L, Arthur HM. Endoglin expression in early development is associated with vasculogenesis and angiogenesis. *Mechanisms of Development*. 2002;110(1–2):193–196.
- 56 Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, et al. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97(6):2626–2631.
- 57 Urness LD, Sorensen LK, Li DY. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nature Genetics*. 2000;26(3):328–331.
- 58 Nakagawa T, Li JH, Garcia G, Mu W, Piek E, Bottinger EP, et al. TGF-beta induces proangiogenic and antiangiogenic factors via parallel but distinct Smad pathways. *Kidney International*. 2004;66(2):605–613.
- 59 Cai J, Pardali E, Sanchez-Duffhues G, ten Dijke P. BMP signaling in vascular diseases. *FEBS Letters*. 2012;586(14):1993–2002.
- 60 Volpert OV, Pili R, Sikder HA, Nelius T, Zaichuk T, Morris C, et al. Id1 regulates angiogenesis through transcriptional repression of thrombospondin-1. *Cancer Cell*. 2002;2(6):473–483.
- 61 International PPHC, Lane KB, Machado RD, Pauculo MW, Thomson JR, Phillips JA, 3rd, et al. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Nature Genetics*. 2000;26(1):81–84.
- 62 Itoh F, Itoh S, Goumans MJ, Valdimarsdottir G, Iso T, Dotto GP, et al. Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. *The EMBO Journal*. 2004;23(3):541–551.
- 63 de Jesus Perez VA, Alastalo TP, Wu JC, Axelrod JD, Cooke JP, Amieva M, et al. Bone morphogenetic protein 2 induces pulmonary angiogenesis via Wnt-beta-catenin and Wnt-RhoA-Rac1 pathways. *The Journal of Cell Biology*. 2009;184(1):83–99.
- 64 Alastalo TP, Li M, de Jesus Perez V, Pham D, Sawada H, Wang JK, et al. Disruption of PPARGgamma/beta-catenin-mediated regulation of apelin impairs BMP-induced mouse and human pulmonary arterial EC survival. *The Journal of Clinical Investigation*. 2011;121(9):3735–3746.
- 65 de Jesus Perez VA, Ali Z, Alastalo TP, Ikeno F, Sawada H, Lai YJ, et al. BMP promotes motility and represses growth of smooth muscle cells by activation of tandem Wnt pathways. *The Journal of Cell Biology*. 2011;192(1):171–188.
- 66 Zerlin M, Julius MA, Kitajewski J. Wnt/Frizzled signaling in angiogenesis. *Angiogenesis*. 2008;11(1):63–69.
- 67 Wright M, Aikawa M, Szeto W, Papkoff J. Identification of a Wnt-responsive signal transduction pathway in primary endothelial cells. *Biochemical and Biophysical Research Communications*. 1999;263(2):384–388.
- 68 Monkley SJ, Delaney SJ, Pennisi DJ, Christiansen JH, Wainwright BJ. Targeted disruption of the Wnt2 gene results in placentation defects. *Development*. 1996;122(11):3343–3353.
- 69 Shu W, Jiang YQ, Lu MM, Morrisey EE. Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development*. 2002;129(20):4831–4842.
- 70 Cohen ED, Ihida-Stansbury K, Lu MM, Panettieri RA, Jones PL, Morrisey EE. Wnt signaling regulates smooth muscle precursor development in the mouse lung via a tenascin C/PDGFR pathway. *The Journal of Clinical Investigation*. 2009;119(9):2538–2549.
- 71 Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes & Development*. 2008;22(10):1276–1312.
- 72 Betsholtz C. Biology of platelet-derived growth factors in development. *Birth Defects Research Part C, Embryo Today: Reviews*. 2003;69(4):272–285.
- 73 Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature*. 2000;408(6808):92–96.
- 74 Grimminger F, Schermuly RT. PDGF receptor and its

- antagonists: role in treatment of PAH. *Advances in Experimental Medicine and Biology*. 2010;661:435–446.
- 75 Dupont J, Holzenberger M. Biology of insulin-like growth factors in development. *Birth Defects Research Part C, Embryo Today: Reviews*. 2003;69(4):257–271.
- 76 Han RN, Post M, Tanswell AK, Lye SJ. Insulin-like growth factor-I receptor-mediated vasculogenesis/angiogenesis in human lung development. *American Journal of Respiratory Cell and Molecular Biology*. 2003;28(2):159–169.
- 77 Salvucci O, Tosato G. Essential roles of EphB receptors and EphrinB ligands in endothelial cell function and angiogenesis. *Advances in Cancer Research*. 2012;114:21–57.
- 78 Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell*. 1998;93(5):741–753.
- 79 Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, et al. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes & Development*. 1999;13(3):295–306.
- 80 Shin D, Garcia-Cardena G, Hayashi S, Gerety S, Asahara T, Stavrakis G, et al. Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Developmental Biology*. 2001;230(2):139–150.
- 81 Baldwin HS. Early embryonic vascular development. *Cardiovascular Research*. 1996;31 Spec No:E34–45.
- 82 Tabruyn SP, Griffioen AW. Molecular pathways of angiogenesis inhibition. *Biochemical and Biophysical Research Communications*. 2007;355(1):1–5.
- 83 Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, et al. Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *The Journal of Clinical Investigation*. 1998;101(5):982–992.
- 84 Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO, et al. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell*. 1998;93(7):1159–1170.
- 85 Tang JR, Karumanchi SA, Seedorf G, Markham N, Abman SH. Excess soluble vascular endothelial growth factor receptor-1 in amniotic fluid impairs lung growth in rats: linking preeclampsia with bronchopulmonary dysplasia. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2012;302(1):L36–46.
- 86 Schwarz M, Lee M, Zhang F, Zhao J, Jin Y, Smith S, et al. EMAP II: a modulator of neovascularization in the developing lung. *The American Journal of Physiology*. 1999;276(2 Pt 1):L365–375.
- 87 Mujahid S, Nielsen HC, Volpe MV. MiR-221 and miR-130a regulate lung airway and vascular development. *PLoS One*. 2013;8(2):e55911.
- 88 De Val S, Black BL. Transcriptional control of endothelial cell development. *Developmental Cell*. 2009;16(2):180–195.
- 89 Swift MR, Weinstein BM. Arterial-venous specification during development. *Circulation Research*. 2009;104(5):576–588.
- 90 Park C, Kim TM, Malik AB. Transcriptional regulation of endothelial cell and vascular development. *Circulation Research*. 2013;112(10):1380–1400.
- 91 Kotch LE, Iyer NV, Laughner E, Semenza GL. Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Developmental Biology*. 1999;209(2):254–267.
- 92 Semenza GL, Agani F, Iyer N, Kotch L, Laughner E, Leung S, et al. Regulation of cardiovascular development and physiology by hypoxia-inducible factor 1. *Annals of the New York Academy of Sciences*. 1999;874:262–268.
- 93 Compennolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, et al. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nature Medicine*. 2002;8(7):702–710.
- 94 Jones PL. Homeobox genes in pulmonary vascular development and disease. *Trends in Cardiovascular Medicine*. 2003;13(8):336–345.
- 95 Volpe MV, Ramadurai SM, Pham LD, Nielsen HC. Hoxb-5 down regulation alters Tenascin-C, FGF10 and Hoxb gene expression patterns in pseudoglandular period fetal mouse lung. *Frontiers in Bioscience: A Journal and Virtual Library*. 2007;12:860–873.
- 96 Ihida-Stansbury K, McKean DM, Gebb SA, Martin JF, Stevens T, Nemenoff R, et al. Paired-related homeobox gene Prx1 is required for pulmonary vascular development. *Circulation Research*. 2004;94(11):1507–1514.
- 97 Lim L, Kalinichenko VV, Whitsett JA, Costa RH. Fusion

- of lung lobes and vessels in mouse embryos heterozygous for the forkhead box f1 targeted allele. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2002;282(5):L1012–1022.
- 98 Seo S, Fujita H, Nakano A, Kang M, Duarte A, Kume T. The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. *Developmental Biology*. 2006;294(2):458–470.
- 99 Lange AW, Haitchi HM, Le Cras TD, Sridharan A, Xu Y, Wert SE, et al. Sox17 is required for normal pulmonary vascular morphogenesis. *Developmental Biology*. 2014;387(1):109–120.
- 100 Minami T, Murakami T, Horiuchi K, Miura M, Noguchi T, Miyazaki J, et al. Interaction between hex and GATA transcription factors in vascular endothelial cells inhibits flk-1/KDR-mediated vascular endothelial growth factor signaling. *The Journal of Biological Chemistry*. 2004;279(20):20626–20635.
- 101 Roman J, McDonald JA. Expression of fibronectin, the integrin alpha 5, and alpha-smooth muscle actin in heart and lung development. *American Journal of Respiratory Cell and Molecular Biology*. 1992;6(5):472–480.
- 102 Hausladen JM, Davis EC, Pierce RA, Mecham RP. Formation of the pulmonary vasculature: elastic fiber proteins as markers of cellular differentiation and vascular development. *Chest*. 1998;114(1 Suppl):6S.
- 103 Shifren A, Durmowicz AG, Knutsen RH, Faury G, Mecham RP. Elastin insufficiency predisposes to elevated pulmonary circulatory pressures through changes in elastic artery structure. *Journal of Applied Physiology*. 2008;105(5):1610–1619.
- 104 Albelda SM. Endothelial and epithelial cell adhesion molecules. *American Journal of Respiratory Cell and Molecular Biology*. 1991;4(3):195–203.
- 105 Sheppard D. Roles of alpha v integrins in vascular biology and pulmonary pathology. *Current Opinion in Cell Biology*. 2004;16(5):552–557.
- 106 Sen P, Dharmadhikari AV, Majewski T, Mohammad MA, Kalin TV, Zabielska J, et al. Comparative analyses of lung transcriptomes in patients with alveolar capillary dysplasia with misalignment of pulmonary veins and in foxf1 heterozygous knockout mice. *PLoS One*. 2014;9(4):e94390.
- 107 You LR, Takamoto N, Yu CT, Tanaka T, Kodama T, Demayo FJ, et al. Mouse lacking COUP-TFII as an animal model of Bochdalek-type congenital diaphragmatic hernia. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(45):16351–16356.
- 108 Tobin CE. Human pulmonic lymphatics; an anatomic study. *The Anatomical Record*. 1957;127(3):611–633.
- 109 Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell*. 1999;98(6):769–778.
- 110 Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science*. 1997;276(5317):1423–1425.
- 111 Yao LC, Testini C, Tvorogov D, Anisimov A, Vargas SO, Baluk P, et al. Pulmonary lymphangiectasia resulting from vascular endothelial growth factor-C overexpression during a critical period. *Circulation Research*. 2014;114(5):806–822.
- 112 Baldwin ME, Halford MM, Roufail S, Williams RA, Hibbs ML, Grail D, et al. Vascular endothelial growth factor D is dispensable for development of the lymphatic system. *Molecular and Cellular Biology*. 2005;25(6):2441–2449.
- 113 Mallory BP, Mead TJ, Wiginton DA, Kulkarni RM, Greenberg JM, Akeson AL. Lymphangiogenesis in the developing lung promoted by VEGF-A. *Microvascular Research*. 2006;72(1–2):62–73.
- 114 Wu X, Liu NF. FOXC2 transcription factor: a novel regulator of lymphangiogenesis. *Lymphology*. 2011;44(1):35–41.
- 115 Yoshimatsu Y, Lee YG, Akatsu Y, Taguchi L, Suzuki HI, Cunha SI, et al. Bone morphogenetic protein-9 inhibits lymphatic vessel formation via activin receptor-like kinase 1 during development and cancer progression. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110(47):18940–18945.
- 116 Kulkarni RM, Greenberg JM, Akeson AL. NFATc1 regulates lymphatic endothelial development. *Mechanisms of Development*. 2009;126(5–6):350–365.
- 117 Treutlein B, Brownfield DG, Wu AR, Neff NF, Mantalas GL, Espinoza FH, et al. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature*. 2014;509(7500):371–375.
- 118 Bland RD, Ling CY, Albertine KH, Carlton DP, MacRitchie AJ, Day RW, et al. Pulmonary vascular dysfunction in preterm lambs with chronic lung disease. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2003;285(1):L76–85.
- 119 Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira

- CM, Rabinovitch M, et al. Prolonged mechanical ventilation with air induces apoptosis and causes failure of alveolar septation and angiogenesis in lungs of newborn mice. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2010;298(1):L23–35.
- 120 Hilgendorff A, Parai K, Ertsey R, Jain N, Navarro EF, Peterson JL, et al. Inhibiting lung elastase activity enables lung growth in mechanically ventilated newborn mice. *American Journal of Respiratory and Critical Care Medicine*. 2011;184(5):537–546.
- 121 Wilson WL, Mullen M, Olley PM, Rabinovitch M. Hyperoxia-induced pulmonary vascular and lung abnormalities in young rats and potential for recovery. *Pediatric Research*. 1985;19(10):1059–1067.
- 122 Rabinovitch M, Gamble WJ, Miettinen OS, Reid L. Age and sex influence on pulmonary hypertension of chronic hypoxia and on recovery. *The American Journal of Physiology*. 1981;240(1):H62–72.
- 123 Koppel R, Han RN, Cox D, Tanswell AK, Rabinovitch M. Alpha 1-antitrypsin protects neonatal rats from pulmonary vascular and parenchymal effects of oxygen toxicity. *Pediatric Research*. 1994;36(6):763–770.
- 124 Todd L, Mullen M, Olley PM, Rabinovitch M. Pulmonary toxicity of monocrotaline differs at critical periods of lung development. *Pediatric Research*. 1985;19(7):731–737.
- 125 Johnson RC, Datar SA, Oishi PE, Bennett S, Maki J, Sun C, et al. Adaptive right ventricular performance in response to acutely increased afterload in a lamb model of congenital heart disease: evidence for enhanced Anrep effect. *American Journal of Physiology Heart and Circulatory Physiology*. 2014;306(8):H1222–1230.
- 126 Sharma S, Aramburo A, Rafikov R, Sun X, Kumar S, Oishi PE, et al. L-carnitine preserves endothelial function in a lamb model of increased pulmonary blood flow. *Pediatric Research*. 2013;74(1):39–47.
- 127 Rabinovitch M, Keane JF, Norwood WI, Castaneda AR, Reid L. Vascular structure in lung tissue obtained at biopsy correlated with pulmonary hemodynamic findings after repair of congenital heart defects. *Circulation*. 1984;69(4):655–667.
- 128 Haworth SG, Reid L. Quantitative structural study of pulmonary circulation in the newborn with pulmonary atresia. *Thorax*. 1977;32(2):129–133.
- 129 Haworth SG, Reid L. Quantitative structural study of pulmonary circulation in the newborn with aortic atresia, stenosis, or coarctation. *Thorax*. 1977;32(2):121–128.
- 130 Haworth SG. Total anomalous pulmonary venous return. Prenatal damage to pulmonary vascular bed and extrapulmonary veins. *British Heart Journal*. 1982;48(6):513–524.
- 131 Fung ME, Thebaud B. Stem cell-based therapy for neonatal lung disease: it is in the juice. *Pediatric Research*. 2014;75(1-1):2–7.
- 132 Baker CD, Balasubramaniam V, Mourani PM, Sontag MK, Black CP, Ryan SL, et al. Cord blood angiogenic progenitor cells are decreased in bronchopulmonary dysplasia. *The European Respiratory Journal*. 2012;40(6):1516–1522.
- 133 Alphonse RS, Vadivel A, Fung M, Shelley WC, Critser PJ, Ionescu L, et al. Existence, functional impairment, and lung repair potential of endothelial colony-forming cells in oxygen-induced arrested alveolar growth. *Circulation*. 2014;129(21):2144–2157.
- 134 Lee C, Mitsialis SA, Aslam M, Vitali SH, Vergadi E, Konstantinou G, et al. Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation*. 2012;126(22):2601–2611.
- 135 Fernandez LG, Le Cras TD, Ruiz M, Glover DK, Kron IL, Laubach VE. Differential vascular growth in postpneumonectomy compensatory lung growth. *The Journal of Thoracic and Cardiovascular Surgery*. 2007;133(2):309–316.
- 136 Chamoto K, Gibney BC, Ackermann M, Lee GS, Lin M, Konerding MA, et al. Alveolar macrophage dynamics in murine lung regeneration. *Journal of Cellular Physiology*. 2012;227(9):3208–2115.
- 137 Ackermann M, Houdek JP, Gibney BC, Ysasi A, Wagner W, Belle J, et al. Sprouting and intussusceptive angiogenesis in postpneumonectomy lung growth: mechanisms of alveolar neovascularization. *Angiogenesis*. 2014;17(3):541–551.
- 138 Dane DM, Yilmaz C, Estrera AS, Hsia CC. Separating in vivo mechanical stimuli for postpneumonectomy compensation: physiological assessment. *Journal of Applied Physiology*. 2013;114(1):99–106.

Transcriptional Mechanisms Regulating Pulmonary Epithelial Maturation:

A Systems Biology Approach

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Abstract

This chapter focuses on the perinatal period of lung growth and differentiation. Advances in high-resolution imaging are integrated with computational approaches to develop a “systems biology” approach to the study of perinatal lung development. Confocal microscopy, three-dimensional imaging, single-cell transcriptomics, and bioinformatics are providing deeper insights to the multiple cell types and molecular mechanisms controlling lung formation and function. Knowledge regarding the transcriptional and signaling networks at the single-cell level provide the framework to understand how cell–cell interactions are integrated to form and maintain the three-dimensional structure of the alveoli necessary for ventilation after birth.

Keywords:

3-dimensional imaging, informatics, systems biology, pathway analysis, molecular regulation, epithelial maturation

Introduction

The lung is an intricate, multicellular machine whose sole function is to transport the oxygen and carbon dioxide needed to maintain cellular respiration of the entire organism. Gas exchange requires the delivery of air through conducting airways that terminate in alveoli wherein gas is diffused across epithelial and endothelial cells to the red blood cells within the pulmonary microvasculature. Lung function is dependent on its remarkable architecture, the tissue being comprised of diverse cell types whose locations and cellular activities are precisely orchestrated both during morphogenesis and in response to environmental challenges throughout life. Major advances in understanding the physiology and anatomy of the lung were made in the late 20th century. The application of histology, stereology, immunohistochemistry, and morphometrics provided an ever deeper understanding of the structure of the alveoli (1). Concomitantly, advances in the clinical care of pre-term infants, including surfactant replacement, improved perinatal survival at earlier gestations, with resultant injury and remodeling of peripheral lung tissue, provide a strong rationale to further understand the biological complexities underlying prenatal and perinatal lung formation, function,

and repair. Recent advances in cell, molecular biology, imaging, and computational sciences afford the opportunity to carefully refine our knowledge regarding the diversity of cells and the genetic programs that determine alveolar structure and function in the perinatal period. This chapter will discuss relatively new technologies and approaches being applied to the study of perinatal lung maturation, with an emphasis on new modalities in lung imaging of RNA expression analysis and a “systems biology” approach to understand the biological processes of building and maintaining the alveoli. The integration of complex data from RNA and DNA sequencing, genomics, proteomics, metabolomics, and lipidomics and the ability to interpret increasingly complex data through the application and computational-systems biology offer an increasingly detailed understanding of the processes generating and maintaining lung structure and function.

Advances in Technologies to Study Pulmonary Maturation – a “Systems Biology” Approach

High throughput technologies are increasingly applied to the study of complex biological systems

Systems Biology to Integrate Complex Data

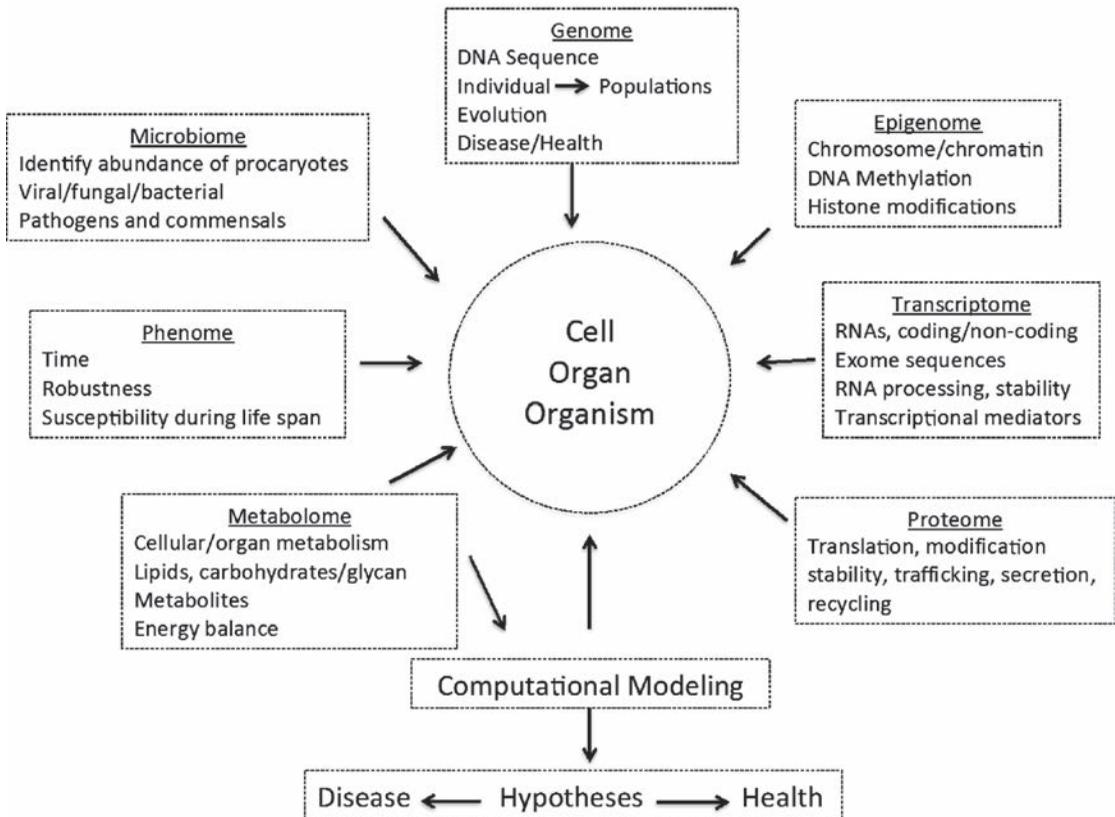


Figure 4-1. Systems biology to integrate complex data.

including organogenesis. “Genomics,” including genome-wide association studies (GWAS), exome and whole genome sequencing, and epigenetics are used to identify disease-related genes and processes (2–7). The “omics” tools of modern science provide multidimensional information from evolution, populations, individuals, organisms, organs, and cells that require the development of new computational approaches needed to interpret ever more complex data (8). Likewise, mass spectroscopy and nuclear magnetic resonance spectroscopy (NMR) enable rapid assessment of proteins, lipids, and metabolic products that can be linked to specific cell types using high-resolution molecular imaging. A great challenge in biology is the integration of detailed quantitative and qualitative data to reveal insights into normal developmental processes and those resulting in disease. Standard, but powerful reductionistic scientific approaches have extended knowledge related to organogenesis and organ function, including the lung (9). Alternatively,

“systems biology” approaches use computational modeling of multidimensional data to model and predict biological mechanisms (10,11) (Figure 4-1). Although the quantitative and qualitative cataloging of data regarding all the measurable molecular components comprising the lung will provide a framework needed to begin to understand its structure and function, such data are not sufficient to understand how the many pieces of the complex puzzle are integrated to build a functional organ like the lung. How multiscaled molecular components of the lung are assembled under the direction of the expression of specific genes, how gene expression is influenced by chromatin, and in turn by transcriptional and post-transcriptional mechanisms that control synthesis and stability of proteins, lipids, and carbohydrates required for cellular functions, remains a considerable challenge in modern biological sciences. Simply stated, it is not enough to identify and quantitate the components of the wiring diagrams that comprise the molecular determinants of a

tissue; discovering how individual components are integrated and assembled, how cellular activities are rheostatically controlled at precise times, and how they are integrated during organogenesis remain a mystery.

Lung Ventilation at Birth Is Dependent on Its Structure

How do the alveoli form? What are the cellular and molecular processes mediating alveolarization and lung maturation? How can static measurements of the cellular and molecular components of the lung be used to predict the dynamic nature of organ function? How important are the dynamic physical forces generated by lung fluid in utero and by the process of ventilation and perfusion to the structures and function of the lung? Although the size and tissue diversity of the conducting airways and large blood vessels have been amenable to quantitative visualization via casting and stereology and more recently by magnetic resonance imaging (MRI) and computerized tomography (CT) scanning, the fine structure of the alveoli have been more challenging to image because of their small size, diverse shapes, and low tissue density (12,13). Recent applications using computer assisted stereology of the developing lung (for example, with STEPanizer) enables rapid quantitation of volumes, lengths, and surfaces of tissue structure (14). X-ray defraction and MRI are unable to resolve alveolar structures at less than 2–3 microns. Imaging technologies, using finite element analysis of high resolution images made by using synchrotron radiation-based X-ray tomograms has furthered the study of the process of alveolarization at a level of resolution of 1.4 μm^3 . This technology is providing insights into the timing and processes involved in prenatal and postnatal growth of the pulmonary acinus and alveoli (13,15,16). With advances in confocal microscopy, immunochemistry, and new procedures to clarify tissue prior to fluorescence imaging, it is now possible to identify specific cell types and their precise anatomic positions during lung maturation (17–21). Changes in lung architecture accompanying the saccular–alveolar period of development in the primate (15,22), rat (23), and mouse (24) all support the concept that alveolar formation begins perinatally in processes that are shared among mammalian species. However, the timing of lung “maturation”

proceeds according to species-specific time lines that are tightly regulated spatially and temporally. Although detailed studies in human alveolarization are limited (25), alveolar growth of the primate lung begins near the time of birth and continues into adulthood (24). In the rat and mouse, alveolar septae are formed primarily after birth within peripheral saccules that were formed by branching morphogenesis (15,26). Septa develop from preformed ridges by lifting off of new septa that further subdivide the saccules into alveoli. In the mouse, the process of alveolarization is most active from postnatal day 4 and continues through day 36. Approximately 5% of the surface area of the peripheral lung is formed prenatally via branching morphogenesis, while 50% of the surface area is created by the process of septation after birth (15). Continued growth of the lung parenchyma occurs after termination of septation, which in the mouse is completed at approximately 36 days postnatally. Thus, there are three major phases in the saccular–alveolar lung maturation in the mouse: (1) the saccular period from E17.5 to PN4 during which branching morphogenesis is completed and epithelial differentiation is induced; (2) the early alveolar period, during which new septa are produced (PN4–21); and (3) a period of isometric lung growth associated with the lengthening of alveolar septa, which continues until maturity. In the early postnatal period of mouse development, septation exceeds lung growth resulting in smaller alveoli. Alveolar size increases thereafter by the isometric growth of tissue that is achieved primarily by elongation, thus increasing the size of the alveoli, a process that continues until completion of lung growth in the mouse. Formation of alveoli is accompanied by the growth and maturation of the pulmonary capillary bed from a double lumen to single lumen alveolar capillary structure that is completed at approximately 21 days of age in the mouse (27). Both vasculogenesis and angiogenesis contribute to the formation of the extensive pulmonary microcirculation characteristic of the mature lung (28–30). Electron micrographic analyses have been a powerful approach to identify the cells contributing to the remarkable architecture during development and at maturity (1,31,32). More recently, use of cell-specific antisera, fluorescence probes, and transgenic mice used for genetic labeling of cells and cell lineages are providing detailed information regarding the

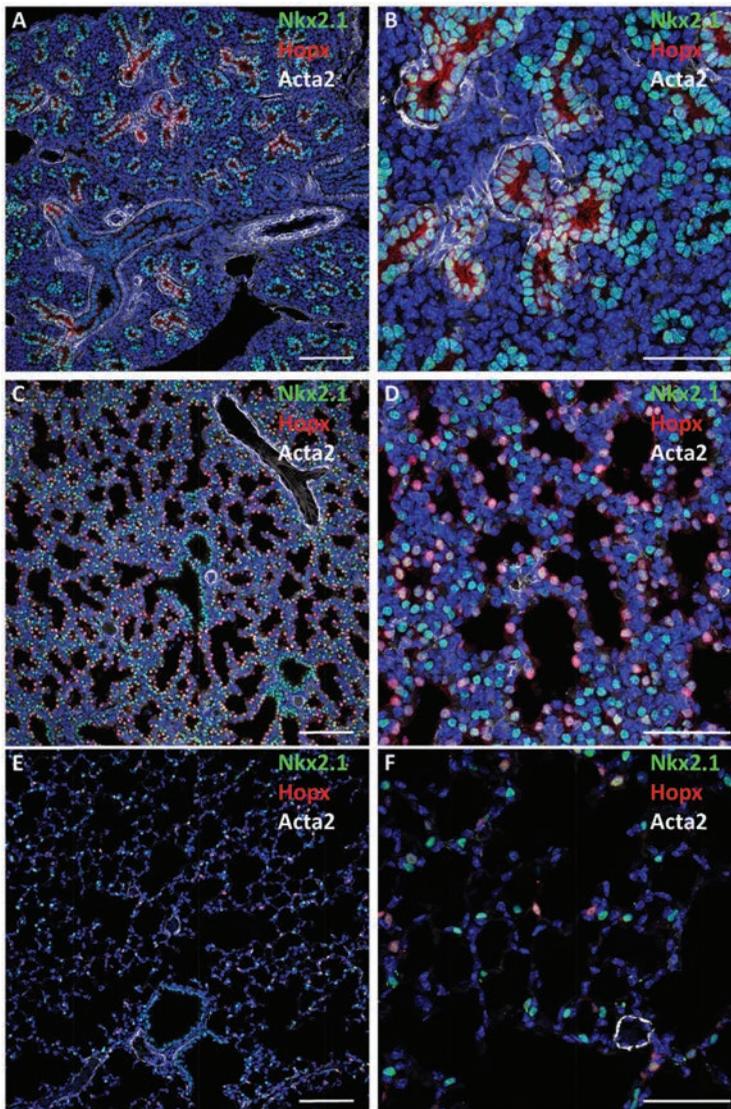


Figure 4-2. Maturation of the lung saccules before birth. Confocal immunofluorescence microscopy was used to image the fetal mouse lung at E16.5 (A, B), E18.5 (C, D), and postnatal lung at PND28 (E, F) demonstrating increasing dilation of the peripheral saccules with advancing age. Nkx2.1 (TTF-1) (green) expressed in epithelial progenitors at E16.5 and 18.5. At PND28, TTF-1 is selectively expressed in cuboidal type II epithelial cells; Hopx (red) is seen in squamous type I cells. As gestation advances, the tubules dilate and the walls of the saccules thin. At PND28, air spaces are prominent and alveolar walls are remarkably thin, enabling efficient gas exchange. α SMA (Acta2) staining of myofibroblasts is shown in white. Bars represent 100 microns (A, C, E) and 40 microns (B, D, F).

cellular and molecular processes mediating branching morphogenesis and alveolarization (Figure 4-2)

Physiological Adaptation to Air-Breathing Depends on Maturation of the Alveolar Epithelium

Perinatal survival is dependent on lung function once the placental circulation ceases at birth. Vital changes in lung and cardiovascular physiology accompany the transition to air-breathing: (1) clearance of lung liquid, (2) enhancement of

pulmonary blood flow during the transition from fetal to postnatal cardiovascular circulation with closure of the foramen ovale and ductus arteriosus, and (3) production and secretion of pulmonary surfactant necessary to reduce surface tension at the gas-liquid interface at the alveolar surfaces. These changes are all required for the perinatal transition. At the cellular level, perinatal lung maturation is linked to the differentiation of the cuboidal alveolar type II epithelial cells that produce and secrete the four surfactant proteins (A, B, C, and D) and lipids (importantly, phosphatidylcholine, the major component of pulmonary surfactant) and the differentiation of more squamous, type I epithelial cells, whose close contact

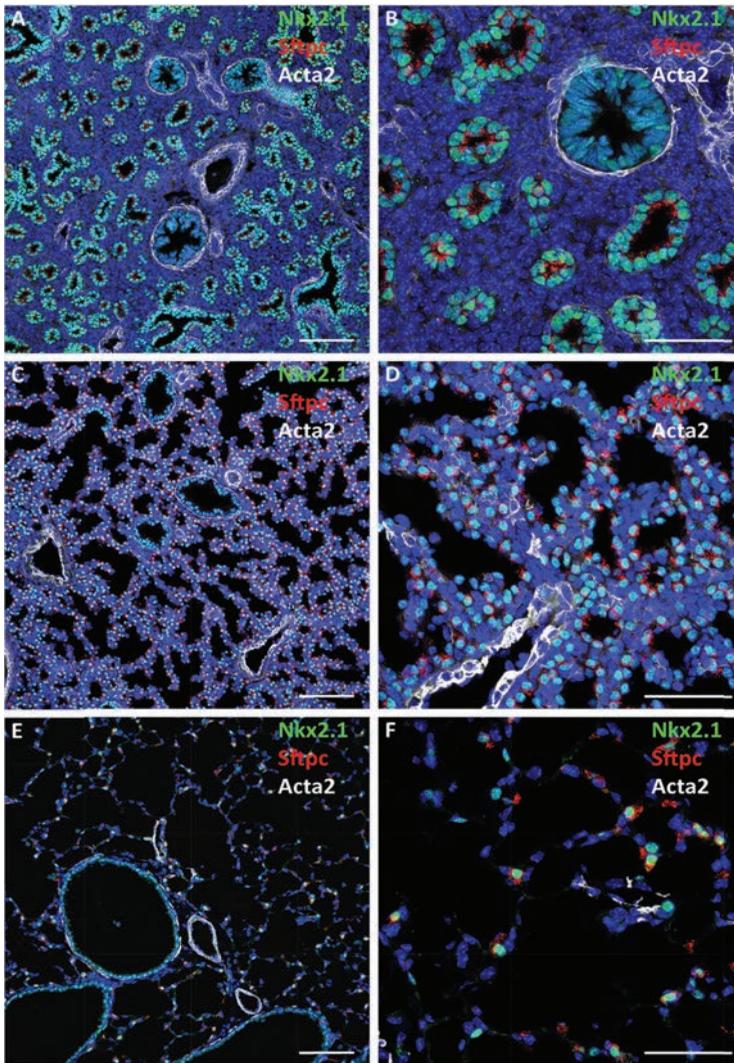


Figure 4-3. Confocal immunofluorescence microscopy shows developmental changes in lung architecture. Staining for proSP-C (red), Nkx2.1 (TTF-1; green), and α SMA (Acta2; white) are shown at E16.5 (A, B), 18.5 (C, D), and adult PND28 (E, F). Dilation of peripheral saccules occurs during maturation in association with increasing expression of the surfactant protein C (Sftpc) in Nkx2.1 stained cells. Bars represent 100 microns (A, C, E) and 40 microns (B, D, F).

with the alveolar capillary endothelial cells enables efficient diffusion of oxygen and carbon dioxide (33, for review) (Figures 4-3 and 4-4). During the latter third of gestation, saccules dilate, mesenchymal components of the lung thin as the pulmonary microvasculature becomes more extensive and is increasingly in close contact with squamous type I epithelial cells lining the peripheral saccules. Dramatic changes in tissue architecture associated with sacculization and alveolarization of the mammalian lung before birth are accompanied by concomitant changes in the differentiation and metabolic functions of the epithelial cells lining the peripheral saccules. These events can be imaged using immunofluorescence confocal microscopy.

The architecture of epithelial and mesenchymal cells contributing to the alveoli and alveolar septa have been studied in detail using electron microscopy (1) and more recently using fluorescence confocal microscopy. In late gestation, the thickness of the lung saccules decreases in association with increasingly prominent microvasculature and the differentiation of type II and type I cells that line the surface of the peripheral lung. In the rodent, alveolarization is marked by the extension of α SMA stained myofibroblasts and pericytes toward the tips of the septa and the invasion of double lumen and capillaries that are covered by squamous type I epithelial cells. The alveolar walls of rodent lungs contain prominent

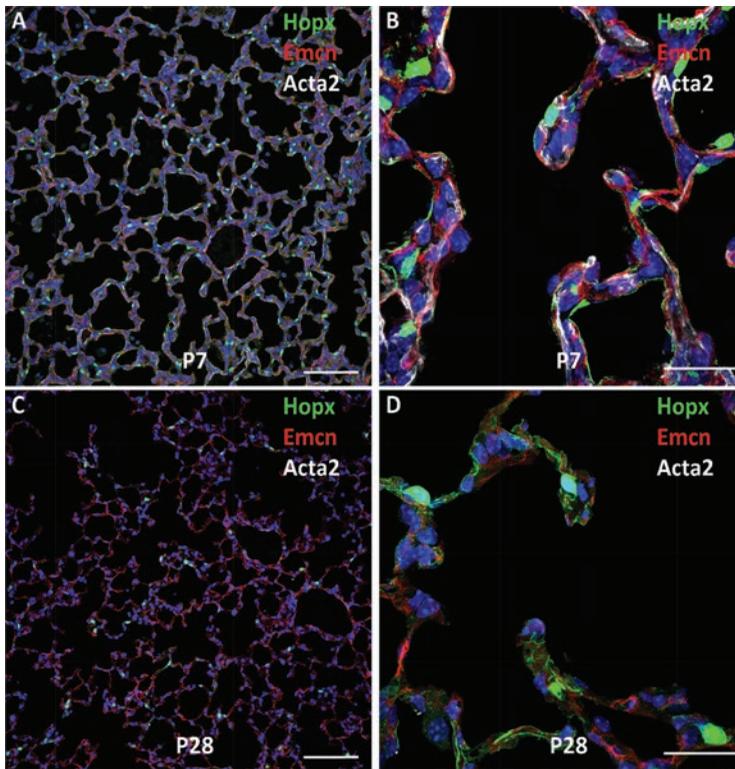


Figure 4-4. Shown are confocal images of mouse lung at PND7 (A, B) and PND28 (C, D). Type I epithelial cells express Hopx (green), alveolar capillary endothelial cells stain for endomucin (Emcn) (red), and myofibroblasts stain for Acta2 (α SMA) in white. Note prominent staining of α SMA during active alveolarization at PND7. Dual capillaries staining for Emcn (red) are seen within the septa. The surfaces of septal and alveolar walls are lined primarily by type I cells that stain for Hopx (green). Bars represent 100 microns (A, C) and 25 microns (B, D).

lipofibroblasts and fibroblasts that stain for neutral lipids. Confocal fluorescence microscopy of some of the distinct cell types comprising alveolar septa in the developing mouse are shown in Figure 4-4.

Integrating Gene Expression Data with Lung Structure During Lung Maturation

A great challenge in lung biology is to integrate structural and genetic data with the cellular processes that determine lung maturation in the perinatal period. Formation of the alveoli requires complex interactions occurring among multiple cell types populating the peripheral lung, including diverse fibroblasts, pericytes, smooth muscle cells, epithelial and endothelial cells that mediate efficient ventilation and perfusion after birth. Proliferation, differentiation, migration, and appropriate placement of cells within the architecture of the peripheral lung saccules are dependent on myriad transcriptional and signaling networks active in each cell that are integrated by interactions with neighboring cells. Precise coordination of cell signaling via direct cell–cell

contact, cell–matrix interactions, and those regulating both paracrine and autocrine cell communication direct the morphogenesis of the peripheral saccules during alveolarization. Ultimately, the spatial organization of the lung is dependent on complex interactions of multiple cell types that function within “ecological” networks. Although analysis of the complex, multicellular interactions is challenging, the application of single-cell transcriptomic analysis, lineage tracing, and cell sorting has begun to shed light on some of the regulatory processes involved in formation of the peripheral lung and maturation of the respiratory epithelium in the perinatal period.

RNA Expression Profiling and Functional Genomics Provide Insights into the Control of Lung Maturation

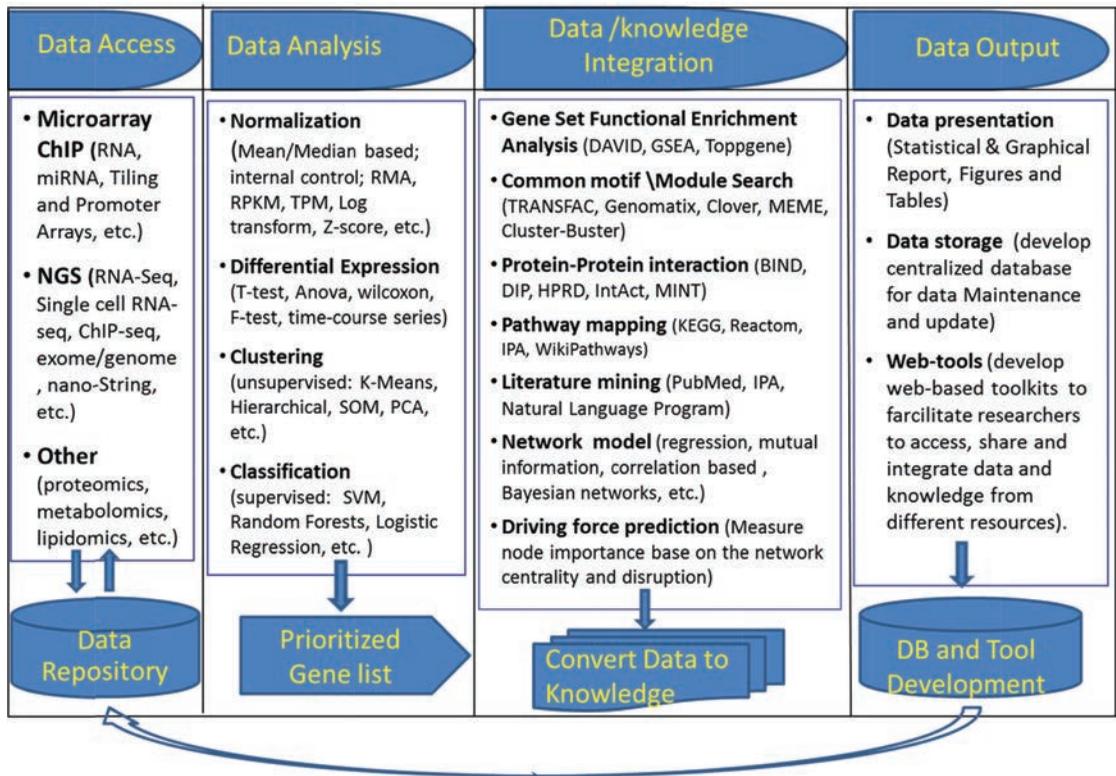
RNA analysis using RT-PCR, RNA microarray, RNA-sequence analyses (RNA-Seq), and chromatin immunoprecipitation (ChIP) are being used to identify genetic and molecular programs mediating

lung maturation, the majority of the data being derived from the study of mouse lung development (34, 35). These and other experiments provide a wealth of expression data whose interpretation and integration with biological functions presents great opportunities for the computational and bioinformatic approaches needed to understand these complex biological systems. Study of gene expression in the lung include the expression profiling of RNAs from whole lung tissue, purified subsets of cells sampled by fluorescence activated cell sorting (FACS) or antibody mediated “panning,” and tissues sampled by laser capture microdissection. Recent advances include single-cell, whole genome analyses using RNA-sequencing (36). Together, these measurements provide rich data resources for the generation of new knowledge and hypotheses regarding lung maturation. A general workflow used in analysis of RNA expression data is given in Table 4-1, the study of which begins with an unbiased assessment of RNA expression data. The workflow consists of four major steps: data access, data analysis, data/knowledge integration, and data output.

Data Access: Data are generated from specifically designed experiments from traditional research, and much of the data are already available in data warehouses. In a systems biology approach, extensive data resources that store and integrate data are available from different technological platforms that can be used to develop a comprehensive model.

Data Analysis: Transcriptomics has evolved rapidly with the advent of microarray and next-generation DNA sequencing technologies for large-scale DNA and RNA profiling. A wide range of statistical approaches have been developed for genome-wide expression data analysis and are readily available. Appropriate choices for these applications depend on the nature of data and experimental design. An understanding of both biology and computational approaches is essential for choosing an analytic pipeline and applying appropriate data mining tools. Some useful approaches for analysis of gene expression profiling include identifying differential expression, “clustering,” and predicting sample classifications using statistical approaches.

Table 4-1. Schema of bioinformatics/systems biology workflow to process high-throughput data. The workflow consists of four major components: data access, data analysis, data/knowledge integration, and data output.



Differentially Expressed Genes: A common approach for transcriptional profiling experiments is to compare gene expression in two different conditions. In experiments without biological replicates, fold change is the simplest way to define differentially expressed genes (i.e., genes with ratios above a fixed cutoff; typically a difference of ± 2 is considered to be significant). False discovery associated with this approach is significant. Replication is essential to enable estimation of variability, which is needed to identify reproducibility of changes among distinct samples. Standard t-tests are useful for detecting significant changes between two groups. Among multiple groups, the ANOVA F-test statistic is appropriate. For data failing to satisfy a normal distribution, nonparametric, rank-based statistics or permutation-based tests are preferred (37,38).

Pattern Recognition and Clustering Analysis: Pattern discovery is widely used to simplify data involving multivariate comparisons; related groups are identified based on expression similarity, which provides an “overview” of the data. Useful dimension-reduction methods include principal components analysis (PCA), independent components analysis (ICA), singular value decomposition (39), and other clustering algorithms. These methods are considered “unsupervised,” meaning that the analysis is derived solely from data and is independent of previous knowledge or classifications. PCA assumes that large variations in the data can be explained by a smaller number of transformed variables. PCA explains the variance-covariance structure of the original data through a few linear combinations of the variables and projects the original data into a new space, for example, in two- or three-dimensional visual representations.

Clustering is a powerful way to explore complex gene expression data and is useful for identifying similarity in expression patterns (40–42). Classical clustering algorithms including K-means, SOM, and “hierarchical” clustering generally emphasize clear group separations; any given entity is assigned to only one cluster. However, many genes have multiple cellular roles and function in cooperation with other interacting partners. Therefore, each gene/RNA/protein can belong to more than one functional class governed by distinct regulatory mechanisms in response to various conditions. Fuzzy heuristic partition (43) considers each gene to be a member

of every cluster, each with variable degrees of membership. In these analyses, “genes” can be assigned to more than one cluster with variable but significant membership, enabling identification of context-dependent regulation. For example, we applied fuzzy clustering by a local approximation of membership algorithm (44) to 194 mRNA microarray samples from 27 distinct mouse models related to lung development and disease. We identified three coexpressed gene clusters that were highly enriched for lung RNAs that influence lipid synthesis, transport, and surfactant homeostasis (45).

Clustering methods are useful in grouping coexpressed genes and provide a basis for the identification of functionally related genes and shared mechanisms by which they are regulated. Alternatively, one can identify their relationship to specific phenotypes in a process used to identify disease-related pathways. Published expression studies of lung RNAs derived after conditional deletion or mutation of specific genes led to the identification a number of transcription factors and signaling molecules that are critical for respiratory adaptation at birth, including NKX2-1 (also termed TTF-1 or thyroid transcription factor-1), FOXA2, C/EBP α , and CNB1. Conditional deletion or mutation of these genes caused phenotypic and biochemical changes similar to those associated with respiratory distress syndrome (RDS), for example, decreased expression of pulmonary surfactant-related lipids and proteins, failure of differentiation of type I and II cells, lack of lamellar bodies, and delayed sacculation (46–50). Meta-analysis of RNA microarray data from these “phenoclusters” showed that although these transcription factors and signaling molecules act via different signaling pathways and bind to distinct cis-elements on transcriptional targets, each is required for normal expression of common transcriptional targets involved in surfactant protein and lipid biosynthesis (e.g., *Abca3*, *Scd1*, *Pon1*, *Sftpa*, *Sftpb*, *Sftpc*, and *Sftpd*), fluid and solute transport (e.g., *Aqp5*, *Scnn1g*, and *Slc34a2*), and innate host defense (e.g., *Lys*, *Sftpa*, *Sftpd*, and *Scgb1a1*), suggesting that FOXA2, CEBP α , CNB1, and TTF-1 (NKX2.1) function in a common transcriptional network regulating genes for perinatal lung maturation and postnatal respiratory adaptation (Figure 4-5A, B).

Data/Knowledge Integration: After identifying differentially expressed genes and coexpressed

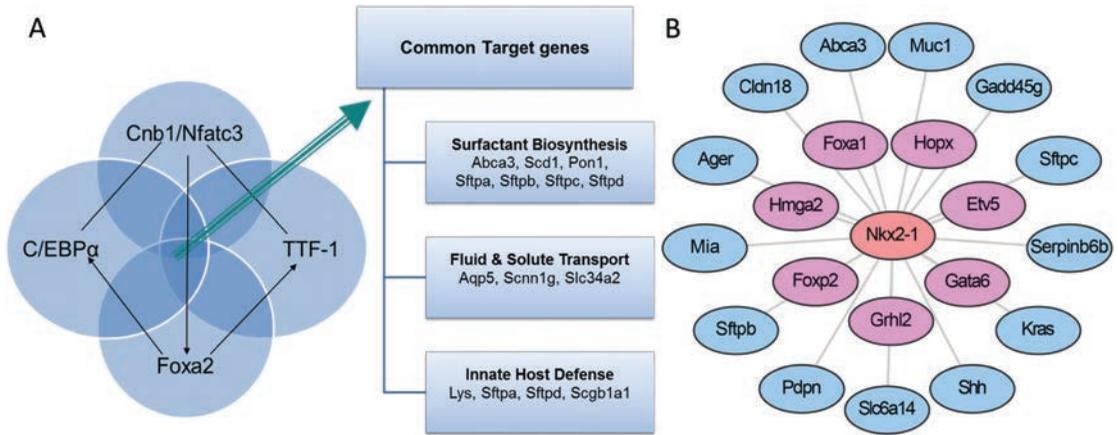


Figure 4-5. NKX2-1 (TTF-1) plays a central role in lung formation and maturation. (A) Meta-analysis of microarrays from mouse models sharing common respiratory distress phenotypes at birth (“phenocluster”). Lung epithelial deletion of *Foxa2*, *Cebpa*, *Cnb1*, and mutation of the mouse *Nkx2-1* gene (encodes TTF-1) identified common transcriptional targets involved in surfactant biosynthesis, fluid and solute transport, and innate defense. (B) An *Nkx2-1* local transcription network was identified as an important transcription factor by statistical “driving force analysis.” The NKX2-1 gene influences expression of numerous genes important for lung epithelial growth, differentiation, and function.

gene groups, general questions to address are: “What is unique about the differentially expressed genes or gene set? Do these genes instruct our understanding of the mechanisms underlying the complex process of lung maturation?” Statistical significance does not directly lead to “biological discovery.” Data integration, via systems biology approaches represents an unbiased way to identify the biological themes that are present in complex data sets.

Gene Set Functional Enrichment Analysis: As each gene is associated with multiple biological annotations from various resources (e.g., gene ontologies, pathways, protein–protein interactions, mouse and human phenotypes, promoter cis-elements), the enrichment of genes in functional categories can be estimated using Fisher’s exact test to compare the occurrence of a given term in the gene set of interest to all available data. Multiple precompiled Web-based functional annotation tools, including Onto-Express (51), GoMiner (52), DAVID (53), GSEA (54), and ToppGene (55), have been developed to identify potential functions of groups of genes.

Biological Knowledge Incorporation: Genome-wide biological knowledge data have been assembled via a number of public/private efforts that include functional annotations and curations of genes/proteins, for example, NCBI Entrez Gene database (<http://www.ncbi.nlm.nih.gov/>), Ensembl (<http://www.ensembl.org/index.html>), GeneCards (<http://www.genecards.org/>),

ENCODE (<http://www.genome.gov/10005107>), Protein Information Resource (PIR: <http://pir.georgetown.edu/>), Kyoto Encyclopedia of Genes and Genomes (KEGG: <http://www.genome.jp/kegg/>), Online Mendelian Inheritance in Man (OMIM: <http://omim.org/>), Human Gene Mutation Database (HGMD, <http://www.hgmd.org>), and Ingenuity knowledge base (IPA: <http://www.ingenuity.com/science/knowledge-base>). These resources provide exceptional depth of present functional knowledge for genes and proteins. Incorporation of knowledge from different genomic and biomedical information resources is useful for “omics” data interpretation. Algorithms for integrating different types of data show promise in combining high-throughput data with knowledge from other clinical or experimental observations.

Network Modeling: A wide range of methods are useful for inferring genome-scale regulatory networks from gene expression RNA datasets (56). A comprehensive assessment of over 30 network inference methods used to analyze microarray datasets from multiple resources was provided by Marbach et al. (57), who concluded that no single method performs optimally across all data sets. In contrast, integrative predictions from multiple inference methods were most useful. Different methods for integration provide complementary advantages and are powerful for optimizing identification of regulatory networks. Such network development and optimization can be achieved via multilevel integration

(i.e., data, knowledge, and method integration, as shown in Table 4-1).

Driving Force Prediction: The identification of essential regulators controlling cell fate and associated biological processes during lung development and maturation is fundamentally important to lung biology and identifies genes to prioritize for experimental validation. An algorithm to quantitatively measure node importance in networks based on node centrality (determine a node connectivity with all other nodes in the network) and/or disruption (determine how the removal of a node in the network affects the network structure) (58, 59) was developed to model regulatory networks important for lung maturation (Guo et al., submitted for publication). Figure 4-5B shows an example of an NKX2-1 subnetwork model based on the single-cell RNA-seq data from E16.5 mouse lung. In that model, TTF-1 (Nkx2-1) is predicted to be an important lung epithelial cell specific driving force functioning via its interactions with other transcription factors, including Gata6, Etv5, Foxp2, Grhl2, and Hopx to regulate target genes, including surfactant proteins, *Abca3*, *Cldn18*, *Shh*, *Kras*, and *Muc1* (Figure 4-5B).

Data Output: Data related to “lung development and disease” has expanded dramatically in the past decade, providing a wealth of data resources useful for both basic science and clinical applications. Given the high volume and complex nature of these data, data storage, maintenance, and utilization becomes a major challenge. A LungMAP consortium (<http://www.lungmap.net/>) provides a freely accessible repository of comprehensive lung development data from different species and developmental time points. This LungMAP is linked to Web-based resources supporting investigations into the processes that regulate lung development. An analytic pipeline (<https://research.cchmc.org/pbge/sincera.html>), relational database, and associated web-tool, termed “LungGENS,” was developed to assist users to query gene expression patterns and cell type RNA signatures in specific pulmonary cells at the single-cell level (<https://research.cchmc.org/pbge/lunggens/default.html>).

Transcriptional Regulatory Network Regulating Lung Surfactant Homeostasis (Static Model): Pulmonary surfactant is required for lung function at birth and throughout life. Lung lipid and surfactant homeostasis requires regulation among multitiered processes, coordinating the synthesis

of surfactant proteins and lipids, their assembly, trafficking, and storage in type II alveolar cells. To generate a transcriptional regulatory network model controlling surfactant homeostasis, we retrieved mRNA microarray samples from a number of distinct experiments in which mouse models were utilized to study lung maturation and function. An algorithm integrating expression profiling with expression-independent knowledge using Gene Ontology (GO) similarity analysis, promoter (gene regulatory sequences) motif, searching, protein-protein interactions, and literature mining were developed to model genetic networks regulating surfactant lipid-related biological processes in lung. A transcription factor (TF)-target gene (TG) similarity matrix was generated by integrating data from different analytic methods. A scoring function was built to rank likely transcription factor-transcription factor regulated targets or TF-TG pairs. Using this strategy, critical components of transcriptional networks directing lipogenesis, lipid trafficking, and surfactant homeostasis in the mouse lung were identified. Within the transcriptional network, SREBP, CEBPA, FOXA2, ETSF, GATA6, and IRF1 were identified as regulatory hubs displaying high connectivity (Figure 4-6). SREBP, FOXA2, and CEBPA were identified as transcription factors forming a regulatory module that controls expression of pulmonary surfactant lipid homeostasis. In turn, this core module comprised of critical transcriptional regulators and their target genes cooperates with other factors to regulate perinatal lung and lipid homeostasis, cell growth, survival, and immune responses (45).

Dynamic Modeling of the Transcriptional Programs Controlling Perinatal Lung Maturation: Genetic, genomic, and bioinformatics methods were used to analyze relationships between the length of gestation and lung maturation in two inbred mouse strains (C57BL/6J and A/J) whose gestational length differed by approximately 30 hr. We were able to identify the genetic regulators that link lung maturation to the timing of birth (60–62). A functional Bayesian statistical approach was used to analyze time-dependent changes in lung mRNAs from each mouse strain from E15.5 to postnatal day 0 (PN0) (63). Dynamic profiling of transcription factors and their targets that changed during lung maturation were matched using STEM (Short Time-series Expression Miner),

a clustering algorithm designed for the analysis of a series of gene expression data sets (64). Through these analyses, we identified both temporal and strain-dependent gene expression patterns during lung maturation, identified key regulators, bioprocesses, and transcriptional networks controlling

lung maturation. Cell adhesion, vasculature development, and lipid metabolism/transport were major bioprocesses induced during the sacculus stage of lung development at E16.5–E17.5 (Figure 4-7). CEBPA, PPARG, VEGFA, CAV1, and CDH1 were found to be key signaling and transcriptional

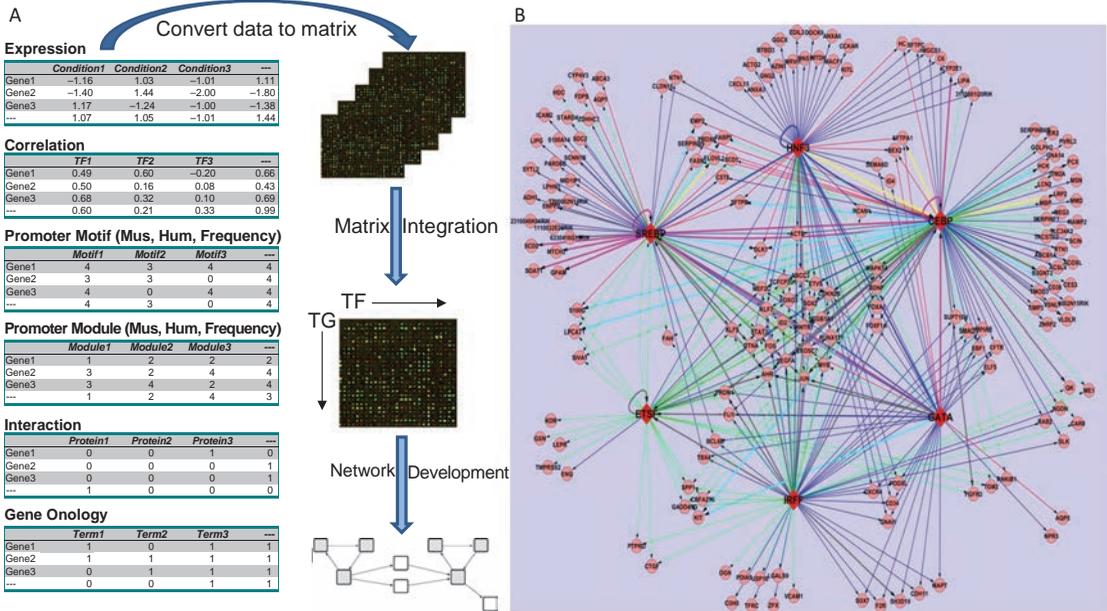


Figure 4-6. Development of a transcriptional regulatory network (TRN) regulating surfactant lipid homeostasis. (A) Work flow for construction of a TRN via data and knowledge integration from multiple independent gene expression profiling studies and expression-independent data (protein interactions, functional annotation, promoter analyses, and literature mining) is shown. We calculated the relative confidence score of TF–TG associations by combining the data. (B) A graphical representation of a subnetwork consisting of predicted TF–TG pairs with confidence cutoff as 0.60 (top 4.5%) and the top 6 TFs with the highest connectivity is shown. The network has 183 nodes and 386 links. Round nodes represent TGs; red diamond nodes represent TFs (Redrawn from Xu et al, *BMC Genomics*, 11:451, 2010).

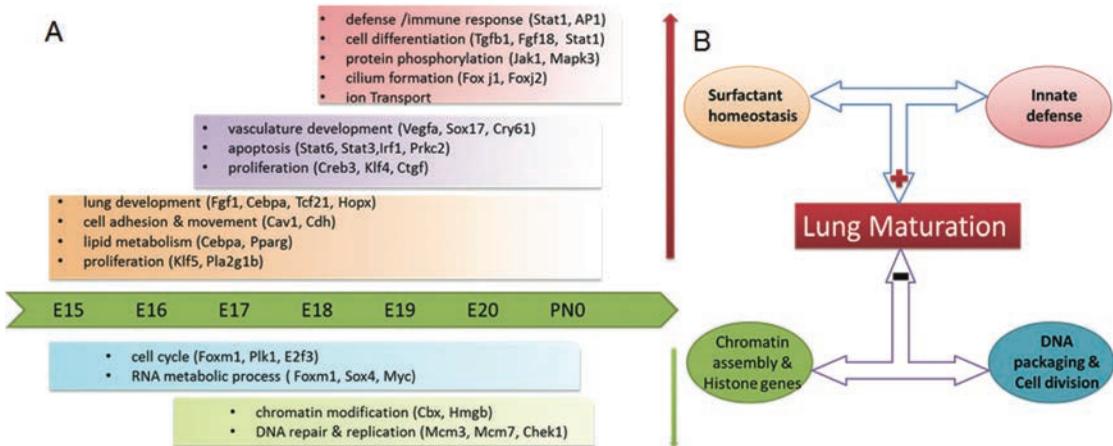


Figure 4-7. Schematized depiction of gestational length and strain dependent effects on lung maturation. (A) Key bioprocesses and regulators controlling lung maturation. (B) Strain effect underlying lung maturation (adapted with permission from Figure 4–9 in Xu. et al., *PLoS One*, 7:e7046, 2012).

regulators of these processes. Innate defense/immune responses were induced at later gestational ages (E18.5–20.5), and STAT1, AP1, and EGFR were important regulators of these responses. Expression of RNAs associated with the cell cycle and chromatin assembly was repressed with advancing prenatal lung maturation, with predicted regulation by FOXM1, PLK1, chromobox, and high mobility group families of transcription factors. Mouse strain-dependent differences in RNA expression patterns were most apparent at E18.5, indicating the earlier maturation of the lung in mice of shorter gestation. At this time, mRNAs regulating surfactant and innate immunity were controlled by the genotype of the dam. These more abundantly expressed genes were in lungs of C58BL6 mice (short gestation) relative to the A/J strain (long gestation), supporting the notion that prior to birth, innate immune responses and surfactant production are critical and “connected” processes that influence cellular behaviors required for respiration and survival after birth (35, 65). Timing of lung maturation and gestational length were determined by the maternal, not paternal, genome in these two mouse strains.

Transcriptional Control of the Genetic Circuits That Regulate Maturation of the Respiratory Epithelium

Changes in lung architecture and cardiopulmonary physiology that accompany the transition to air breathing are mediated by dramatic changes in gene expression that controls cell proliferation and differentiation in pulmonary epithelial cells (35, 36). The identification of the surfactant proteins and genes controlling surfactant lipid homeostasis provided molecular tools (antibodies, cDNAs, and gene promoters) useful for a further exploration of the genetic basis of lung epithelial maturation (33). The precise orchestration of gene expression controlling cell proliferation and differentiation directs the behavior of cells critical for lung function. RNA microarray and RNA-sequence studies utilized in concert with cell lineage tracing, gene deletion, and gene addition experiments have helped elucidate the processes regulating lung maturation. High rates of cell proliferation occur during the period of embryonic lung formation as peripheral lung structures are formed by the process of

branching morphogenesis occurring from E9.5 to approximately E17.5 in the developing mouse. During the saccular period of perinatal lung maturation before birth, cell proliferation decreases in concert with increasing expression of a multiplicity of genes associated with epithelial cell differentiation (35). In the mouse lung, proliferation of peripheral lung cells again increases in the early postnatal period, when the processes of septation and lung growth begin to form the alveolar structures characteristic of the more mature lung. Expression of genes associated with pulmonary maturation, including those encoding the surfactant-associated proteins, particularly those related to host defense, fluid and electrolyte transport, and surfactant homeostasis are induced during this late saccular period in lung development prior to birth (E17.5 to birth) (35, 66). Bioinformatic analyses of the changes in expression of RNAs accompanying the perinatal period provides insight into the regulatory network controlling lung function at birth (35, 45) (Figures 4-6 and 4-7). Although relatively limited data are available at the level of single cells, the ability to identify the genetic processes in specific lung cells provided by single-cell analysis have been integrated with dynamic changes in RNAs that occur during whole tissue development that, together, begin to identify the genetic programs in respiratory epithelial cells, during perinatal lung function (36).

Single-Cell Genomics to Identify Cell-Specific Gene Signatures and Functions During Lung Maturation

While analysis of whole-lung RNA expression profiles have provided an increasingly detailed framework for understanding the integrative processes directing lung maturation, the diversity of cell types, their unique gene expression patterns and functions, and the dynamic interactions of individual cells with their neighbors has been limited by technological constraints in isolating single cells and in the analysis of small quantities of RNAs. Analytic pipelines for single-cell transcriptome analysis and microfluidic separation with the Fluidigm C1 apparatus (67), was developed for analysis of individual lung cells to determine the hierarchical relationships among lung cells during sacculation. This new technology and the analytic strategy identifies a

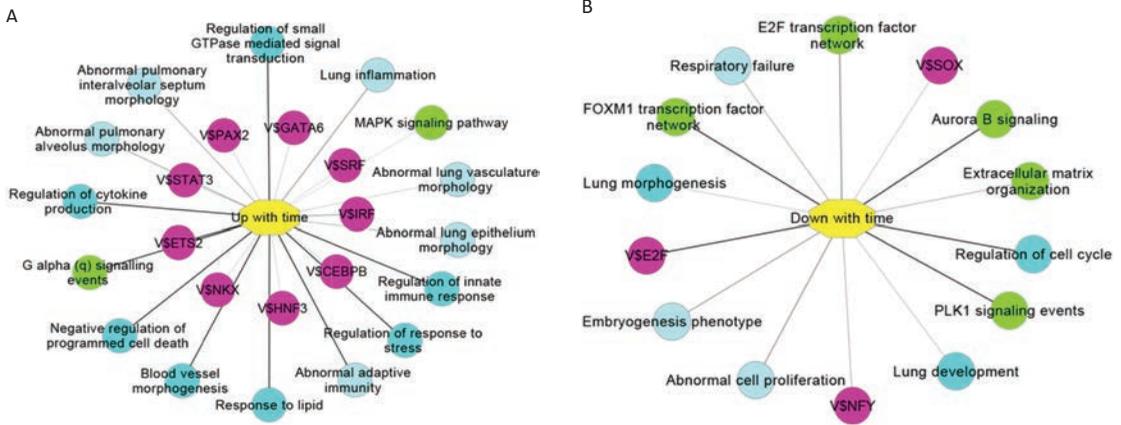


Figure 4-8. Identification of dynamic bioprocesses and key regulators during maturation of Rhesus Macaque lung. Gene functions increasing (A) or decreasing (B) with advancing gestation are shown. Gene sets’ functional enrichment analysis revealed distinct bioprocesses, pathways, and mouse phenotypes associated with the distinct gene sets. Purple color indicates shared transcriptional enhancer binding sites in related genes. Green indicates biological processes.

remarkable diversity of lung cell types, cell-specific gene signatures, key regulators, and bioprocesses. Single-cell RNA profiling provides the framework to delineate cell signaling and communications among distinct cell types and the identification of cell-specific transcriptional regulatory networks, which in turn provides the opportunity to link gene expression with the physiology, function, and phenotype of individual lung cells during development. An analytic pipeline and database of single-cell RNA expression data and its relationship to changes in lung morphogenesis during lung maturation are available at (<https://research.cchmc.org/pbge/sincera.html>).

The Fetal Rhesus Macaque for Study of Lung

Maturation: Although extensive anatomic data regarding lung structure are available for primate models (22, 24), the molecular and genetic programs mediating lung formation and maturation have not been extensively studied. We have utilized the Rhesus primate to assess gene expression during late gestation. Transcriptomic profiling of lung RNA from fetal Rhesus macaque from gestational day 105, 130, and 150 of a 165-day gestation was performed to identify genes and processes controlling formation of the primate lung. The ontogenic changes in gene expression were evaluated for functional enrichment analyses to predict important regulators and the distinct biological processes associated with lung maturation. Importantly, the maturational processes in the primate lung included “blood vessel morphogenesis,” “regulation of innate immune response,”

“response to lipid,” G alpha, and MAPK mediated signaling pathways, whose expression increased with advancing gestational age. Genes and pathways controlling cell cycle, extracellular matrix organization, lung development, and morphogenesis were inversely correlated with advancing gestation age (Figure 4-8). These studies support the concept that the enhanced lung cell differentiation and decreased cell proliferation that occurs with advancing gestation are generally shared between mouse and Rhesus primate.

Transgenic Mice for the Study of Lung Maturation

While bioinformatics analyses of large-scale expression data generate hypotheses regarding perinatal lung maturation, direct experimental validations using in vitro and in vivo models are required to identify molecular mechanisms that will provide the insights needed to develop new diagnostic and therapeutic interventions for prevention and treatment of lung disease. Identification of genes specific for subtypes of respiratory cells, including cells of the microvasculature, pulmonary mesenchymal, and subsets of epithelial cells, have been useful as a molecular “toolkit” to identify cells and processes involved in lung formation and maturation. Gene promoters specific for lung cell types have been highly useful for lineage tracing and for the deletion or mutation of genes critical for lung development. A summary of strategies for manipulating the mouse genome

in the lung was recently provided (68). Both tetracycline and tamoxifen regulatable gene constructs have enabled temporal control of gene editing in various pulmonary cells. Constructs containing regulatory regions of genes selectively expressed in subsets of respiratory epithelial cells make possible the addition, deletion, or mutation of genes for study of lung biology in the perinatal period. Targeted insertion into regulatory regions of genes selectively expressed in subsets of respiratory epithelial cells (for example, *Scgb1a1* (targeting non-ciliated bronchiolar cells); *Foxj1* (ciliated cells); *SFTPC* and *Sftpc* (type II epithelial and progenitor cells); and *Aqa5* (type I epithelial cells) have been useful reagents). Targeted insertion or the use of promoter regions of genes expressed widely in the early embryonic respiratory epithelium (for example, using *Shh* or *Nkx2-1*, *Sox9*, *Sox2*, and *Id2* regulatory regions) have been used to direct gene expression in respiratory epithelial cells. Temporal control of gene addition and deletion is accomplished by the inclusion of either tetracycline or tamoxifen controllable gene constructs. Thus at present, genetic toolkits useful for targeting the diverse cell types in the peripheral lung of the mouse are well developed for experimental use by the field (46, 47, 49, 50, 66, 69–80).

NKX2-1 (aka TTF-1) Plays a Central Role in Lung Formation and Maturation

The important role of Thyroid Transcription Factor-1 (NKX2-1/TTF-1) in the regulation of lung epithelial-specific gene expression was initially identified by its requirement for the transcriptional regulation of genes selectively expressed in the developing and mature lung, including those encoding *SFTPC*, *SFTPA*, *SFTPB*, and *SCGB1A1* (81). NKX2-1 is a nuclear transcription factor expressed in foregut endodermal cells committed to form the primordial lung buds at E8–9 and in varying levels in epithelial cells of the trachea, bronchi, and peripheral lung tubules throughout mouse development (82). Deletion of *Nkx2-1* in the mouse caused severe lung hypoplasia, thyroid agenesis, and tracheal–esophageal fistula (83). Like many transcription factors critical for early determination of cell fate during embryogenesis, NKX2-1 plays a critical role in the maturation of the respiratory epithelium prior

to birth (47). While complete deletion of *Nkx2-1* caused severe pulmonary hypoplasia, hypomorphic mutations in NKX2-1 locus caused by mutation of phosphorylation sites regulating its activity rescued lung formation, but resulted in delayed lung maturation and respiratory failure at birth (47). Defects in sacculation and decreased expression of a number of genes critical for surfactant homeostasis, fluid and electrolyte transport, and innate host defense indicated the importance of these biological processes in pulmonary “maturation.” Analysis of lung RNA microarray data from the *Nkx2-1^{PM}* mice provided insights into both cellular and physiologic processes required for adaptation to air-breathing at birth. Correlation of genes whose expression was decreased by inhibition of NKX2-1 activity with target genes whose regulatory regions bound NKX2-1 protein in chromatin immunoprecipitation experiments (ChIP) was used to predict transcriptional targets of NKX2-1 and its coactivators (71). Thus, *NKX2-1* plays an important role in lung maturation mediated by its interactions with other transcription factors to regulate target genes (Figures 4-5 and 4-6). Many of these genes were subsequently validated by direct experimentation to identify their roles in lung epithelial cell function. Not surprisingly, *NKX2-1* and other genes within the proposed network are involved in the pathogenesis of lung disease in human patients. For example, mutations in *NKX2-1* and its target genes, including *ABCA3*, *SFTPA*, *SFTPC*, *SFTPB*, *SFTPD*, and *SCL34AC*, have been identified as the cause of severe lung diseases in the human (33, for review). Haploinsufficient mutations in *NKX2-1* cause a “thyroid, brain, pulmonary” syndrome associated with hypothyroidism, central nervous system disorders, and interstitial lung disease. Figure 4-5 depicts a subnetwork in which NKX2-1 interacts with other transcription factors and signaling networks to regulate lipid homeostasis, fluid and electrolyte transport, and innate host defense in the developing lung. The temporal, spatial, and stochastic control of the activities of these transcription factors serves to differentiate and maintain respiratory epithelial structure and function prior to and after birth. Recently, a long noncoding RNA (lnc RNA) called NANCI was identified as a critical regulator of the NKX2-1 gene locus, deletion of NANCI partially phenocopies the haploinsufficiency of NKX2-1 (84).

Mesenchymal-Epithelial Cross-Talk Plays a Critical Role in Lung Maturation

The recognition that glucocorticoids (GCs) play an important role in perinatal lung maturation and function was provided by the observation that antenatal steroid administration to the fetal sheep protected lambs from respiratory distress after preterm birth. These seminal experiments resulted in clinical trials in which antenatal glucocorticoids were shown to decrease respiratory distress syndrome (RDS) in preterm infants (85). Antenatal treatments with glucocorticoids are now standard medical practice for women at risk of preterm delivery (86, 87). Although in clinical use for more than 40 years, only recently were the molecular and cellular mechanisms underlying their efficacy identified. Initial *in vitro* studies suggested that the actions of glucocorticoids (GCs) were mediated by their activity on respiratory epithelial cells. Deletion of the GC receptor (*Nr3c1*), a transcription factor required for many of the effects of glucocorticoids, inhibited lung maturation in the mouse, causing perinatal respiratory failure at birth (88). While deletion of *Nr3c1* in embryonic respiratory epithelial cells did not alter lung structure and function, its deletion in cells of the splanchnic mesenchyme inhibited lung maturation and caused respiratory failure at birth, decreasing expression of genes associated with lung maturation and increasing those associated with cell proliferation (88). Likewise, expression of genes encoding proteins selectively expressed in respiratory epithelial cells were decreased, indicating the important role of glucocorticoid signaling in the developing lung mesenchyme for the regulation of respiratory epithelial cell differentiation.

Conclusions and the Future

Pulmonary “maturation” in late gestation depends on precisely choreographed interactions

among multiple cell types, the timing varying among diverse mammalian species. As such, “maturation” is a relative term because growth and differentiation of the lung is not completed at birth. Historically, clinical lung “maturity” has been linked to pulmonary surfactant function in the early postnatal period. Although postnatal ventilation and survival of extremely preterm infants is now possible, lung structure and function are not mature, and pulmonary tissues are subject to injury and remodeling after birth. Understanding the processes involved in the maturation of lung structure and function will inform the development of potential therapies designed to protect the preterm lung from injury/remodeling after birth, seeking to preserve lung architecture necessary for ventilation throughout life. The complexity of the processes by which multiple cell types interact to form the lung presents a daunting task for present-day and future molecular–cellular biology and physiology.

Systems biology provides a process by which diverse and complex data can be integrated to support hypothesis generation and testing. Advances in high-throughput screening technologies will provide an increasing wealth of data regarding the biological processes governing normal lung morphogenesis and differentiation. Understanding the cellular and molecular processes that determine normal lung formation and function will provide the framework needed to interpret the “omic” data derived from pathological tissues from patients with significant lung disease.

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References

- 1 Weibel ER. On the tricks alveolar epithelial cells play to make a good lung. *Am J Respir Crit Care Med.* 2015;191(5):504–513.
- 2 Consortium EP. An integrated encyclopedia of DNA elements in the human genome. *Nature.* 2012;489(7414):57–74.
- 3 Gerstein MB, Rozowsky J, Yan KK, Wang D, Cheng C, Brown JB, et al. Comparative analysis of the transcriptome across distant species. *Nature.* 2014;512(7515):445–448.
- 4 Ramos EM, Hoffman D, Junkins HA, Maglott D, Phan L, Sherry ST, et al. Phenotype-Genotype Integrator (PheGenI): synthesizing genome-wide association study (GWAS) data with existing genomic resources. *Eur J Hum Genet.* 2014;22(1):144–147.

- 5 Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet.* 2009;10(1):57–63.
- 6 Hindorff LA, MacArthur J, Morales J, Junkins HA, Hall PN, Klemm AK, et al. A catalog of published genome-wide association studies (April 2, 2015). Available from: <http://www.genome.gov/gwastudies>.
- 7 Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, et al. The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. *Nucleic Acids Res.* 2014;42(Database issue):D1001–1006.
- 8 Schadt EE, Linderman MD, Sorenson J, Lee L, Nolan GP. Computational solutions to large-scale data management and analysis. *Nat Rev Genet.* 2010;11(9):647–657.
- 9 Hogan BL, Barkauskas CE, Chapman HA, Epstein JA, Jain R, Hsia CC, et al. Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. *Cell Stem Cell.* 2014;15(2):123–138.
- 10 Bunyavanich S, Schadt EE. Systems biology of asthma and allergic diseases: a multiscale approach. *J Allergy Clin Immunol.* 2015;135(1):31–42.
- 11 Kitano H. Computational systems biology. *Nature.* 2002;420(6912):206–210.
- 12 Massaro GD, Massaro D. Formation of pulmonary alveoli and gas-exchange surface area: quantitation and regulation. *Annu Rev Physiol.* 1996;58:73–92.
- 13 Schittny JC, Mund SI, Stampanoni M. Evidence and structural mechanism for late lung alveolarization. *Am J Physiol Lung Cell Mol Physiol.* 2008;294(2):L246–254.
- 14 Tschanz SA, Burri PH, Weibel ER. A simple tool for stereological assessment of digital images: the STEPanizer. *J Microsc.* 2011;243(1):47–59.
- 15 Mund SI, Stampanoni M, Schittny JC. Developmental alveolarization of the mouse lung. *Dev Dyn.* 2008;237(8):2108–16.
- 16 Tsuda A, Filipovic N, Habertur D, Dickie R, Matsui Y, Stampanoni M, et al. Finite element 3D reconstruction of the pulmonary acinus imaged by synchrotron X-ray tomography. *J Appl Physiol* (1985). 2008;105(3):964–976.
- 17 Amos WB, White JG. How the confocal laser scanning microscope entered biological research. *Biol Cell.* 2003;95(6):335–342.
- 18 Inerot S, Heinegard D, Olsson SE, Telhag H, Audell L. Proteoglycan alterations during developing experimental osteoarthritis in a novel hip joint model. *J Orthop Res.* 1991;9(5):658–673.
- 19 Kherlopian AR, Song T, Duan Q, Neimark MA, Po MJ, Gohagan JK, et al. A review of imaging techniques for systems biology. *BMC Syst Biol.* 2008;2:74.
- 20 St Croix CM, Shand SH, Watkins SC. Confocal microscopy: comparisons, applications, and problems. *Biotechniques.* 2005;39(6 Suppl):S2–5.
- 21 Vielreicher M, Schurmann S, Detsch R, Schmidt MA, Buttgerit A, Boccaccini A, et al. Taking a deep look: modern microscopy technologies to optimize the design and functionality of biocompatible scaffolds for tissue engineering in regenerative medicine. *J R Soc Interface.* 2013;10(86):20130263.
- 22 Ten Have-Opbroek AA, Plopper CG. Morphogenetic and functional activity of type II cells in early fetal rhesus monkey lungs. A comparison between primates and rodents. *Anat Rec.* 1992;234(1):93–104.
- 23 Hislop A, Howard S, Fairweather DV. Morphometric studies on the structural development of the lung in *Macaca fascicularis* during fetal and postnatal life. *J Anat.* 1984;138 (Pt 1):95–112.
- 24 Hyde DM, Blozis SA, Avdalovic MV, Putney LF, Dettorre R, Quesenberry NJ, et al. Alveoli increase in number but not size from birth to adulthood in rhesus monkeys. *Am J Physiol Lung Cell Mol Physiol.* 2007;293(3):L570–579.
- 25 Thurlbeck WM. Lung growth and alveolar multiplication. *Pathobiol Annu.* 1975;5:1–34.
- 26 Metzger RJ, Klein OD, Martin GR, Krasnow MA. The branching programme of mouse lung development. *Nature.* 2008;453(7196):745–750.
- 27 Burri PH, Hlushchuk R, Djonov V. Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. *Dev Dyn.* 2004;231(3):474–488.
- 28 Galambos C, Levy H, Cannon CL, Vargas SO, Reid LM, Cleveland R, et al. Pulmonary pathology in thyroid transcription factor-1 deficiency syndrome. *Am J Respir Crit Care Med.* 2010;182(4):549–554.
- 29 Burri PH. The postnatal growth of the rat lung. 3. Morphology. *Anat Rec.* 1974;180(1):77–98.
- 30 Roth-Kleiner M, Berger TM, Tarek MR, Burri PH, Schittny JC. Neonatal dexamethasone induces premature microvascular maturation of the alveolar capillary network. *Dev Dyn.* 2005;233(4):1261–1271.
- 31 Burri PH. Fetal and postnatal development of the lung. *Annu Rev Physiol.* 1984;46:617–628.

- 32 Ten Have-Opbroek AA. Lung development in the mouse embryo. *Exp Lung Res.* 1991;17(2):111–130.
- 33 Whitsett JA, Wert SE, Weaver TE. Diseases of pulmonary surfactant homeostasis. *Annu Rev Pathol.* 2015;10:371–393.
- 34 Cuna A, Halloran B, Faye-Petersen O, Kelly D, Crossman DK, Cui X, et al. Alterations in gene expression and DNA methylation during murine and human lung alveolar septation. *Am J Respir Cell Mol Biol.* 2015;53(1):60–73.
- 35 Xu Y, Wang Y, Besnard V, Ikegami M, Wert SE, Heffner C, et al. Transcriptional programs controlling perinatal lung maturation. *PLoS One.* 2012;7(8):e37046.
- 36 Treutlein B, Brownfield DG, Wu AR, Neff NF, Mantalas GL, Espinoza FH, et al. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature.* 2014;509(7500):371–375.
- 37 Thas O, Clement L, Rayner JC, Carvalho B, Van Criekinge W. An omnibus consistent adaptive percentile modified Wilcoxon rank sum test with applications in gene expression studies. *Biometrics.* 2012;68(2):446–454.
- 38 Tseng GC, Wong WH. Tight clustering: a resampling-based approach for identifying stable and tight patterns in data. *Biometrics.* 2005;61(1):10–16.
- 39 Holter NS, Mitra M, Maritan A, Cieplak M, Banavar JR, Fedoroff NV. Fundamental patterns underlying gene expression profiles: simplicity from complexity. *Proc Natl Acad Sci U S A.* 2000;97(15):8409–8414.
- 40 Kaminski N, Friedman N. Practical approaches to analyzing results of microarray experiments. *Am J Respir Cell Mol Biol.* 2002;27(2):125–132.
- 41 Krajewski P, Bocianowski J. Statistical methods for microarray assays. *J Appl Genet.* 2002;43(3):269–278.
- 42 Slonim DK. From patterns to pathways: gene expression data analysis comes of age. *Nat Genet.* 2002;32 Suppl:502–508.
- 43 Gasch AP, Eisen MB. Exploring the conditional coregulation of yeast gene expression through fuzzy k-means clustering. *Genome Biol.* 2002;3(11):RESEARCH0059.
- 44 Fu L, Medico E. FLAME, a novel fuzzy clustering method for the analysis of DNA microarray data. *BMC Bioinformatics.* 2007;8:3.
- 45 Xu Y, Zhang M, Wang Y, Kadambi P, Dave V, Lu LJ, et al. A systems approach to mapping transcriptional networks controlling surfactant homeostasis. *BMC Genomics.* 2010;11:451.
- 46 Dave V, Childs T, Xu Y, Ikegami M, Besnard V, Maeda Y, et al. Calcineurin/Nfat signaling is required for perinatal lung maturation and function. *J Clin Invest.* 2006;116(10):2597–2609.
- 47 DeFelice M, Silberschmidt D, DiLauro R, Xu Y, Wert SE, Weaver TE, et al. TTF-1 phosphorylation is required for peripheral lung morphogenesis, perinatal survival, and tissue-specific gene expression. *J Biol Chem.* 2003;278(37):35574–35583.
- 48 Lin S, Ikegami M, Xu Y, Bosserhoff AK, Malkinson AM, Shannon JM. Misexpression of MIA disrupts lung morphogenesis and causes neonatal death. *Dev Biol.* 2008;316(2):441–455.
- 49 Martis PC, Whitsett JA, Xu Y, Perl AK, Wan H, Ikegami M. C/EBPalpha is required for lung maturation at birth. *Development.* 2006;133(6):1155–1164.
- 50 Wan H, Xu Y, Ikegami M, Stahlman MT, Kaestner KH, Ang SL, et al. Foxa2 is required for transition to air breathing at birth. *Proc Natl Acad Sci U S A.* 2004;101(40):14449–14454.
- 51 Khatri P, Draghici S, Ostermeier GC, Krawetz SA. Profiling gene expression using onto-express. *Genomics.* 2002;79(2):266–270.
- 52 Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, Sunshine M, et al. GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol.* 2003;4(4):R28.
- 53 Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao Y, Lane HC, et al. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* 2003;4(5):P3.
- 54 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A.* 2005;102(43):15545–15550.
- 55 Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res.* 2009;37(Web Server issue):W305–311.
- 56 De Smet R, Marchal K. Advantages and limitations of current network inference methods. *Nat Rev Microbiol.* 2010;8(10):717–729.
- 57 Marbach D, Costello JC, Kuffner R, Vega NM, Prill RJ, Camacho DM, et al. Wisdom of crowds for robust gene network inference. *Nat Methods.* 2012;9(8):796–804.

- 58 Borgatti SP, Mehra A, Brass DJ, Labianca G. Network analysis in the social sciences. *Science*. 2009;323(5916):892–895.
- 59 Hahn MW, Kern AD. Comparative genomics of centrality and essentiality in three eukaryotic protein-interaction networks. *Mol Biol Evol*. 2005;22(4):803–806.
- 60 Besnard V, Wert SE, Ikegami M, Xu Y, Heffner C, Murray SA, et al. Maternal synchronization of gestational length and lung maturation. *PLoS One*. 2011;6(11):e26682.
- 61 Murray SA, Morgan JL, Kane C, Sharma Y, Heffner CS, Lake J, et al. Mouse gestation length is genetically determined. *PLoS One*. 2010;5(8):e12418.
- 62 Xu J, Liu M, Xia Z. Asian medicine: Call for more safety data. *Nature*. 2012;482(7383):35.
- 63 Angelini C, Cuttillo L, De Canditiis D, Mutarelli M, Pensky M. BATS: a Bayesian user-friendly software for analyzing time series microarray experiments. *BMC Bioinformatics*. 2008;9:415.
- 64 Ernst J, Bar-Joseph Z. STEM: a tool for the analysis of short time series gene expression data. *BMC Bioinformatics*. 2006;7:191.
- 65 Whitsett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nat Immunol*. 2015;16(1):27–35.
- 66 Xu Y, Saegusa C, Schehr A, Grant S, Whitsett JA, Ikegami M. C/EBP α is required for pulmonary cytoprotection during hyperoxia. *Am J Physiol Lung Cell Mol Physiol*. 2009;297(2):L286–298.
- 67 Flatz L, Roychoudhuri R, Honda M, Filali-Mouhim A, Goulet JP, Kettaf N, et al. Single-cell gene-expression profiling reveals qualitatively distinct CD8 T cells elicited by different gene-based vaccines. *Proc Natl Acad Sci U S A*. 2011;108(14):5724–5729.
- 68 Rawlins EL, Perl AK. The a“MAZE”ing world of lung-specific transgenic mice. *Am J Respir Cell Mol Biol*. 2012;46(3):269–282.
- 69 Bridges JP, Xu Y, Na CL, Wong HR, Weaver TE. Adaptation and increased susceptibility to infection associated with constitutive expression of misfolded SP-C. *J Cell Biol*. 2006;172(3):395–407.
- 70 Lian X, Yan C, Yang L, Xu Y, Du H. Lysosomal acid lipase deficiency causes respiratory inflammation and destruction in the lung. *Am J Physiol Lung Cell Mol Physiol*. 2004;286(4):L801–807.
- 71 Maeda Y, Chen G, Xu Y, Haitchi HM, Du L, Keiser AR, et al. Airway epithelial transcription factor NK2 homeobox 1 inhibits mucous cell metaplasia and Th2 inflammation. *Am J Respir Crit Care Med*. 2011;184(4):421–429.
- 72 Matsuzaki Y, Xu Y, Ikegami M, Besnard V, Park KS, Hull WM, et al. Stat3 is required for cytoprotection of the respiratory epithelium during adenoviral infection. *J Immunol*. 2006;177(1):527–537.
- 73 Metzger DE, Xu Y, Shannon JM. Elf5 is an epithelium-specific, fibroblast growth factor-sensitive transcription factor in the embryonic lung. *Dev Dyn*. 2007;236(5):1175–1192.
- 74 Miller LA, Wert SE, Clark JC, Xu Y, Perl AK, Whitsett JA. Role of Sonic hedgehog in patterning of tracheal-bronchial cartilage and the peripheral lung. *Dev Dyn*. 2004;231(1):57–71.
- 75 Mucenski ML, Nation JM, Thitoff AR, Besnard V, Xu Y, Wert SE, et al. Beta-catenin regulates differentiation of respiratory epithelial cells in vivo. *Am J Physiol Lung Cell Mol Physiol*. 2005;289(6):L971–979.
- 76 Wan H, Dingle S, Xu Y, Besnard V, Kaestner KH, Ang SL, et al. Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis. *J Biol Chem*. 2005;280(14):13809–13816.
- 77 Wan H, Luo F, Wert SE, Zhang L, Xu Y, Ikegami M, et al. Kruppel-like factor 5 is required for perinatal lung morphogenesis and function. *Development*. 2008;135(15):2563–2572.
- 78 Xu Y, Ikegami M, Wang Y, Matsuzaki Y, Whitsett JA. Gene expression and biological processes influenced by deletion of Stat3 in pulmonary type II epithelial cells. *BMC Genomics*. 2007;8:455.
- 79 Xu Y, Liu C, Clark JC, Whitsett JA. Functional genomic responses to cystic fibrosis transmembrane conductance regulator (CFTR) and CFTR (Δ 508) in the lung. *J Biol Chem*. 2006;281(16):11279–11291.
- 80 Xu Y, Clark JC, Aronow BJ, Dey CR, Liu C, Wooldridge JL, et al. Transcriptional adaptation to cystic fibrosis transmembrane conductance regulator deficiency. *J Biol Chem*. 2003;278(9):7674–7682.
- 81 Bohinski RJ, Di Lauro R, Whitsett JA. The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol Cell Biol*. 1994;14(9):5671–5681.
- 82 Lazzaro D, Price M, de Felice M, Di Lauro R. The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in

- restricted regions of the foetal brain. *Development*. 1991;113(4):1093–1104.
- 83 Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, et al. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev*. 1996;10(1):60–69.
- 84 Herriges MJ, Swarr DT, Morley MP, Rathi KS, Peng T, Stewart KM, et al. Long noncoding RNAs are spatially correlated with transcription factors and regulate lung development. *Genes Dev*. 2014;28(12):1363–1379.
- 85 Liggins GC, Howie RN. A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. *Pediatrics*. 1972;50(4):515–525.
- 86 Brownfoot FC, Gagliardi DI, Bain E, Middleton P, Crowther CA. Different corticosteroids and regimens for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst Rev*. 2013;8:CD006764.
- 87 Roberts D, Dalziel S. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst Rev*. 2006(3):CD004454.
- 88 Habermehl D, Parkitna JR, Kaden S, Brugger B, Wieland F, Grone HJ, et al. Glucocorticoid activity during lung maturation is essential in mesenchymal and less in alveolar epithelial cells. *Mol Endocrinol*. 2011;25(8):1280–1288.

Environmental Effects on Lung Morphogenesis and Function:

Tobacco Products, Combustion Products, and Other Sources of Pollution

Cindy T. McEvoy and Eliot R. Spindel

Abstract

The fetal and neonatal lung are very sensitive to environmental conditions, which can alter lung development, leading to reduced lung function and increased risk of respiratory illness later in life. This is magnified in that the human lung primarily develops during prenatal life and infancy, after which it increases in size but not complexity. Therefore, early life events can lead to permanent structural changes that translate into lifelong alterations in pulmonary function with increased risk of respiratory disease and, potentially, earlier deterioration of lung function during the normal aging process. The effect of these exposures is dependent on individual susceptibilities, particularly genetic polymorphism and epigenetic changes, as well as the timing, duration, and level of exposures. In utero and early postnatal environmental factors that have been linked to changes in lung development include maternal use of tobacco products during pregnancy, secondhand tobacco smoke exposure, nicotine, and environmental pollution including air pollution and indoor wood-/cookstove exposures. This chapter will focus on how these exposures lead to altered lung morphology and the resulting clinical consequences with particular focus on the effects of in utero tobacco product exposure. Future environmental trends likely to influence lung development include e-cigarette usage and climate change.

Keywords:

Lung development, pulmonary function, smoking, secondhand smoke exposure, indoor air pollution, nicotine, tobacco, pregnancy, e-cigarettes, climate change

Introduction

The fetal and neonatal lung are very sensitive to suboptimal environmental conditions, which can alter lung development/morphogenesis, leading to reduced/altered lung function and an increased risk of respiratory illness later in life. This is magnified by the fact that in humans, the lung develops during prenatal life and infancy, after which it increases in size but not in complexity. Therefore, early life events can lead to permanent structural changes that translate into lifelong altered pulmonary function with an increased risk of respiratory disease and may contribute to early deterioration of lung function during the normal aging process. The effect of these influences is dependent on individual susceptibilities, particularly genetic predispositions and epigenetic changes, as well as the timing, duration, and level of exposures. In utero and early postnatal

environmental factors that have been linked to changes in lung structure include maternal use of tobacco products during pregnancy, secondhand tobacco smoke exposure, and exposure to environmental pollution including air pollution and indoor wood-/cookstove related exposures. This chapter will focus on how environmental exposures lead to altered lung morphology and the clinical consequences that follow, with particular focus on the effects of in utero tobacco product exposure. Future trends likely to influence environmental effects on lung development include e-cigarette usage and climate change.

As discussed in the opening chapters of this book, lung development can be roughly divided into the pseudoglandular, canalicular, saccular, and alveolar stages of development (1,2). During the pseudoglandular period that lasts from 5 to 17 weeks gestation, the majority of conducting airway

lung branching occurs. During the canalicular stage, from 17–27 weeks, differentiation of airway epithelium begins, and the majority of vascular development for the conducting airways occurs. There is also a beginning of formation of airspace development with some development of respiratory bronchioles. During the saccular period, from 27–approximately 36 weeks, the terminal respiratory sacs develop and begin to be separated by secondary crests as forerunners to alveoli. The alveolar period then extends from approximately 36 weeks to birth, and alveolization continues after birth for at least 3–4 years (1,2). Thus, depending on the time of environmental exposure, different aspects of lung development will be affected with different resulting sequelae. It is also important to note that immune phenomenon that may play a role in asthma sensitivity will be linked to stages of immune development as well as stages of lung development.

The Effects of In Utero Tobacco Product Exposure on Lung Development/Morphogenesis and Clinical Outcomes

Maternal smoking during pregnancy is the largest preventable cause of low birth weight (LBW), prematurity, and perinatal mortality (3,4). Maternal smoking during pregnancy is also the largest preventable cause of childhood respiratory illness, and children whose mothers smoked during pregnancy show lifetime decreases in pulmonary function and increased respiratory illnesses and asthma (5–7). Maternal smoking is estimated to cause 10% of direct medical expenditures in the first year of life (8), and Stoddard and Gray (9) estimated that approximately 20% of expenditures for childhood respiratory illness are caused by maternal smoking, amounting to \$660 million annually in 1997 dollars.

The decreases in offspring pulmonary function caused by maternal smoking during pregnancy have been very clearly documented (10–17). One of the initial reports indicating a connection between maternal smoking and children's respiratory function was from Tager et al. (10), who reported decreases of 7–10% in the forced expiratory volume in one second (FEV₁) in children 1–5 years of age with smoking mothers. Hanrahan et al. (15) examined pulmonary function of infants shortly after birth (~4.2 weeks) as a function of

maternal smoking during pregnancy and found a significant decrease in maximal expiratory flow at functional residual capacity (V_{max}FRC). Similarly, Hoo et al. (17) found a significantly decreased time to peak tidal expiratory flow to expiratory time ratio (TPTEF: TE) in premature infants studied at a corrected gestational age of 36 weeks whose mothers smoked during pregnancy. This demonstrates that the changes in pulmonary function tests after in utero tobacco smoke exposure are not caused just by exposures at the end of the gestation. These deficits appear permanent, as Cunningham et al. (18) performed tests on 8,800 nonsmoking schoolchildren ages 8–12 and found reduced forced expiratory flows in children whose mothers smoked during pregnancy. A recent prospective study with a 21-year follow-up has now extended the decreases in FEV₁ and FEF₂₅₋₇₅ (forced expiratory flows between 25% and 75% of FVC) in males (7).

Mechanisms Underlying Effects of Tobacco Products and Nicotine on Lung Development

Although epidemiologic studies clearly show that in utero tobacco smoke exposure leads to decreased pulmonary function in offspring, there are multiple questions regarding mechanism. There are multiple potential mediators of the effects of tobacco smoke exposure on lung development. Tobacco smoke is commonly described as containing 3500–5000 different chemicals, many of them toxic and carcinogenic (19,20). For components of tobacco smoke to affect the fetus, they have to be absorbed by the maternal lung, enter the bloodstream, and either affect placental function or cross the placenta and affect fetal development. Components in tobacco smoke that meet these criteria include the many toxins such as aromatic hydrocarbons, carbon monoxide, and nicotine. Although all these undoubtedly affect lung development, experimental data suggest that nicotine is the primary mediator of the decreases in offspring pulmonary function caused by maternal smoking during pregnancy.

Role of Nicotine in Modifying Lung Development

In rodents, exposure to prenatal tobacco smoke leads to abnormal lung development. In a rat model, Collins et al. (21) showed that prenatal cigarette

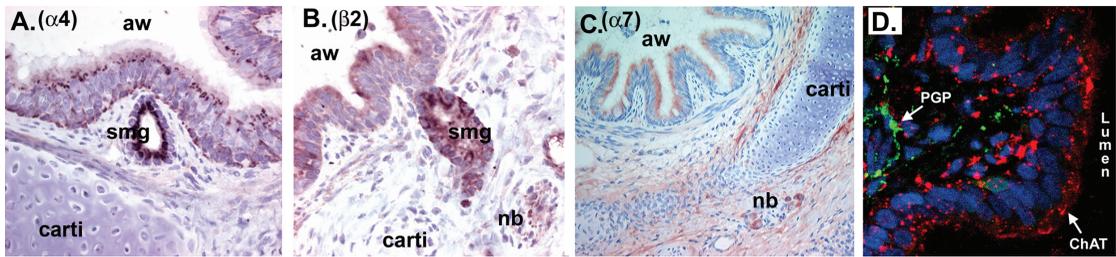


Figure 5-1. Expression of nAChR in fetal monkey airway (134 days gestation). (A) $\alpha 4$ nAChR immunostaining (purple) in airway epithelial cells and submucosal glands (200x); (B) $\beta 2$ immunostaining (purple) in airway epithelial cells and submucosal glands (200x) (C) $\alpha 7$ (red) immunostaining in airway epithelial cells and airway fibroblasts (100x). (D) Choline acetyltransferase (ChAT) immunostaining in red, PGP a nerve fiber marker in green. ChAT, the enzyme that synthesizes ACh is clearly present in airway epithelial cells and distinct from nerve fibers. aw = airway, smg = submucosal gland.

smoke caused reduced lung volume, decreased number of saccules and septal crests, and decreased elastin fibers in fetal lungs. Maritz and coworkers (22–24) then showed that prenatal plus early post-natal nicotine exposure by itself caused similar changes, including decreased alveolar septation, decreased elastin, and increased lamellar bodies in rat pup lungs. The data from Maritz and coworkers were the first suggestion that nicotine was the critical component in tobacco smoke to alter lung development. Providing an explanation for the actions of nicotine on lung development, Spindel and coworkers demonstrated extensive expression of nicotinic acetylcholine receptors (nAChR) in fetal monkey lung (25) as part of a cholinergic autocrine loop in which developing airway epithelium synthesized and secreted acetylcholine (ACh) that could then interact with the nAChR in the developing lung (26).

The developing lung expresses high levels of nAChR (Figure 5-1). The nAChR are ligand-gated ion channels composed of five homologous subunits arranged around a central ion channel, such that binding of acetylcholine (ACh) allows ion flow through the channel (primarily calcium and sodium) (27,28). Ligand binding also activates kinase cascades by a not yet understood mechanism (27,29). ACh is the endogenous ligand for nAChR, and nicotine is an exogenous ligand for nAChR that can disrupt normal development. Fourteen genes that code for neuronal nicotinic subunits have been identified to date; 4 β subunits and 10 α subunits. nAChR can be heteromers composed of both α and β subunits, or homomers composed of one type of α subunit. Nicotinic receptors composed of $\alpha 4$ and $\beta 2$ subunits ($\alpha 4\beta 2$) are the most common heteromeric nAChR and receptors composed only of $\alpha 7$ subunits are

the most common homomeric nAChR. Depending on subunit structure, nicotine can have no effect, strongly or weakly activate, or strongly or weakly inhibit. Thus smoking enables nicotine crossing the placenta to inappropriately activate or inhibit nAChR in the developing lung and influence pulmonary morphogenesis.

Treatment of pregnant rhesus monkeys with low levels of nicotine designed to simulate the nicotine exposure of pregnant human smokers increased levels of $\alpha 7$ nAChR in airway epithelial cells and fibroblasts in fetal monkey lung (Figure 5-2) and increased collagen in a similar distribution (25,30,31). As observed in studies in rodents, prenatal nicotine exposure decreased levels of elastin (Figure 5-2). In the rat model, nicotine caused lung hypoplasia, reduced surface complexity of developing alveoli, caused discordant growth of the airways and alveoli, and increased the number of alveolar type II epithelial cells. Consistent with increased type II cells, the expression of surfactant protein B (SP-B) and SP-A were increased (25).

Nicotine-induced changes in lung structure translated into alterations in pulmonary function in the newborn rhesus monkeys similar to those measured in children born to smoking mothers (Figure 5-3) (32). In the pregnant rhesus model, dams were infused subcutaneously with nicotine at 1.5 mg/kg/day or saline from days 26 to 160 of gestation (term is 165 days). Cesarean sections were done at 160 days and pulmonary function measured at 24 hours of age. Nicotine significantly decreased lung volume and forced expiratory volume (FEV 0.2), peak tidal expiratory flow during tidal breathing, and mean mid-expiratory flow (MMEF or FEF 25%–75%) when compared to saline controls. Pulmonary resistance was significantly

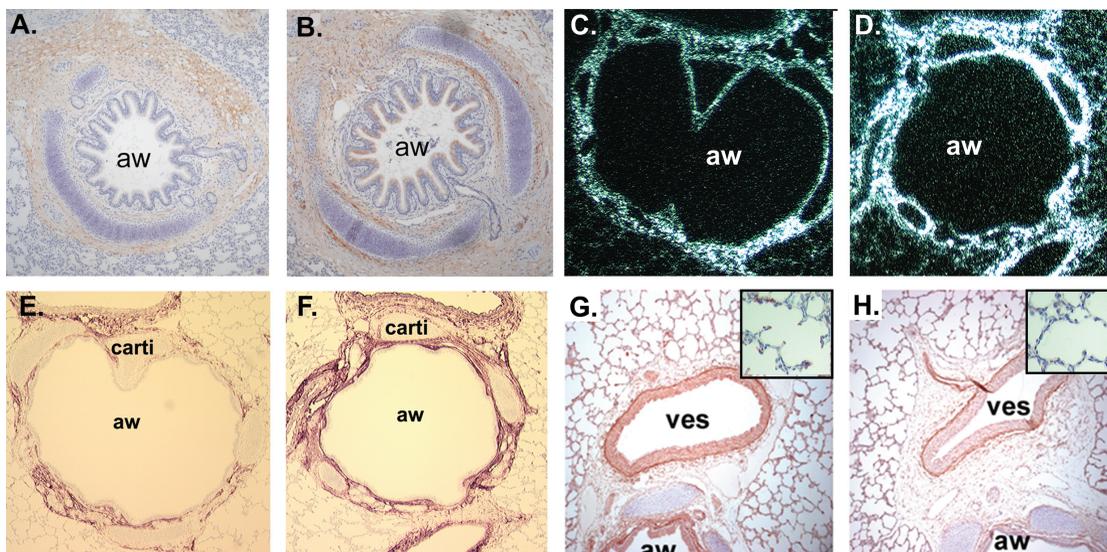


Figure 5-2. Prenatal nicotine exposure increases $\alpha 7$ nAChR and collagen expression but decreases elastin expression in fetal monkey lung (134 days gestation). (A) $\alpha 7$ nAChR immunostaining of 134-day fetal lung. Chromogen = AEC (red), 100X. (B) $\alpha 7$ nAChR immunostaining of 134-day nicotine-exposed lung. (C, D) In situ hybridization showing collagen $\alpha 1$ (III) mRNA expression in lung from control and nicotine-exposed animals (100X). (E, F) Collagen III immunostaining in lungs from control and nicotine-exposed animals. Chromogen = AEC (red), 100x. aw = airway, carti = cartilage. (G, H) Elastin immunostaining in airway associated vessels from control and nicotine-exposed animals. Insert boxes show elastin staining in tips of secondary septi (400x). Modified from Sekhon et al. (30,31).

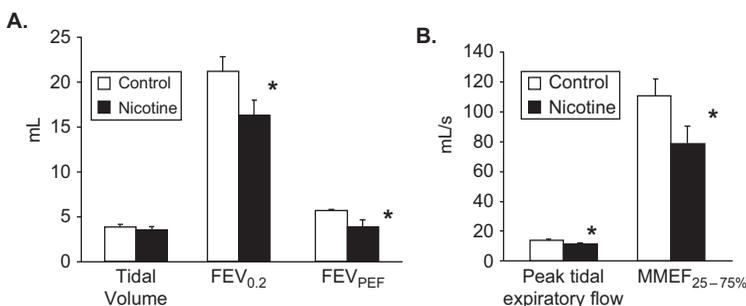


Figure 5-3. Effect of prenatal nicotine exposure on tidal and forced-expiratory flows and volumes of newborn monkeys. Values are means \pm SE. FEV_{0.2} (forced expiratory volume during first 200 milliseconds, FEV_{PEF} (forced expiratory volume at peak expiratory flow), MMEF_{25-75%} (mean mid-expiratory flow). * $p < .05$ compared to control. Data modified from Sekhon et al. (32).

increased while static and dynamic lung compliance decreased, but not significantly (Figure 5-3) (32). Increased collagen and decreased elastin caused by prenatal nicotine exposure likely underlies the decreased compliance caused by nicotine (Figure 5-2).

Changes in pulmonary function caused by prenatal nicotine exposure were similar to those seen in human infants exposed to maternal smoking during pregnancy. These findings strongly suggest that nicotine is the component in tobacco smoke that impairs normal lung development and leads to decreased pulmonary function in offspring of women who smoked during pregnancy.

Studies in mice provide a potential mechanism by which prenatal nicotine exposure leads to decreased expiratory flow. In embryonic murine lung explants cultured in vitro, nicotine stimulated lung branching and caused dysanaptic lung growth in a dose-dependent fashion. Effects of nicotine were dependent on the presence of the $\alpha 7$ nAChR receptors (33). In a murine model of in utero nicotine exposure in which pregnant mice were treated with nicotine from gestation day 7 to postnatal day 14, pre- and postnatal nicotine exposure significantly decreased forced expiratory flows in the offspring, findings similar to those in humans and monkeys (34). Effects of nicotine were blocked in $\alpha 7$ nAChR gene deleted

mice (34). When mice were exposed to nicotine during gestation days 7–21, gestation days 14 to postnatal day 7, and postnatal days 3–15, only exposure from prenatal day 14 to postnatal day

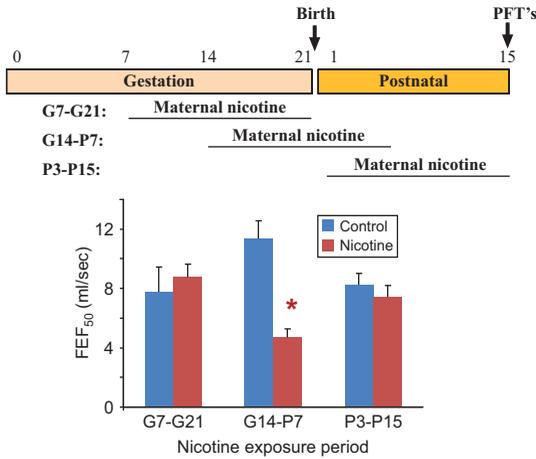


Figure 5-4. The critical period for perinatal nicotine exposure to affect pulmonary function in mice. C57BL6/J mice were mated and pregnant/lactating females treated with nicotine (2 mg/kg/day) for 2-week windows of exposure from gestation day 7 to gestation day 21, gestation day 14 to postnatal day 7 and from postnatal day 3 to postnatal day 15. On postnatal day 15, mice were evaluated by pulmonary function testing as previously described (34). Only wild-type mice treated with nicotine from gestation day 14 to postnatal day 7 had significant decreases in forced expiratory flows. Error bars denote \pm SD, * $p < 0.05$ compared to control, $n = 6-8$ animals in each group. (FEF₅₀ = forced expiratory flows at 50 msec.) Similar effects were also seen for FEF₇₅. Modified from Wongtrakool et al. *Am J Respir Cell Mol Biol.* 2012;46:695–702.

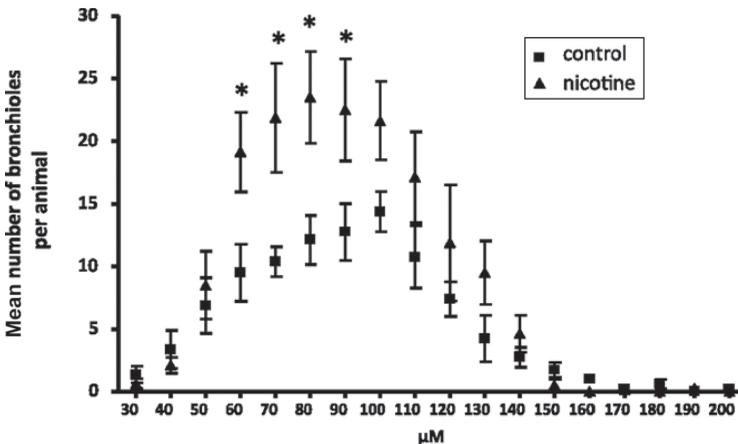


Figure 5-5. Adult mice exposed to prenatal nicotine have significantly greater numbers of small bronchioles compared with unexposed control animals. C57BL6/J female mice were administered either water or water containing 100 $\mu\text{g}/\text{mL}$ nicotine before timed breeding and throughout gestation. Lungs were from age-matched offspring at 8 weeks of age. Whole lungs were embedded and sectioned in an isotropic, uniform random manner to create unbiased samples for examination. Airway diameter was measured for each visible bronchus and bronchiole. Mice exposed to prenatal nicotine had significantly greater numbers of small bronchioles, with diameters between 60 and 90 μm ($n = 8$ per group). Error bars denote SD. * $P < 0.05$ compared with control. Reproduced with permission of the American Thoracic Society, Copyright 2014; from Wongtrakool et al. *Am J Physiol Lung Cell Mol Physiol.* 2007;293:L611–L618.

7 decreased forced expiratory flows in the offspring (Figure 5-4) (34). This time period in mouse lung development includes the pseudo-glandular, canalicular, and sacular periods, but occurs before most of the alveolar period (35), a finding supporting a primary effect of nicotine on airway growth. Stereologic analysis of airway size and diameter showed an increased number of airways of small diameter after nicotine exposure (Figure 5-5). These data support the concept that prenatal nicotine exposure decreased forced expiratory flows by simulating epithelial cell growth and altering airway morphogenesis to resulting in longer and more torturous airways; thus forcing airflows through narrower tubes. After prenatal nicotine, exposure to methacholine caused increased airflow resistance in adulthood, even in the absence of allergic sensitization, consistent with a link between maternal smoking during pregnancy and increased risk of childhood asthma.

Thus, based on data from animal models, the effects of smoking during pregnancy on pulmonary function in the offspring are likely mediated by nicotine acting to stimulate nicotinic receptors expressed in the developing lung, mediated in part by $\alpha 7$ -nAChR. Effects of nicotine are associated with increased collagen production and altered airway geometry at critical periods in the latter part of pregnancy.

Clinical Sequela of In Utero Tobacco Exposure

Multiple studies have shown increased lower respiratory illness in infants born to mothers who smoke (36–39). Taylor and Wadsworth (38) studied 12,743 children and found significantly increased bronchitis and hospital admissions for lower respiratory illness in children from smoking mothers. Tager et al. (40) found increased lower respiratory illness with prenatal smoke exposure but not with postnatal exposure. Increased wheezing and asthma in children born to mothers who smoked during pregnancy have been repeatedly documented (11,41–46). Prenatal exposure is more deleterious than postnatal exposure. A large meta-analysis drawing data from 79 prospective studies (47) estimated the effects of prenatal and postnatal smoking by the mother, the father, or any other household member during three different age ranges of the offspring: 0–2, 3–4, and 5–18 years of age. Prenatal maternal smoking had the strongest effect on asthma in children ≤ 2 years old. A pooled analysis of eight European birth cohorts (48) demonstrated that maternal smoking during pregnancy increased the risk of wheeze and asthma among children who were not exposed to maternal smoking after birth. A recent study also showed a highly significant interaction between LBW, maternal smoking during pregnancy, and risk of the offspring developing asthma (45).

There appears to be a direct link between decreased indices of offspring pulmonary function (primarily decreases in forced expiratory flows in the small airways) caused by maternal smoking during pregnancy and increased rates of respiratory illness. Decreased pulmonary function in infants was correlated with increased rates of respiratory illness (40,49–55). A prospective birth cohort study of 802 healthy babies in Norway (52), with follow-up from the newborn period through 10 years of age, demonstrated that infants with measurements of TPTEF: TE at or below the median shortly after birth have a history of asthma (24.3% vs. 16.2%, $p = 0.01$; OR of 1.58); have current asthma (14.6% vs. 7.5%, $p = 0.005$; OR of 2.10); and have severe bronchial hyperresponsiveness (9.1% vs. 4.9%, $p = 0.05$) at 10 years of age (52). From this same cohort, infants whose passive respiratory compliance was at or below the median shortly after birth were more likely to have a history of asthma

(27.4% vs. 14.8%, $p = 0.001$; OR of 2.18) and current asthma (15.0% vs. 7.7%, $p = 0.009$; OR of 2.01). Similarly, Tager et al. (40) showed that the decreased VmaxFRC seen with prenatal smoke exposure correlated directly with increased lower respiratory illnesses (LRIs). An increased risk of wheezing in the first years of life was reported in children with a decreased ratio of TPTEF: TE measured in the first week of life (53) or at 3 months of age (50,53), and in those with reduced VmaxFRC at 1, 3, and 6 months of age (40,50,54,56). Another population-based longitudinal study demonstrated that infants less than 6 months of age with a decreased VmaxFRC developed wheezing and lower respiratory tract illnesses in the first year of life (40). In addition, results from the Tucson Children's Respiratory Study demonstrated that children in the lowest quartile of pulmonary function continue to have decreased pulmonary functions as young adults, putting them at increased risk for developing chronic obstructive pulmonary disease (COPD) as adults (55,57).

These findings emphasize the importance of in utero or early life interventions to promote optimal lung development and function. A recent double-blind study (58) randomized pregnant smokers to daily vitamin C (500 mg/day) versus placebo during pregnancy and demonstrated that offspring born to women who received vitamin C had significantly improved newborn pulmonary function tests and decreased wheezing through 1 year of age. These findings suggest that in utero interventions offer the potential to influence factors related to the fetal origins of childhood and adult respiratory disease.

Implications for Nicotine Replacement Therapy During Pregnancy

A variety of nicotine replacement products, including nicotine-containing gums, patches, lozenges, and sprays, are currently available to potentially reduce the craving for smoking and thereby potentially decrease the pregnant smoker's exposure to other toxins present in cigarette smoke. Unfortunately, there is a strong body of evidence that nicotine itself is an important mediator of the adverse effects on the fetal lung. There is no evidence supporting a safe dose for nicotine replacement therapy during pregnancy. In addition, there is insufficient

evidence from randomized trials of nicotine replacement therapy (NRT) to determine whether or not NRT is effective or safe to promote smoking cessation during pregnancy and whether it has positive or negative impacts on birth outcomes. Coleman et al. randomized 1,050 pregnant smokers to active nicotine patches versus placebo patches. Both groups also received behavioral cessation support. There was no difference in the rate of abstinence from the quit date until delivery between the two groups, although the compliance of both groups was low. A recent Cochrane review (59) examined the safety and efficacy of six trials of NRT in 1,745 pregnant smokers. No significant differences for smoking cessation or other important birth-related outcomes were demonstrated between randomized groups.

Secondhand Tobacco Smoke Exposure

Despite the implementation of laws requiring smoke-free public and working places, secondhand tobacco exposure remains a significant burden to those who do not actively smoke. This is particularly true for developing fetuses and young children who spend the majority of time in their home environment, where secondhand smoke (SHS) exposure is present. Multinational studies report that up to 40% of pregnant nonsmokers are exposed to SHS due to smokers in their home environment. SHS consists of exhaled smoke as well as sidestream smoke that is released from a burning cigarette between inhalations, both of which have very similar compositions. Although the composition of SHS changes as it is diluted with ambient air in the environment and interacts with other compounds, SHS continues to contain significant amounts of nicotine (60). The effects of SHS exposure during pregnancy on lung development will likely depend on the relative amounts of nicotine and other toxins crossing the placenta. There is evidence from a recent meta-analysis that exposure of pregnant nonsmokers to SHS can reduce the mean birth weight of offspring by up to 33 grams and increase the risk of birth weight by less than 2,500 grams (61).

Because most pregnant nonsmokers who are exposed prenatally to a smoking home will likely continue this exposure postnatally, it is difficult to identify the specific effects of prenatal and

postnatal SHS exposure on fetal lung development and postnatal respiratory health. A population-based cohort of 5,619 7-year-old Toronto children was evaluated for the longitudinal association between maternal smoke exposure in pregnancy (active smoker and passive exposure) and childhood asthma development by parental report (62). The children whose mothers smoked or were exposed to home secondhand smoke during pregnancy were more likely to develop asthma with an adjusted hazard ratio of 1.3. This association persisted for children of nonsmoking mothers exposed to SHS in the home during pregnancy after adjusting for SHS exposure from birth to age 7 years.

The impact of perinatal SHS exposure on airway obstruction and hyperresponsiveness has been investigated in a rat model (63) in which pregnant Sprague-Dawley rats were exposed to filtered air or to sidestream smoke in utero and/or postnatally for 4 hours per day, 7 days per week from day 3 of gestation until birth. The pups were exposed to filtered air containing sidestream smoke for 7–10 weeks postnatally. Rats exposed both prenatally and postnatally to sidestream smoke had significantly lower dynamic compliance and significantly greater reactivity to methacholine than those exposed to the other three treatments. Both pre- and postnatal exposure to sidestream smoke was needed to change pulmonary function (63). The effect of perinatal SHS was studied by Joad et al. at the California National Primate Center in a nonhuman primate model, in which pregnant rhesus monkeys were exposed to filtered air or SHS for 6 hours per day. Exposure for 5 days per week started at 50 days of gestational age and continued postnatally until 3 months of age altered with intrinsic airway responsiveness and alveolar attachments (64).

E-cigarettes and Pregnancy

Electronic cigarettes (E-cigarettes) are tobacco-free nicotine delivery devices in which burning tobacco is replaced by a battery-operated atomizer that produces an aerosol from a liquid containing nicotine and flavoring. The liquid used to solubilize nicotine contains carrier solvents such as glycerol and/or propylene glycol, which deliver the nicotine and flavorings directly into the respiratory tract. Potential effects of e-cigarettes on lung development will include exposure to nicotine and

production of toxins from the carriers. E-cigarette use during pregnancy exposes the mother and fetus to nicotine and potential toxicants in an aerosol and includes the potential risks associated with secondhand exposure to e-cigarette vapor. Aerosols from e-cigarettes contain toxic and carcinogenic carbonyl compounds such as formaldehyde, acetaldehyde, and acrolein (65–68). In particular, levels of formaldehyde were comparable to those measured in regular cigarette smoke. Levels of these compounds vary dramatically, depending on the brand or model of e-cigarette and the formulation of the aerosol liquids. E-cigarette vapor and secondhand e-cigarette vapor may affect lung development in a manner similar to smoking and are therefore likely risks to the infants of mothers using e-cigarettes.

A major concern with e-cigarettes is their potential to deliver nicotine during pregnancy. Progression toward nicotine dependence associated with e-cigarettes is still unclear. Although there appears to be a learning curve for effective “vaping” (69,70), experienced users of e-cigarettes achieve levels of nicotine and cotinine similar to individuals using conventional cigarettes (69–71). E-cigarettes have the potential to deliver as much nicotine as conventional cigarettes and therefore to alter lung development in similar fashion. It is likely that e-cigarettes will prove to be as addictive as conventional cigarettes. Advertising conveys the concept that e-cigarette usage is safe, but their use may lead to addiction in vulnerable individuals who will continue use of e-cigarettes during pregnancy. Use of e-cigarettes in adolescents is a particular concern because they may be at greater risk to develop nicotine addiction and to continue the use of e-cigarettes during pregnancy (72–75).

Environmental Pollution

Increased environmental air pollution is associated with increased risk of childhood asthma, respiratory infections, and indices of reduced lung function (76–79). Various types of air pollution, including particulate matter, ozone, nitrogen oxides (NO), sulfur oxides, and carbon monoxide (CO) have been associated with altered lung development. Environmental air pollution is associated with increased risk of premature delivery and intrauterine growth restriction (80–82). Effects on birth weight have been summarized by Proietti et al. (83). For most of the

epidemiologic studies on the effects of air pollution on lung development, it is difficult to differentiate prenatal from early childhood effects.

In a study by Jedrychowski et al. (84), children in Poland exposed to the highest quartile of PM_{2.5} (inhalable material <2.5 μm in diameter) had significant decreases in FVC, FEV₁, and FEV_{0.5}. The increased exposure to PM_{2.5} was in turn associated with increased wheezing at 2 years of age, though this effect was no longer significant by 4 years of age (85). Mortimer et al. (86) identified negative associations among pulmonary function with prenatal and early exposures to PM₁₀, NO, and CO in asthmatic children, although associations were for specific subgroups of children (86). Exposure to increased levels of CO during pregnancy increased allergic sensitization in children with asthma (87). In a retrospective study of 37,401 children born in British Columbia by Clark et al. (88), the incidence of asthma in 3- to 4-year-olds was correlated with estimated levels of in utero and first year of life exposures to air pollution. There was an increase in asthma risk with increased exposure to NO, CO, and PM₁₀.

The mechanisms by which exposure to air pollution alter lung development and lead to increased respiratory disease are not completely clear, although animal models have been developed to examine effects of both particulate and gaseous pollution. Fedulov et al. (77) showed that exposure of pregnant mice to diesel exhaust particles, as well as to inert particles, resulted in increased airway reactivity in the offspring. Plopper, Schelegle, and colleagues at the California National Primate Research Center developed models of ozone exposure to infant monkeys to determine how ozone affects lung development and leads to lung disease. Ozone is a strong oxidant and is a major component of air pollution in cities. Ozone is formed by the interaction of sunlight with hydrocarbons and nitrogen oxides produced during combustion. Exposure of infant monkeys to ozone caused decreased lung branching, hyperplastic airway epithelium, alterations in alveolar development, and smooth muscle remodeling (89). The combination of ozone and allergen increased the innervation and increased CD25+ cells of the airway epithelium (90,91), providing a potential link between ozone and asthma. Miller and coworkers have suggested that early exposure to ozone causes long lasting changes in innate

immunity that may be regulated by changes in miRNAs, potentially miR-149 (92). Auten et al. (93) reported that prenatal exposure to diesel exhaust particles further enhanced airway reactivity and abnormalities in alveolar development. Thus, there is likely considerable interaction between multiple pollutants among pre- and post-natal exposures.

Indoor Air Pollution: Biomass Fuel Exposure, Wood Smoke, and Cookstoves

Over half of the world's population (an estimated 3 billion people) and 90% of households in rural areas of developing countries burn unprocessed biomass fuel, typically wood, charcoal, dried animal dung, and agricultural residues. Incomplete combustion results in gaseous air pollutants and fine particulate matter (PM) that linger in the cooking area. Women and children in low- and middle-income countries are disproportionately impacted by exposure to high levels of indoor air pollution related to their role in cooking for the family. Significant increases in respiratory diseases including doubling of the risk of pneumonia and other acute lower respiratory infections in children <5 years of age (94) and an increased risk of COPD, asthma, and tuberculosis in adults have been reported (95). A recent meta-analysis (95) extracting data from 25 studies demonstrated an overall pooled OR that indicated a significant association of solid biomass fuels with acute respiratory infections in children (OR 3.53, 95% CI 1.94 to 6.43). No significant association with asthma was noted; however, only four appropriate studies in children were available for analysis (95). Although tobacco smoking is an established risk factor for COPD, 25–45% of patients with COPD have never smoked. Emerging evidence suggests that biomass exposure may be among the biggest risk factors for COPD globally (96).

The relationship between biomass use and adverse pregnancy outcomes/abnormal lung development is only partially understood. Because smoke from biomass cooking and heating produces many of the same pollutants found in tobacco smoke and outdoor air pollution, there is good reason to expect a relationship between exposure to biomass smoke and adverse lung development. Like tobacco, smoke from biomass

combustion produces a large number of health-damaging air pollutants, including PM, carbon monoxide, nitrogen oxides, formaldehyde, benzene, 1,3 butadiene, polycyclic aromatic hydrocarbons, and many other toxic organic compounds (97). Carbon monoxide binds to hemoglobin-forming carboxyhemoglobin, which reduces the ability of blood to carry oxygen with a potential to cause intrauterine growth restriction (IUGR), reduced birth weight, or perinatal mortality. There is evidence for probable in utero effects of biomass exposure. Analysis of 3,559 childbirths in Zimbabwe demonstrated that babies born to mothers cooking with wood, dung, or straw were 175 grams lighter compared to babies born to mothers using liquefied petroleum gas, natural gas, or electricity (98). A population-based cohort of 11,728 live born infants were followed from birth through 6 months of age in India, and exposure to biomass fuel was associated with an adjusted 49% increased risk of LBW, a 34% increased incidence of respiratory illness, and a 12% increased risk of 6-month infant mortality (99). The mean BW was 104.5 grams lower in infants born to women from biomass using households (99). A study in India demonstrated a significant increase in stillbirths associated with cooking with biomass fuels (OR = 1.44; 95% CI: 1.04–1.97) (100). A significant increase in severe stunting (height at 2 standard deviations below the median of an international reference population recommended by the World Health Organization) and moderate to severe anemia (hemoglobin < 9.9 g/dL) was associated with biomass fuel use in India (101). IUGR has been associated with reduced lung function in infants (102), children (103), and adults (104), indicating it may influence lung function throughout life. Indeed, exposure to biomass smoke was associated with deficits in lung function (FEV₁, FVC, FEV₁/FVC, and forced expiratory flow at 25–75% of FVC), an effect that can be detected as early as the late teenage years, suggesting a detrimental effect of biomass smoke exposure on lung growth in early life (105).

PM in biomass smoke may increase the chance of an adverse pregnancy outcome by reducing the mother's lung function and increasing maternal risk for chronic and acute respiratory diseases that may reduce oxygen delivery to the fetus. In biomass burning households, the PM₁₀ and PM_{2.5} often exceed guideline levels of mean

24-hour concentration, especially during cooking (106). Histologic sections of adult lungs have shown that ambient PM penetrates into and is retained in the walls of small airways. Even in nonsmokers, long-term exposure to high levels of ambient particulate pollutants is associated with small airway remodeling that may produce chronic airflow obstruction (107). A controlled trial in households in Mexico randomized to Patsari stoves (that reduce indoor emissions) versus traditional open fires demonstrated that the Patsari stove was associated with significantly lower risk of respiratory symptoms, including cough and wheezing, and a lower FEV1 decline over a 1-year follow-up compared to open fire use in women over 20 years of age (108). This reduction of respiratory symptoms and of slower lung function decline was comparable to smoking cessation (108). Polycyclic aromatic hydrocarbons (PAHs) are also important potential toxins present in biomass combustion. Some of the individual PAHs are classified as Class II carcinogens, and benzo[a]pyrene is classified as a class I carcinogen. As a whole, PACs have been associated with immunotoxicity (109). Women who cook exclusively with wood or kerosene have higher creatinine-adjusted hydroxyl-PAH levels in their urine samples compared to women who cook with liquefied petroleum gas or coal briquettes (109). In a subset of the women in the previous trial who were randomized to the use of the Patsari stove versus open fire cooking, use of the Patsari stove also significantly reduced carbon monoxide and polycyclic aromatic hydrocarbon exposures (110).

Although the relationship between indoor air pollution and increased respiratory disease appears well established, our understanding of the underlying molecular and cellular events culminating in the pulmonary response is limited and evolving. Exposure to wood smoke PM in cell cultures is associated with increased expression and production of proinflammatory cytokines, oxidatively damaged DNA, and oxidative stress (111). An emerging hypothesis is that chronic PM exposure causes oxidative stress, reduced immunity, and subsequent infection. The oxidative potential of PM collected during the burning of wood and mixed biomass was evaluated with a validated, synthetic respiratory tract lining fluid. Incubation of this fluid with PM for 4 hours caused large losses of ascorbate and reduced

glutathione, both physiologically relevant antioxidants (112). Short-term controlled exposure to high concentrations of wood smoke with intermittent exercise was associated with increased arterial stiffness and decreased heart rate variability in humans (113).

Studies in humans with regard to mechanisms have been less clear cut. A recent study in humans examining high inhalation exposure to wood combustion for a 1-week period demonstrated limited systemic effects in terms of inflammation, monocyte activation, and oxidative stress to DNA (114). Controlled woodstove exposure for 3-hour periods for 2-week intervals in atopic subjects did not alter markers of oxidative stress, DNA damage, cell adhesion, cytokines, or microvascular function (115). Also, short-term exposure to wood smoke at a concentration normally found in a residential area with a high density of burning woodstoves caused only mild inflammatory responses (116). Nevertheless, the role of environmental smoke will likely continue to be an area of active investigation.

Genetic Susceptibility

Both genetic polymorphisms and epigenetic factors regulating sensitivity of the lung to environmental exposures are likely strong factors that modify the effects of the environment or the outcome of programming and subsequent lung function in the offspring. Underlying the increased genetic susceptibility to environmental effects on lung development may be genetic polymorphisms that alter coding regions of genes either through deletions or single nucleotide polymorphisms (117). Most often these represent mutations in genes that either defend against the specific effector or genes that mediate the effects of the specific effector. Genetic polymorphisms can occur in either the mother, the fetus, or both. This concept is well illustrated by genetic polymorphisms that mediate the sensitivity of the fetus to maternal smoking during pregnancy.

Effects of maternal smoking during pregnancy are strongly mediated by nicotine. A polymorphism in the $\alpha 5$ nAChR, in which amino acid 398 is changed from Asp to Asn, increases the risk of smoking-related diseases such as lung cancer, COPD, and the degree of nicotine addiction (118). This same polymorphism in the mother increases the sensitivity of the fetus to the

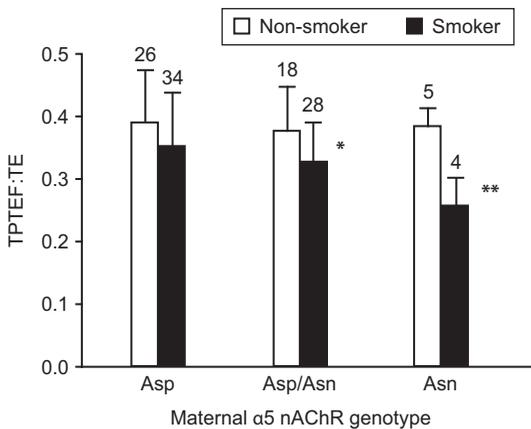


Figure 5-6. Effect of maternal $\alpha 5$ nAChR (rs16969968) genotype on sensitivity of fetus to maternal smoking during pregnancy. Infants whose mothers were homozygous for the risk allele of $\alpha 5$ single-nucleotide polymorphism (SNP) rs16969968 in which amino acid 398 of the $\alpha 5$ nAChR is changed from Asp to Asn showed the largest decrease in TPTEF:TE (ratio of time to peak tidal expiratory flow to expiratory time) if mothers smoked during pregnancy. Error bars show standard deviation. Asp = mothers homozygous for nonrisk allele (Asp/Asp), Asp/Asn = heterozygotes, Asn = mothers homozygous for risk allele (Asn/Asn). * $p < .01$, ** $p < .001$ compared to nonsmokers of same genotype by t-test. (Modified from McEvoy et al. JAMA. 2014;311:2074–2082.)

effects of maternal smoking during pregnancy on lung development. As shown in Figure 5-6, infants whose mothers smoked during pregnancy had diminished pulmonary function if their mothers were heterozygous or homozygous for the $\alpha 5$ risk allele. This polymorphism in the mother increased the severity of IUGR caused by maternal smoking (119). Thus, maternal genotype can affect the sensitivity of the fetus to tobacco smoke.

Polymorphisms in the glutathione transferases (GST) (120), enzymes critical for antioxidant defenses, are examples of how the infant's genotype makes the child more sensitive to effects of environment on lung health. Deletions in GST M1 increase the sensitivity of children with asthma to ozone (121). Infants with deletions in GST M1 and GST T1 are more sensitive to respiratory effects of maternal smoking during pregnancy (122,123).

In addition to genetic mechanisms, epigenetic mechanisms can influence environmental effects on lung development. This was demonstrated by Rehan et al. (124), who showed that exposure of mice to prenatal nicotine increased airway reactivity and increased DNA methylation. Increased airway reactivity and the alterations in

DNA methylation were transmitted to subsequent generations even without continued nicotine exposure. Epigenetic changes also likely mediate the effects of maternal smoking on human lung development as well because maternal smoking changes DNA methylation in placenta, cord blood, and epithelial cells of the offspring (125,126).

Environmental Effects of Climate Change and Lung Development

While the potential for climate change to affect lung development and disease is speculative, climate change is likely to alter patterns of air pollution and allergens. It is therefore reasonable to speculate that changes related to global warming would affect lung development and disease. The American Thoracic Society (127) and the European Respiratory Society (128) have issued position papers outlining the potential effects of climate change on respiratory health and its potential effects on lung development. Higher temperatures may result in more air pollution greater energy use for air conditioning (127,128) and increased PM associated with forest fires that may be more common (129). Higher temperatures combined with increased energy needs may result in higher levels of ground-level ozone (130). Thus, increases in PM and ozone may occur with climate change and bring about adverse effects on lung development and respiratory systems.

In addition, environmental pollution and climate change are likely to increase pollen associated with higher temperatures and changing rain patterns (127,129,130). Increased temperatures and flooding may also be associated with increased mold (130). The combination of increased pollution and increased allergens (pollen and mold) has the potential for increasing asthma incidence.

Future Prospects

It is difficult to forecast the effect of environmental changes or the burden of lung disease in the future. Increasing population and climate change are likely to cause increased air pollution. Decreasing tobacco use and more use of less-polluting indoor stoves may decrease environmental pollution. The impact of E-cigarettes

on human health is an evolving experience. It is hoped that data regarding its import will result in regulation that may minimize its impact in infant health. There will undoubtedly be more personalized-medicine approaches brought to bear on the potential effects of the environment. New genomic techniques, be it SNP analysis or

direct DNA sequence analysis, will allow for the identification of individuals most at risk for the effects of smoking, air pollution, or allergic stimuli. Targeted efforts to lessening the environmental risk or specific therapeutic interventions, including in utero therapies, may moderate lung disease in the future.

References

- 1 Hislop AA. Airway and blood vessel interaction during lung development. *J Anat.* 2002;201:325–334.
- 2 Merkus PJ, Ten Have-Opbroek AA, Quanjer PH. Human lung growth: a review. *Pediatr Pulmonol.* 1996;21:383–397.
- 3 Salihu HM, Aliyu MH, Pierre-Louis BJ, Alexander GR. Levels of excess infant deaths attributable to maternal smoking during pregnancy in the United States. *Matern Child Health J.* 2003;7:219–227.
- 4 Dietz PM, England LJ, Shapiro-Mendoza CK, Tong VT, Farr SL, Callaghan WM. Infant morbidity and mortality attributable to prenatal smoking in the U.S. *Am J Prev Med.* 2010;39:45–52.
- 5 U.S. Department of Health and Human Services. The Health Consequences of Involuntary Exposure to Tobacco Smoke: A Report of the Surgeon General – Executive Summary. 1–27. 2006. Atlanta, GA: Centers for Disease Control and Prevention.
- 6 Best D. From the American Academy of Pediatrics: Technical report—Secondhand and prenatal tobacco smoke exposure. *Pediatrics.* 2009;124:e1017–e1044.
- 7 Hayatbakhsh MR, Sadasivam S, Mamun AA, Najman JM, O’Callaghan MJ. Maternal smoking during and after pregnancy and lung function in early adulthood: A prospective study. *Thorax.* 2009;64:810–814.
- 8 Leung GM, Ho LM, Lam TH. The economic burden of environmental tobacco smoke in the first year of life. *Arch Dis Child.* 2003;88:767–771.
- 9 Stoddard JJ, Gray B. Maternal smoking and medical expenditures for childhood respiratory illness. *Am J Public Health.* 1997;87:205–209.
- 10 Tager IB, Weiss ST, Munoz A, Rosner B, Speizer FE. Longitudinal study of the effects of maternal smoking on pulmonary function in children. *NEJM.* 1983;309:699–703.
- 11 Brown RW, Hanrahan JP, Castile RG, Tager IB. Effect of maternal smoking during pregnancy on passive respiratory mechanics in early infancy. *Pediatr Pulmonol.* 1995;19:23–28.
- 12 Stick SM, Burton PR, Gurrin L, Sly PD, LeSouef PN. Effects of maternal smoking during pregnancy and a family history of asthma on respiratory function in newborn infants. *Lancet.* 1996;348:1060–1064.
- 13 Lodrup Carlsen KC, Jaakkola JJ, Nafstad P, Carlsen KH. In utero exposure to cigarette smoking influences lung function at birth. *Eur Respir J.* 1997;10:1774–1779.
- 14 Li YF, Gilliland FD, Berhane K, McConnell R, Gauderman WJ, Rappaport EB, et al. Effects of in utero and environmental tobacco smoke exposure on lung function in boys and girls with and without asthma. *Am J Respir Crit Care Med.* 2000;162:2097–2104.
- 15 Hanrahan JP, Tager IB, Segal MR, Tosteson TD, Castile RG, Van Vunakis H, et al. The effect of maternal smoking during pregnancy on early infant lung function. *Am Rev Respir Dis.* 1992;145:1129–1135.
- 16 Centers for Disease Control and Prevention. Cigarette use among high school students—United States, 1991–2007. *Morb Mortal Wkly Rep.* 2008;57:686–688.
- 17 Hoo AF, Henschen M, Dezateux C, Costeloe K, Stocks J. Respiratory function among preterm infants whose mothers smoked during pregnancy. *Am J Respir Crit Care Med.* 1998;158:700–705.
- 18 Cunningham J, Dockery DW, Speizer FE. Maternal smoking during pregnancy as a predictor of lung function in children. *Am J Epidemiol.* 1994;139:1139–1152.
- 19 Talhout R, Schulz T, Florek E, van BJ, Wester P, Opperhuizen A. Hazardous compounds in tobacco smoke. *Int J Environ Res Public Health.* 2011;8:613–628.
- 20 Hecht SS. Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst.* 1999;91:1194–1210.
- 21 Collins MH, Moessinger AC, Kleinerman J, Bassi J, Rosso P, Collins AM, et al. Fetal lung hypoplasia associated with maternal smoking: a morphometric analysis. *Pediatr Res.* 1985;19:408–412.
- 22 Maritz GS, Dennis H. Maternal nicotine exposure during gestation and lactation interferes with alveolar development in the neonatal

- lung. *Reprod Fertil Dev.* 1998;10:255–261.
- 23 Maritz GS. Maternal nicotine exposure during gestation and lactation of rats induce microscopic emphysema in the offspring. *Exp Lung Res.* 2002;28:391–403.
- 24 Maritz GS, Woolward K. Effect of maternal nicotine exposure on neonatal lung elastic tissue and possible consequences. *S Afr Med J.* 1992;81:517–519.
- 25 Sekhon HS, Jia Y, Raab R, Kuryatov A, Pankow JF, Whitsett JA, et al. Prenatal nicotine increases pulmonary alpha7 nicotinic receptor expression and alters fetal lung development in monkeys. *J Clin Invest.* 1999;103:637–647.
- 26 Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, et al. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology.* 2004;145:2498–2506.
- 27 Papke RL. Merging old and new perspectives on nicotinic acetylcholine receptors. *Biochem Pharmacol.* 2014;89:1–11.
- 28 Hurst R, Rollema H, Bertrand D. Nicotinic acetylcholine receptors: from basic science to therapeutics. *Pharmacol Ther.* 2013;137:22–54.
- 29 Kabbani N, Nordman JC, Corgiat BA, Veltri DP, Shehu A, Seymour VA, et al. Are nicotinic acetylcholine receptors coupled to G proteins? *Bioessays.* 2013;35:1025–1034.
- 30 Sekhon HS, Keller JA, Proskocil BJ, Martin EL, Spindel ER. Maternal nicotine exposure upregulates collagen gene expression in fetal monkey lung. Association with alpha7 nicotinic acetylcholine receptors. *Am J Respir Cell Mol Biol.* 2002;26:31–41.
- 31 Sekhon HS, Proskocil BJ, Clark JA, Spindel ER. Prenatal nicotine exposure increases connective tissue expression in foetal monkey pulmonary vessels. *Eur Respir J.* 2004;23:906–915.
- 32 Sekhon HS, Keller JA, Benowitz NL, Spindel ER. Prenatal nicotine exposure alters pulmonary function in newborn rhesus monkeys. *Am J Respir Crit Care Med.* 2001;164:989–994.
- 33 Wongtrakool C, Roser-Page S, Rivera HN, Roman J. Nicotine alters lung branching morphogenesis through the alpha7 nicotinic acetylcholine receptor. *Am J Physiol Lung Cell Mol Physiol.* 2007;293:L611–L618.
- 34 Wongtrakool C, Wang N, Hyde DM, Roman J, Spindel ER. Prenatal nicotine exposure alters lung function and airway geometry through alpha7 nicotinic receptors. *Am J Respir Cell Mol Biol.* 2012;46:695–702.
- 35 Ten Have-Opbroek AA. The development of the lung in mammals: an analysis of concepts and findings. *Am J Anat.* 1981;162:201–219.
- 36 Rantakallio P. Relationship of maternal smoking to morbidity and mortality of the child up to the age of five. *Acta Paediatr Scand.* 1978;67:621–631.
- 37 Fergusson DM, Horwood LJ, Shannon FT. Parental smoking and respiratory illness in infancy. *Arch Dis Child.* 1980;55:358–361.
- 38 Taylor B, Wadsworth J. Maternal smoking during pregnancy and lower respiratory tract illness in early life. *Arch Dis Child.* 1987;62:786–791.
- 39 Margolis PA, Keyes LL, Greenberg RA, Bauman KE, LaVange LM. Urinary cotinine and parent history (questionnaire) as indicators of passive smoking and predictors of lower respiratory illness in infants. *Pediatr Pulmonol.* 1997;23:417–423.
- 40 Tager IB, Hanrahan JP, Tosteson TD, Castile RG, Brown RW, Weiss ST, et al. Lung function, pre- and post-natal smoke exposure, and wheezing in the first year of life. *Am Rev Respir Dis.* 1993;147:811–817.
- 41 Hu FB, Persky V, Flay BR, Zelli A, Cooksey J, Richardson J. Prevalence of asthma and wheezing in public schoolchildren: association with maternal smoking during pregnancy. *Ann Allergy Asthma Immunol.* 1997;79:80–84.
- 42 Zlotkowska R, Zejda JE. Fetal and postnatal exposure to tobacco smoke and respiratory health in children. *Eur J Epidemiol.* 2005;20:719–727.
- 43 Lannero E, Pershagen G, Wickman M, Nordvall L. Maternal smoking during pregnancy increases the risk of recurrent wheezing during the first years of life (BAMSE). *Respir Res.* 2006;7:3.
- 44 Alati R, Al MA, O'Callaghan M, Najman JM, Williams GM. In utero and postnatal maternal smoking and asthma in adolescence. *Epidemiology.* 2006;17:138–144.
- 45 Bjerg A, Hedman L, Perzanowski M, Lundback B, Ronmark E. A strong synergism of low birth weight and prenatal smoking on asthma in schoolchildren. *Pediatrics.* 2011;127:e905–e912.
- 46 Pattenden S, Antova T, Neuberger M, Nikiforov B, De SM, Grize L, et al. Parental smoking and children's respiratory health: independent effects of prenatal and postnatal exposure. *Tob Control.* 2006;15:294–301.
- 47 Burke H, Leonardi-Bee J, Hashim A, Pine-Abata H, Chen Y, Cook DG, et al. Prenatal and passive smoke exposure and

- incidence of asthma and wheeze: systematic review and meta-analysis. *Pediatrics*. 2012;129:735–744.
- 48 Neuman A, Hohmann C, Orsini N, Pershagen G, Eller E, Kjaer HF, et al. Maternal smoking in pregnancy and asthma in preschool children: a pooled analysis of eight birth cohorts. *Am J Respir Crit Care Med*. 2012; 186:1037–1043.
- 49 Adler A, Tager IB, Brown RW, Ngo L, Hanrahan JP. Relationship between an index of tidal flow and lower respiratory illness in the first year of life. *Pediatr Pulmonol*. 1995;20:137–144.
- 50 Martinez FD, Morgan WJ, Wright AL, Holberg CJ, Taussig LM. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. *New Engl J Med*. 1988;319:1112–1117.
- 51 Dezateux C, Stocks J, Dundas I, Fletcher ME. Impaired airway function and wheezing in infancy. The influence of maternal smoking and a genetic predisposition to asthma. *Am J Respir Crit Care Med*. 1999;159:403–410.
- 52 Haland G, Carlsen KC, Sandvik L, Devulapalli CS, Munthe-Kaas MC, Pettersen M, et al. Reduced lung function at birth and the risk of asthma at 10 years of age. *N Engl J Med*. 2006;355:1682–1689.
- 53 Yuksel B, Greenough A, Giffin F, Nicolaidis KH. Tidal breathing parameters in the first week of life and subsequent cough and wheeze. *Thorax*. 1996;51:815–818.
- 54 Young S, Arnott J, O’Keeffe PT, Le Souef PN, Landau LI. The association between early life lung function and wheezing during the first 2 yrs of life. *Eur Respir J*. 2000;15:151–157.
- 55 Martinez FD. The origins of asthma and chronic obstructive pulmonary disease in early life. *Proc Am Thorac Soc*. 2009;6:272–277.
- 56 Turner SW, Palmer LJ, Rye PJ, Gibson NA, Judge PK, Cox M, et al. The relationship between infant airway function, childhood airway responsiveness, and asthma. *Am J Respir Crit Care Med*. 2004;169:921–927.
- 57 Holberg CJ, Wright AL, Martinez FD, Morgan WJ, Taussig LM. Child day care, smoking by caregivers, and lower respiratory tract illness in the first 3 years of life. *Pediatrics*. 1993;91: 885–892.
- 58 McEvoy CT, Schilling D, Clay N, Jackson K, Go MD, Spitale P, et al. Vitamin C supplementation for pregnant smoking women and pulmonary function in their newborn infants: a randomized clinical trial. *JAMA*. 2014;311:2074–2082.
- 59 Coleman T, Chamberlain C, Davey MA, Cooper SE, Leonardi-Bee J. Pharmacological interventions for promoting smoking cessation during pregnancy. *Cochrane Database Syst Rev*. 2012;9:CD010078.
- 60 Joya X, Manzano C, Alvarez AT, Mercadal M, Torres F, Salat-Batlle J, et al. Transgenerational exposure to environmental tobacco smoke. *Int J Environ Res Public Health*. 2014;11:7261–7274.
- 61 Nieuwenhuijsen MJ, Dadvand P, Grellier J, Martinez D, Vrijheid M. Environmental risk factors of pregnancy outcomes: a summary of recent meta-analyses of epidemiological studies. *Environ Health*. 2013;12:6.
- 62 Simons E, To T, Moineddin R, Stieb D, Dell SD. Maternal second-hand smoke exposure in pregnancy is associated with childhood asthma development. *J Allergy Clin Immunol Pract*. 2014;2:201–207.
- 63 Joad JP, Ji C, Kott KS, Bric JM, Pinkerton KE. In utero and postnatal effects of sidestream cigarette smoke exposure on lung function, hyperresponsiveness, and neuroendocrine cells in rats. *Toxicol Appl Pharmacol*. 1995;132:63–71.
- 64 Joad JP, Kott KS, Bric JM, Peake JL, Pinkerton KE. Effect of perinatal secondhand tobacco smoke exposure on in vivo and intrinsic airway structure/function in non-human primates. *Toxicol Appl Pharmacol*. 2009;234:339–344.
- 65 Goniewicz ML, Knysak J, Gawron M, Kosmider L, Sobczak A, Kurek J, et al. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tob Control*. 2014;23:133–139.
- 66 Cheng T. Chemical evaluation of electronic cigarettes. *Tob Control*. 2014;23(Suppl 2): ii11–ii17.
- 67 Kosmider L, Sobczak A, Fik M, Knysak J, Zaciera M, Kurek J, et al. Carbonyl compounds in electronic cigarette vapors – effects of nicotine solvent and battery output voltage. *Nicotine Tob Res*. 2014.
- 68 Uchiyama S, Ohta K, Inaba Y, Kunugita N. Determination of carbonyl compounds generated from the E-cigarette using coupled silica cartridges impregnated with hydroquinone and 2,4-dinitrophenylhydrazine, followed by high-performance liquid chromatography. *Anal Sci*. 2013;29:1219–1222.
- 69 Etter JF. Levels of saliva cotinine in electronic cigarette users. *Addiction*. 2014;109: 825–829.
- 70 Schroeder MJ, Hoffman AC. Electronic cigarettes and nicotine clinical pharmacology. *Tob Control*. 2014;23(Suppl 2): ii30–ii35.
- 71 Flouris AD, Chorti MS, Poulianiti KP, Jamurtas AZ,

- Kostikas K, Tzatzarakis MN, et al. Acute impact of active and passive electronic cigarette smoking on serum cotinine and lung function. *Inhal Toxicol.* 2013;25:91–101.
- 72 Dutra LM, Glantz SA. Electronic cigarettes and conventional cigarette use among US adolescents: a cross-sectional study. *JAMA Pediatr.* 2014.
- 73 Kmietowicz Z. E-cigarettes are "gateway devices" for smoking among young people, say researchers. *BMJ.* 2014;348:g2034.
- 74 Edwards R, Carter K, Peace J, Blakely T. An examination of smoking initiation rates by age: results from a large longitudinal study in New Zealand. *Aust N Z J Public Health.* 2013;37:516–519.
- 75 Breslau N, Peterson EL. Smoking cessation in young adults: age at initiation of cigarette smoking and other suspected influences. *Am J Public Health.* 1996;86:214–220.
- 76 Graf N, Johansen P, Schindler C, Wuthrich B, Ackermann-Liebrich U, Gassner M, et al. Analysis of the relationship between pollinosis and date of birth in Switzerland. *Int Arch Allergy Immunol.* 2007;143:269–275.
- 77 Fedulov AV, Leme A, Yang Z, Dahl M, Lim R, Mariani TJ, et al. Pulmonary exposure to particles during pregnancy causes increased neonatal asthma susceptibility. *Am J Respir Cell Mol Biol.* 2008;38:57–67.
- 78 Kajekar R. Environmental factors and developmental outcomes in the lung. *Pharmacol Ther.* 2007;114:129–145.
- 79 Wang L, Pinkerton KE. Air pollutant effects on fetal and early postnatal development. *Birth Defects Res C Embryo Today.* 2007;81:144–154.
- 80 Pereira G, Belanger K, Ebisu K, Bell ML. Fine particulate matter and risk of preterm birth in Connecticut in 2000–2006: a longitudinal study. *Am J Epidemiol.* 2014;179:67–74.
- 81 Bobak M. Outdoor air pollution, low birth weight, and prematurity. *Environ Health Perspect.* 2000;108:173–176.
- 82 Shah PS, Balkhair T. Air pollution and birth outcomes: a systematic review. *Environ Int.* 2011;37:498–516.
- 83 Proietti E, Roosli M, Frey U, Latzin P. Air pollution during pregnancy and neonatal outcome: a review. *J Aerosol Med Pulm Drug Deliv.* 2013;26:9–23.
- 84 Jedrychowski WA, Perera FP, Maugeri U, Mroz E, Klimaszewska-Rembiasz M, Flak E, et al. Effect of prenatal exposure to fine particulate matter on ventilatory lung function of preschool children of non-smoking mothers. *Paediatr Perinat Epidemiol.* 2010;24:492–501.
- 85 Jedrychowski WA, Perera FP, Maugeri U, Mrozek-Budzyn D, Mroz E, Klimaszewska-Rembiasz M, et al. Intrauterine exposure to polycyclic aromatic hydrocarbons, fine particulate matter and early wheeze. Prospective birth cohort study in 4-year olds. *Pediatr Allergy Immunol.* 2010;21:e723–e732.
- 86 Mortimer K, Neugebauer R, Lurmann F, Alcorn S, Balmes J, Tager I. Air pollution and pulmonary function in asthmatic children: effects of prenatal and lifetime exposures. *Epidemiology.* 2008;19:550–557.
- 87 Mortimer K, Neugebauer R, Lurmann F, Alcorn S, Balmes J, Tager I. Early-lifetime exposure to air pollution and allergic sensitization in children with asthma. *J Asthma.* 2008;45:874–881.
- 88 Clark NA, Demers PA, Karr CJ, Koehoorn M, Lencar C, Tamburic L, et al. Effect of early life exposure to air pollution on development of childhood asthma. *Environ Health Perspect.* 2010;118:284–290.
- 89 Fanucchi MV, Plopper CG, Evans MJ, Hyde DM, Van Winkle LS, Gershwin LJ, et al. Cyclic exposure to ozone alters distal airway development in infant rhesus monkeys. *Am J Physiol Lung Cell Mol Physiol.* 2006;291:L644–L650.
- 90 Kajekar R, Pieczarka EM, Smiley-Jewell SM, Schelegle ES, Fanucchi MV, Plopper CG. Early postnatal exposure to allergen and ozone leads to hyperinnervation of the pulmonary epithelium. *Respir Physiol Neurobiol.* 2007;155:55–63.
- 91 Miller LA, Gerriets JE, Tyler NK, Abel K, Schelegle ES, Plopper CG, et al. Ozone and allergen exposure during postnatal development alters the frequency and airway distribution of CD25+ cells in infant rhesus monkeys. *Toxicol Appl Pharmacol.* 2009;236:39–48.
- 92 Clay CC, Maniar-Hew K, Gerriets JE, Wang TT, Postlethwait EM, Evans MJ, et al. Early life ozone exposure results in dysregulated innate immune function and altered microRNA expression in airway epithelium. *PLoS One.* 2014;9:e90401.
- 93 Auten RL, Gilmour MI, Krantz QT, Potts EN, Mason SN, Foster WM. Maternal diesel inhalation increases airway hyperreactivity in ozone-exposed offspring. *Am J Respir Cell Mol Biol.* 2012;46:454–460.
- 94 O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than

- 5 years: global estimates. *Lancet*. 2009;374:893–902.
- 95 Po JY, FitzGerald JM, Carlsten C. Respiratory disease associated with solid biomass fuel exposure in rural women and children: systematic review and meta-analysis. *Thorax*. 2011;66:232–239.
- 96 Eisner MD, Anthonisen N, Coultas D, Kuenzli N, Perez-Padilla R, Postma D, et al. An official American Thoracic Society public policy statement: novel risk factors and the global burden of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2010;182:693–718.
- 97 Bruce N, Perez-Padilla R, Albalak R. Indoor air pollution in developing countries: a major environmental and public health challenge. *Bull World Health Organiz*. 2000;78:1078–1092.
- 98 Mishra V, Dai X, Smith KR, Mika L. Maternal exposure to biomass smoke and reduced birth weight in Zimbabwe. *Ann Epidemiol*. 2004;14:740–747.
- 99 Tielsch JM, Katz J, Thulasiraj RD, Coles CL, Sheeladevi S, Yanik EL, et al. Exposure to indoor biomass fuel and tobacco smoke and risk of adverse reproductive outcomes, mortality, respiratory morbidity and growth among newborn infants in south India. *Int J Epidemiol*. 2009;38:1351–1363.
- 100 Mishra V, Retherford RD, Smith KR. Cooking smoke and tobacco smoke as risk factors for stillbirth. *Int J Environ Health Res*. 2005;15:397–410.
- 101 Mishra V, Retherford RD. Does biofuel smoke contribute to anaemia and stunting in early childhood? *Int J Epidemiol*. 2007;36:117–129.
- 102 Dezateux C, Lum S, Hoo AF, Hawdon J, Costeloe K, Stocks J. Low birth weight for gestation and airway function in infancy: exploring the fetal origins hypothesis. *Thorax*. 2004;59:60–66.
- 103 Rona RJ, Gulliford MC, Chinn S. Effects of prematurity and intrauterine growth on respiratory health and lung function in childhood. *BMJ*. 1993;306:817–820.
- 104 Lawlor DA, Ebrahim S, Davey SG. Association of birth weight with adult lung function: findings from the British Women's Heart and Health Study and a meta-analysis. *Thorax*. 2005;60:851–858.
- 105 Kurmi OP, Devereux GS, Smith WC, Semple S, Steiner MF, Simkhada P, et al. Reduced lung function due to biomass smoke exposure in young adults in rural Nepal. *Eur Respir J*. 2013;41:25–30.
- 106 Regalado J, Perez-Padilla R, Sansores R, Paramo Ramirez JI, Brauer M, Pare P, et al. The effect of biomass burning on respiratory symptoms and lung function in rural Mexican women. *Am J Respir Crit Care Med*. 2006;174:901–905.
- 107 Churg A, Brauer M, del Carmen Avila-Casado M, Fortoul TI, Wright JL. Chronic exposure to high levels of particulate air pollution and small airway remodeling. *Environ Health Perspect*. 2003;111:714–718.
- 108 Romieu I, Riojas-Rodriguez H, Marron-Mares AT, Schilmann A, Perez-Padilla R, Masera O. Improved biomass stove intervention in rural Mexico: impact on the respiratory health of women. *Am J Respir Crit Care Med*. 2009;180:649–656.
- 109 Adetona O, Li Z, Sjodin A, Romanoff LC, Aguilar-Villalobos M, Needham LL, et al. Biomonitoring of polycyclic aromatic hydrocarbon exposure in pregnant women in Trujillo, Peru—comparison of different fuel types used for cooking. *Environ Int*. 2013;53:1–8.
- 110 Riojas-Rodriguez H, Schilmann A, Marron-Mares AT, Masera O, Li Z, Romanoff L, et al. Impact of the improved Patsari biomass stove on urinary polycyclic aromatic hydrocarbon biomarkers and carbon monoxide exposures in rural Mexican women. *Environ Health Perspect*. 2011;119:1301–1307.
- 111 Danielsen PH, Brauner EV, Barregard L, Sallsten G, Wallin M, Olinski R, et al. Oxidatively damaged DNA and its repair after experimental exposure to wood smoke in healthy humans. *Mutat Res*. 2008;642:37–42.
- 112 Kurmi OP, Dunster C, Ayres JG, Kelly FJ. Oxidative potential of smoke from burning wood and mixed biomass fuels. *Free Radic Res*. 2013;47:829–835.
- 113 Unosson J, Blomberg A, Sandstrom T, Muala A, Boman C, Nystrom R, et al. Exposure to wood smoke increases arterial stiffness and decreases heart rate variability in humans. *Part Fibre Toxicol*. 2013;10:20.
- 114 Jensen A, Karottki DG, Christensen JM, Bonlokke JH, Sigsgaard T, Glasius M, et al. Biomarkers of oxidative stress and inflammation after wood smoke exposure in a reconstructed Viking Age house. *Environ Mol Mutagen*. 2014;55(8):652–661.
- 115 Forchhammer L, Moller P, Riddervold IS, Bonlokke J, Massling A, Sigsgaard T, et al. Controlled human wood smoke exposure: oxidative stress, inflammation and microvascular function. *Part Fibre Toxicol*. 2012;9:7.
- 116 Riddervold IS, Bonlokke JH, Olin AC, Gronborg TK, Schlunssen V, Skogstrand K, et al. Effects of wood smoke

- particles from wood-burning stoves on the respiratory health of atopic humans. *Part Fibre Toxicol.* 2012;9:12.
- 117 Yang IA, Fong KM, Zimmerman PV, Holgate ST, Holloway JW. Genetic susceptibility to the respiratory effects of air pollution. *Postgrad Med J.* 2009;85:428–436.
- 118 Bierut LJ. Convergence of genetic findings for nicotine dependence and smoking related diseases with chromosome 15q24-25. *Trends Pharmacol Sci.* 2010;31:46–51.
- 119 Leermakers ET, Taal HR, Bakker R, Steegers EA, Hofman A, Jaddoe VW. A common genetic variant at 15q25 modifies the associations of maternal smoking during pregnancy with fetal growth: the generation R study. *PLoS One.* 2012;7:e34584.
- 120 Bolt HM, Thier R. Relevance of the deletion polymorphisms of the glutathione S-transferases GSTT1 and GSTM1 in pharmacology and toxicology. *Curr Drug Metab.* 2006;7:613–628.
- 121 Romieu I, Ramirez-Aguilar M, Sienra-Monge JJ, Moreno-Macias H, Del Rio-Navarro BE, David G, et al. GSTM1 and GSTP1 and respiratory health in asthmatic children exposed to ozone. *Eur Respir J.* 2006;28:953–959.
- 122 Gilliland FD, Li YF, Dubeau L, Berhane K, Avol E, McConnell R, et al. Effects of glutathione S-transferase M1, maternal smoking during pregnancy, and environmental tobacco smoke on asthma and wheezing in children. *Am J Respir Crit Care Med.* 2002;166:457–463.
- 123 Murdzoska J, Devadason SG, Khoo SK, Landau LI, Young S, Goldblatt J, et al. In utero smoke exposure and maternal and infant GST genes on airway responsiveness and lung function in infancy. *Am J Respir Crit Care Med.* 2010;181:64–71.
- 124 Rehan VK, Liu J, Sakurai R, Torday JS. Perinatal nicotine-induced transgenerational asthma. *Am J Physiol Lung Cell Mol Physiol.* 2013;305:L501–L507.
- 125 Breton CV, Byun HM, Wenten M, Pan F, Yang A, Gilliland FD. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med.* 2009;180:462–467.
- 126 Joubert BR, Haberg SE, Bell DA, Nilsen RM, Vollset SE, Middtun O, et al. Maternal smoking and DNA methylation in newborns: in utero effect or epigenetic inheritance? *Cancer Epidemiol Biomarkers Prev.* 2014;23:1007–1017.
- 127 Pinkerton KE, Rom WN, Akpınar-Elci M, Balmes JR, Bayram H, Brandli O, et al. An official American Thoracic Society workshop report: climate change and human health. *Proc Am Thorac Soc.* 2012;9:3–8.
- 128 Ayres JG, Forsberg B, Annesi-Maesano I, Dey R, Ebi KL, Helms PJ, et al. Climate change and respiratory disease: European Respiratory Society position statement. *Eur Respir J.* 2009;34:295–302.
- 129 Rice MB, Thurston GD, Balmes JR, Pinkerton KE. Climate change. A global threat to cardiopulmonary health. *Am J Respir Crit Care Med.* 2014;189:512–519.
- 130 Forsberg B, Braback L, Keune H, Kobernus M, Kraye von KM, Yang A, et al. An expert assessment on climate change and health – with a European focus on lungs and allergies. *Environ Health.* 2012;11 (Suppl 1):S4.

Congenital Malformations of the Lung

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Abstract

Congenital lung malformations are a heterogeneous group of abnormalities resulting from defective foregut specification, branching morphogenesis and cell proliferation, survival, and differentiation. Advancements in radiologic imaging and routine investigations in utero have resulted in a shift from postnatal to prenatal diagnoses. Prenatal diagnosis provides an opportunity to follow congenital malformations sequentially to better understand pathophysiological mechanisms. Genetic analysis of patients with hereditary lung malformations has also shed light on the molecular mechanisms underlying aberrant lung organogenesis. In this chapter, an overview of the five stages of lung development is given followed by discussion of the congenital lung malformations that result from defects in early and late lung morphogenesis. The malformations are described followed by a discussion of the associated syndromes, etiology, and pathogenesis with a focus toward the underlying cellular and molecular mechanisms. Clinical presentations, diagnosis, treatments, and outcomes are summarized, including radiographic and pathologic images of the most common malformations. The anomalies are presented in a format designed to provide clinicians caring for fetal and neonatal patients as well as scientists interested in lung development with a concise, up-to-date overview of congenital lung malformations and the deregulated cellular and molecular processes underlying their pathogenesis.

Keywords:

Lung malformations, tracheoesophageal fistula, agenesis, dysplasia, congenital pulmonary airway malformation, pulmonary sequestration, pulmonary hypoplasia, pulmonary lymphangiectasia, congenital lobar emphysema, pleuropulmonary blastoma

Overview of Human Lung Development

Human lung development is normally divided into five overlapping, chronological stages of organogenesis, which describe the structural and histologic changes that occur during morphogenesis and maturation of the lung (1–3). These five stages include the embryonic, pseudoglandular, canalicular, saccular, and alveolar stages of lung development, which extend throughout gestation and into the postnatal period (Table 6-1). Human lung development is initiated during the early embryonic period of gestation (3–7 weeks gestation) as a small saccular outgrowth of the ventral foregut endoderm, called the respiratory diverticulum. During the subsequent pseudoglandular stage of lung development (5–17 weeks gestation), formation of the conducting airways, that is, the tracheobronchial tree, occurs by elongation and repetitive branching of the primitive bronchial tubules. By the end of this period, the terminal bronchioles have divided into two or more respiratory bronchioles, which will subdivide

again into small clusters of short acinar tubules and buds. These peripheral structures will become the adult pulmonary acinus, consisting of respiratory bronchiole, alveolar duct, and alveoli. Vascularization of the surrounding mesenchyme with formation of the air–blood barrier, that is, the alveolar–capillary respiratory membrane, occurs during the canalicular stage of lung development (16–26 weeks gestation). Cytodifferentiation of bronchiolar and alveolar epithelial cells is also initiated during this stage. Enlargement and expansion of the peripheral air spaces occurs during the saccular stage of lung development (24–38 weeks gestation), resulting in the formation of primitive sac-like alveoli separated by thick interalveolar septa. Formation of thin secondary alveolar septa and remodeling of the capillary bed occurs during the alveolar stage of lung development (36 weeks gestation to 2 years of age), giving rise to the mature alveolar organization of the adult lung.

While definitive alveoli are found in the human lung by 36 weeks of gestation, 85–90% of all alveoli are formed within the first 6 months of

Table 6-1. Summary of human lung development.

Developmental Stage	Major Developmental Events
Embryonic	Lung bud arises from ventral foregut endoderm
3–7 wk. gestation	Branching morphogenesis initiated Primary, secondary, and tertiary bronchi form Trachea and esophagus separate Pulmonary arteries bud off 6th pair of aortic arches Pulmonary veins develop as outgrowths of left atrium Autonomic innervation extends to trachea and bronchi
Pseudoglandular	Branching morphogenesis continues
5–17 wk. gestation	Tracheobronchial tree formed by 17 wk. Cartilage and glands develop in conducting airways Airway smooth muscle extends to bronchioles Basal, ciliated, mucus, and neuroendocrine cells differentiate Acinar tubules form in peripheral lung Pulmonary arterial development parallels airway branching Pulmonary lymphatics arise from pulmonary veins Autonomic innervation parallels airway branching Pleuroperitoneal cavity closes
Canalicular	Acinar tubules lengthen and subdivide
16–26 wk. gestation	Mesenchyme begins to thin/condense Primitive alveolar capillary network forms Alveolar type I/type II cells differentiate Surfactant synthesized and stored in type II cells
Saccular	Acinar tubules expand into thin-walled, fluid-filled saccules
24–38 wk. gestation	Mesenchyme thins further to form primary alveolar septa Alveolar septa contain well-formed, double capillary network Elastin deposited at future sites of septal crest formation Type I cells flatten and elongate Surfactant synthesized and secreted by type II cells Fetal breathing initiated/gas exchange feasible
Alveolar	Alveolar surface area for gas exchange increases
36 wk. gestation–2 yr.	Secondary alveolar septa subdivide saccules into true alveoli Alveolar septa thin further with loss of connective tissue Double capillary network fuses into a single network Fibroblasts proliferate and differentiate Collagen, elastin, and fibronectin deposited Surfactant production and secretion increase in type II cells

life (4). After 6 months, alveolar formation occurs at a slower pace until about 8 years of age, when further growth of the lung becomes proportional to growth of the body (5,6). Overall, the number of alveoli increases by about sixfold between birth and adulthood, that is, from an average of 150 million alveoli (range: 110–174 million) in the term lung (7) to 480 million alveoli (range: 274–790 million) in the adult human lung (8).

Congenital Malformations Associated with Lung Initiation and Formation

These are a group of rare abnormalities that are caused by defective budding, branching, differentiation, and/or separation of the primitive lung from the foregut during early lung development. Often these lesions result in obstruction of the

airway, which subsequently causes secondary cystic or dysplastic changes in the lung (9–11). Pulmonary vascular abnormalities also cause obstructive malformations of the lung and conducting airways during development, and it is common to see overlapping, combination, or hybrid lesions composed of more than one abnormality.

In general, there are four different categories of congenital malformations associated with the early period of lung formation: (1) lung/foregut abnormalities, including trachea–esophageal fistula (TEF), tracheal agenesis, and bronchogenic cysts; (2) conducting airway abnormalities, such as tracheal and bronchial stenosis and malacia; (3) pulmonary or parenchymal abnormalities, such as pulmonary agenesis, acinar dysplasia, and alveolar capillary dysplasia; and (4) associated vascular abnormalities, including agenesis of the pulmonary artery, aberrant pulmonary arteries, anomalous pulmonary venous drainage, and pulmonary arteriovenous malformations.

Multiple congenital malformations in other organ systems are commonly found in conjunction with these early lesions and include musculoskeletal, cardiovascular, gastrointestinal (GI), and genitourinary (GU) abnormalities. In some cases, chromosomal disorders or single gene mutations have been associated with these malformations. Many of these mutated genes are important for the maintenance of self-renewing progenitor/stem cells, for cell proliferation and differentiation, or for migration and adhesion, that is, processes important for morphogenesis and differentiation of the lung, as well as other organs.

Lung/Foregut Abnormalities

Tracheoesophageal Fistulas

Most congenital malformations of the tracheobronchial tree arise during formation of the respiratory diverticulum and branching morphogenesis of the lung. One of the most critical events in the formation of the respiratory system is the initial separation of the primitive foregut into respiratory and digestive tracts. This process begins during week 3 of gestation and is complete by week 6 of the embryonic period of lung development. Failure of this process to proceed normally results in a variety of defects, including formation of a tracheoesophageal fistula (TEF).

TEF is one of the most commonly encountered abnormalities of the trachea with an incidence of 1 in 3,500 live births and is usually found in combination with various forms of esophageal atresia (EA) (12–14). The most common combination is EA with a lower, or distal, TEF, in which the upper, or proximal, esophagus ends in a blind pouch, while the lower, or distal, esophagus originates from the trachea. This combination, known as a Type C fistula, accounts for 87% of all TEF cases (15). Other combinations include (1) isolated EA without a fistula (Type A); (2) EA with a proximal fistula connecting the proximal esophagus and the trachea with the distal esophagus forming a blind pouch (Type B); (3) EA with a double fistula, where both proximal and distal portions of the esophagus join the trachea at separate points along its length (Type D); and (4) an isolated, or common, fistula without EA (Type E), which may be difficult to diagnose if the fistula is small. Rarely, the trachea may fail to separate from the esophagus along its entire length. Interestingly, these anomalies do not interfere with cellular differentiation, so that the ventral, or anterior, tracheal segments will contain ciliated cells and cartilaginous rings, while the dorsal, or posterior, esophageal segments contain stratified squamous epithelium and muscle (16).

Defects in development of the esophagus and/or trachea disrupt breathing and feeding in the newborn infant. Infants often present with respiratory distress, secondary to airway obstruction caused by excess secretions or mucus, and/or aspiration of gastric contents into the lung through the fistula. Excessive salivation, vomiting, cough, choking, and cyanosis after feeding are seen in the immediate postnatal period. In comparison, children with an isolated TEF usually present with recurring pneumonia, aspiration, and/or persistent cough with failure to thrive. Diagnosis of EA/TEF is confirmed by endoscopic and/or radiologic examination. TEF in low-birth-weight infants is often associated with a variety of other malformations, which carries a high risk for a poor outcome. In comparison, survival from an isolated TEF is almost 100% in term infants.

TEFs are often associated with multiple organ abnormalities (~50% of all cases), the most common being GI malformations and congenital heart defects (17). For example, familial EA/TEF occurs in 30–40% of those with Feingold syndrome [Online Mendelian Inheritance of Man

(OMIM #164280), which is also characterized by duodenal atresia, digital abnormalities, and microcephaly (17). TEFs with EA are found in 50–80% of subjects with VATER/VACTERL syndrome (OMIM #192350), which is comprised of multiple developmental abnormalities, including vertebral defects, anal atresia, cardiac defects, TEF, renal malformations, and limb defects (18). In general, the incidence of cardiac malformations associated with EA/TEF is 13%, while that for skeletal defects is 11%; anal atresia, 10%; and renal malformations, 5% (17).

Although the majority (90%) of EA/TEF cases arise as sporadic events with a low risk of recurrence (17), multiple genetic abnormalities and syndromes involving chromosomal disorders or single gene mutations have been associated with EA/TEF. Chromosomal anomalies have been reported in about 6–10% of EA/TEF patients in association with trisomy 18 (Edwards syndrome) and trisomy 21 (Down syndrome, OMIM #190685) (15,17,19), while single gene mutations have been reported in several other syndromes associated with EA/TEF (12,14,15). For example, Feingold syndrome (familial EA/TEF) is caused by heterozygous mutations in the oncogene, *MYCN* (OMIM *16840), which is downstream of SHH signaling and regulates cell proliferation; while AEG syndrome (anophthalmia, esophageal, genital; OMIM #206900), in which the incidence of EA is 100% with or without TEF is caused by heterozygous mutations in the *SOX2* gene (OMIM *184429), a transcription factor essential for maintenance of self-renewing progenitor/stem cells. EA/TEF also occurs in 10% of patients with CHARGE syndrome (coloboma of the eye, heart defects, choanal atresia, retarded growth, genital hypoplasia, and ear anomalies, OMIM #214800), which is caused by heterozygous mutations in the transcriptional regulator, *CHD7* (OMIM *608892), an important nuclear cofactor for *SOX2* activity. Opitz G/BBB syndrome (OMIM #145410), in which the prevalence of EA/TEF is 44%, is caused by a heterozygous deletion on chromosome 22 (autosomal dominant inheritance) or by heterozygous mutations in the *MID1* gene (OMIM *300552; X-linked inheritance), a microtubule-associated protein involved in the cell cycle. VATER/VACTERL syndrome is associated with heterozygous mutations in the transcription factor, *HOXD13* (OMIM *142989), a gene that is downstream of the SHH signaling

pathway and is important for cell adhesion processes. X-linked VACTERL with or without hydrocephalus (OMIM #314390) is associated with mutations or deletions of the nuclear localization factor, *ZIC3* (OMIM *300265), while one patient with VACTERL plus hydrocephalus (OMIM #276950) was found to have a mutation in the phosphatase encoding gene, *PTEN* (OMIM *601728.0030). Both of these latter genes influence cell migration, adhesion, proliferation, and growth.

Tracheal Agenesis

Congenital tracheal agenesis is a rare fatal malformation that involves either partial or complete absence of the trachea below the larynx, with or without a TEF. Often the trachea ends in a blind sac just below the larynx, and the lungs may be connected directly to the esophagus via the bronchi or bronchoesophageal fistulas (20). Classification of anatomical variations in this malformation is based on the length of the remaining tracheal segment and the presence or absence of an esophageal fistula (16,20–22). The most common variation is complete absence of the trachea below the larynx with the right and left main stem bronchi forming a common airway that is connected to the esophagus (Figure 6-1). Other variations include (1) tracheal agenesis with both the right and left main stem bronchi arising from the esophagus, (2) agenesis of the proximal trachea with an intact distal trachea, with or without an esophageal fistula, (3) a short segment of proximal trachea connected to the esophagus, and (4) proximal and distal segments of the trachea, linked by a short atretic, or a chord-like, fibrous band of tissue. Infants with tracheal atresia are cyanotic with severe respiratory distress and cannot be intubated or ventilated. As for EA/TEF, other congenital anomalies are present in most cases, including additional lower respiratory tract malformations, such as abnormal lobe formation, and complex cardiac defects with a high incidence of abnormal vessels. Associated malformations often overlap with those seen in VACTERL or TARCD (tracheal agenesis/atresia, radial ray defect, complex congenital cardiac abnormalities, and duodenal atresia) (22).

Congenital Bronchogenic Cysts

Congenital bronchogenic cysts, also known as foregut duplications, are commonly found in the

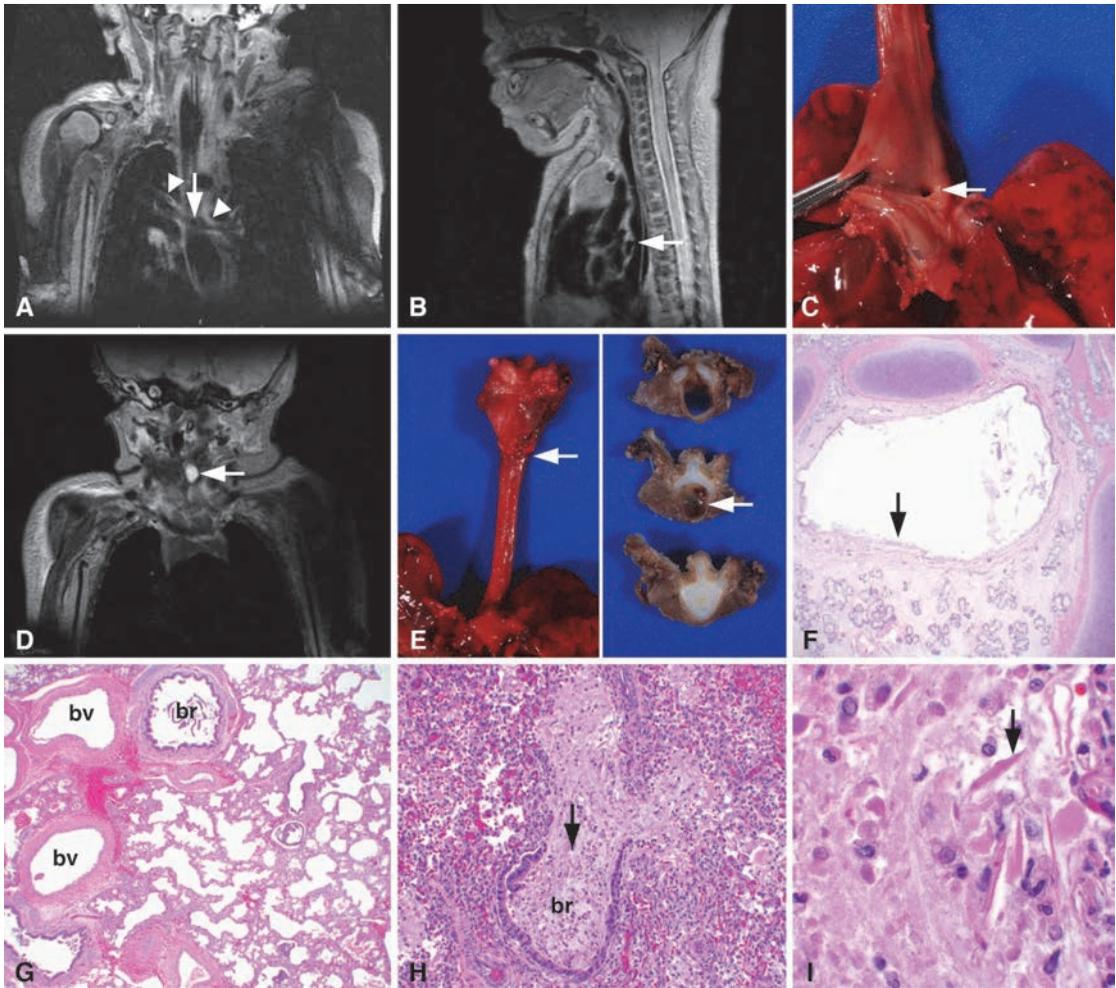


Figure 6-1. Tracheal agenesis with bronchoesophageal fistula. An infant boy was born at 36 weeks gestation after a pregnancy complicated by idiopathic polyhydramnios, a prenatal evaluation showing normal chromosome karyotype, and a fetal echocardiogram demonstrating a moderately dilated right ventricle with normal function, mild right ventricular hypertrophy, and a mildly dilated right atrium, pulmonary valve annulus, and main pulmonary artery. The infant had respiratory failure at delivery with resuscitation complicated by difficulty in intubation and ventilation. MRI showed a small fistulous connection between the carina and air distended esophagus (A–B, arrows) that was confirmed at autopsy (C, arrow). The carina was continuous with the right and left main stem bronchi distally (A, arrowheads), but there was tracheal agenesis proximally with a fluid-filled structure in the midneck at the level of the glottis (D, arrow). Tracheal agenesis was confirmed at autopsy showing absence of the trachea below the larynx (E, left arrow), with the lower portion of the larynx terminating in a cystic blind-ended sac (E, right arrow; laryngotracheal cross sections shown proximal to distal from top to bottom). The cystic sac was lined by respiratory type epithelium transitioning to squamous epithelial metaplasia (F, arrow), consistent with trachea agenesis with a blind-ended sac just below the larynx. The lungs were developed (G, br = bronchiole; bv = blood vessels) because the fistulous connection between the carina and esophagus (A–C, arrows) allowed amniotic fluid into the lung. Histologic features of aspiration pneumonia including intraluminal mucus with inflammatory cells (H, arrow) and numerous squames (I, arrow), as well as features of pulmonary hypertension, were present at autopsy, resulting in respiratory failure as the immediate cause of death at 3 days of age. As is common with bronchoesophageal fistulas and tracheal agenesis, multiple other congenital anomalies were identified by radiography and at autopsy, including multilevel anomalies of the thoracic vertebrae, absence of the right superior vena cava with persistence of the left superior vena cava, Meckel's diverticulum, and a bifid thumb. This constellation of anomalies is consistent with VATER/VACTERL association. Original magnifications: 4x (F–G), 20x (H), 100x (I).

mediastinum and are caused by abnormal budding of the ventral foregut during the embryonic stage of lung development (Figure 6-2). Bronchogenic cysts may also be found in the peripheral lung, most commonly in the lower lobes, and arise from

abnormal branching of the tracheobronchial tree at a later time (11,21,23–27). Approximately two-thirds are located in the mediastinum and one-third in the parenchyma. Although bronchogenic cysts are rare, with an incidence of 1 per 68,000

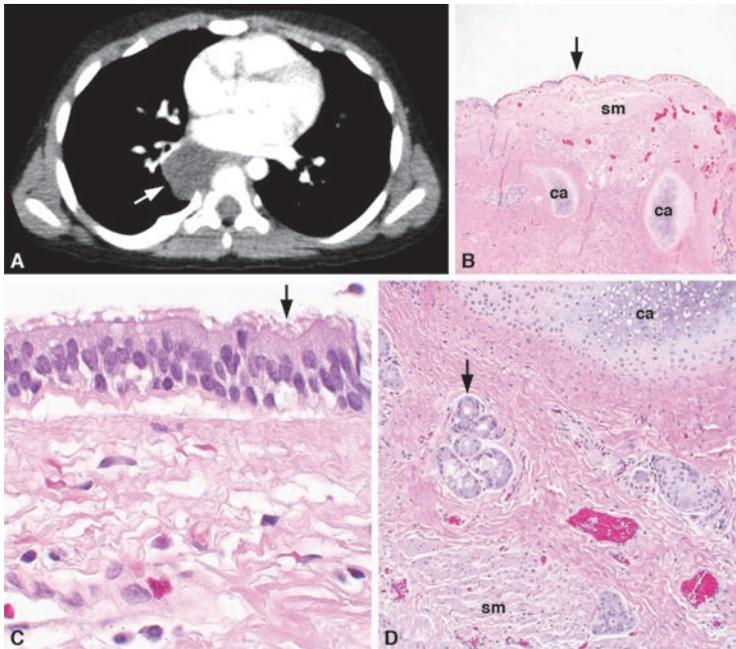


Figure 6-2. Congenital bronchogenic cyst. A 3-year-old boy had a right posterior mediastinal, hypodense, fluid-filled, 3-cm mass just inferior and posterior to the right hilum by CT imaging (A, arrow). No evidence of extension into the adjacent neural foramen was identified, and the mass did not enhance with intravenous contrast nor was there an aberrant vessel to the mass. The radiographic imaging favored the diagnosis of a foregut duplication cyst. At the time of surgery, the lesion was confirmed to be in the posterior mediastinum and to lack a connection to any surrounding structures. The lesion was resected with gross examination, revealing a unilocular, fluid-filled cyst. Histologic examination demonstrated that the cyst was lined by a pseudostratified, ciliated, columnar, respiratory epithelium (B–C, arrows) and that the cyst wall (B and D) contained cartilage (ca), smooth muscle (sm), and mucus glands (D, arrow), confirming the diagnosis of a bronchogenic cyst. Original magnifications: 4x (B), 20x (D), 100x (C).

(26), they are the most common primary cysts of the mediastinum (21,28).

Bronchogenic cysts found in the mediastinum can arise at any point along the tracheobronchial tree, commonly in paratracheal, carinal, hilar, or paraesophageal locations with the carinal location being the most frequent (21,26). Typically, they do not communicate with the conducting airway, but may be attached to the trachea or bronchus by a strand of tissue. Bronchogenic cysts found in the lungs as peripheral cysts, usually in the medial third of the lung, may or may not communicate with the tracheobronchial tree (21). Bronchogenic cysts are unilocular, typically solitary, thin-walled cystic cavities that are filled with fluid or mucus. They are lined with bronchial epithelium composed of pseudostratified, ciliated, columnar, or cuboidal epithelial cells, and their walls contain cartilage, smooth muscle, and mucus glands (Figure 6-2B–D). On chest X-ray, they appear as round or oval masses with smooth walls and may either be fluid-filled, air-filled, or have distinct air-fluid levels, unless infected (Figure 6-2A) (28). Most bronchogenic cysts are asymptomatic, but communication between an intrapulmonary cyst and the tracheobronchial tree may result in rapid expansion of the cyst, causing respiratory distress and cyanosis in the neonate, or if obstructed, atelectasis of the distal lung may

occur. Mediastinal cysts may cause compression of the trachea, bronchi, and/or esophagus and can present as wheezing, stridor, dyspnea, and/or dysphagia. Intrapulmonary cysts may present as recurrent infection in older children and more rarely with hemoptysis or pneumothorax. Treatment is surgical excision (28).

Conducting Airway Abnormalities

Tracheal Stenosis

Congenital tracheal stenosis is a rare malformation in which the trachea is narrowed, due either to intrinsic abnormalities in cartilage formation or to external compression by abnormal vessel formation or vascular rings (21,29). Narrowing of the trachea by compression results in local obstruction to the passage of air, while cartilage deformities may cause obstruction of the airway on both inspiration and expiration. The major underlying causes of intrinsic tracheal stenosis are diffuse or focal abnormalities in cartilaginous ring formation, either due to posterior fusion of the C-shaped rings or formation of a complete cartilaginous sleeve (16). Several types of intrinsic stenosis have been described: (1) segmental stenosis with local narrowing of the trachea; (2) generalized or complete stenosis of the trachea, which is associated with other anomalies excluding heart

and lung disease; and (3) tracheal stenosis with marked heart and lung disease (30,31). Segmental stenosis, which can occur anywhere in the tracheobronchial tree, is seen in 50% of cases, while generalized or complete stenosis is found in 30% of cases (32). Interestingly, the latter malformation has been reported in children with craniosynostosis, including Apert (OMIM #101200) and Pfeiffer (OMIM #101600) syndromes (33–42). These syndromes involve abnormal fusion of skeletal or osseous structures and are associated with autosomal dominant, heterozygous mutations in the fibroblast growth factor receptor, *FGFR2* (OMIM *176943) (35,37,38,42). Craniosynostosis, cleft palate, and tracheal stenosis, with thickened, cartilaginous tracheal sleeves and atelectasis of the distal lung, have also been observed in transgenic mice wherein *Fgfr2* has been mutated or partially deleted (43–45). These malformations represent mesenchymal defects in which the cells do not respond normally to FGF signaling, affecting both chondrogenesis and osteogenesis.

Recently, tracheal abnormalities consistent with intrinsic tracheal stenosis have been reported in young children with cystic fibrosis (CF; OMIM #219700), as well as in genetic animal models of CF caused by mutations or deletion of the *CFTR* gene (OMIM *602421). Examination of the conducting airway in neonatal CF pigs demonstrated luminal narrowing of both the trachea and main stem bronchi, as well as the presence of irregularly shaped cartilage rings, hypoplastic submucosal glands, and abnormal airway smooth muscle (46). Likewise, analysis of previously published morphometric data and chest CT scans from children with CF (less than 2 weeks old) showed alterations in the circular shape of the trachea, as well as a reduction in the size of the lumen (46). Subsequent functional studies demonstrated air trapping and airflow obstruction in young children with CF (47), as well as in the neonatal CF pig (48). Previous studies in *Cftr* knockout and/or mutated mice also revealed disrupted or incomplete tracheal rings with tracheal stenosis in the upper trachea, as well as altered breathing patterns in both newborn and adult mutant mice (49,50).

As for TEF and tracheal agenesis, patients with congenital tracheal stenosis have additional malformations, including other airway and lung abnormalities, esophageal and diaphragmatic

abnormalities, as well as cardiovascular, skeletal, GU, and GI tract anomalies (16,31). Infants with tracheal stenosis present with respiratory distress, stridor, cyanosis, cough, and difficulty feeding. Older patients may present with recurrent pneumonia. Diagnosis is confirmed by bronchoscopy and radiologic imaging.

Extrinsic tracheal stenosis is caused by external compression of the trachea, usually associated with abnormally situated blood vessels, termed vascular rings (20,31). These can include a double aortic arch, a right aortic arch with a left ligamentum arteriosum, an aberrant (retroesophageal) right subclavian artery, a right aortic arch with aberrant left subclavian artery, an anomalous left innominate or carotid artery, or a pulmonary artery sling (retrotracheal), which is found in 20% of tracheal stenosis cases (31,32). In the case of pulmonary artery sling, the left pulmonary artery originates from the right pulmonary artery, encircling and compressing the right main stem bronchus and distal trachea.

Tracheomalacia

Congenital tracheomalacia occurs when there is an absence or abnormality of the cartilaginous rings with hypotonia of the membranous or muscular posterior wall (the trachealis muscle), which causes the trachea to collapse on expiration, obstructing the airway. It is the most common congenital abnormality of the trachea with an estimated incidence of 1 per 1,445 infants (51). Tracheomalacia can also be caused by external compression from cardiovascular structures, tumors, lymph nodes, or other masses. Primary tracheomalacia is caused by congenital immaturity of the tracheal cartilage and may be associated with other lung/foregut defects, such as TEF, EA, and bronchopulmonary dysplasia (51). Tracheomalacia is often seen in connective tissue disorders, which result in the formation of abnormal cartilaginous structures, as well as in many other genetic syndromes (51).

In infants, the airway cartilage is normally soft, so that all infants have some degree of collapse on expiration, that is, when external pressure on the trachea is greater than the internal pressure. In the normal trachea, the cartilage-to-soft tissue ratio is 4.5:1, a ratio that remains constant throughout childhood. In tracheomalacia, there is a reduction in this ratio, in some instances as low as 2:1 (20,51). In secondary, or acquired

tracheomalacia, normal cartilage undergoes degeneration, which may be caused by prolonged positive pressure ventilation or by infection or inflammation. Tracheomalacia may also be a frequent complication of surgical repair of EA/TEF. Symptoms include wheeze, cough, stridor, dyspnea, tachypnea, cyanosis, and recurrent infection. Most affected infants improve by 6–12 months of age as the structural integrity of the trachea is gradually restored by further cartilage development and growth of the trachea (16).

Bronchial Atresia, Bronchial Stenosis, and Bronchomalacia

Congenital bronchial atresia is a rare anomaly and is often identified as an incidental finding on a chest X-ray in asymptomatic older children or adults, appearing as a hyperinflated or hyperlucent area that may compress adjacent tissue and cause a mediastinal shift (Figure 6-3). The most commonly affected lobe is the left upper lobe, but bronchial atresia of the right upper and lower lobes has also been reported. The segmental bronchus is the most common site of atresia, but subsegmental and lobar bronchi can also be affected along with multiple segments (21,29). The lung distal to the obstruction may be hypoplastic, often with regions of microcystic maldevelopment (9), emphysema, or hyperinflation. Air may enter the affected lobe via collateral airways,

causing mild overinflation or air trapping. Mucus may accumulate in the distal bronchial segments, causing a mucus plug or a mucus-filled cyst. Often there is a loss of bronchi and vessels in the affected lobe, as well as an absence of segmentation and interlobular septa. It has been suggested that bronchial atresia is a secondary process rather than a primary developmental failure, and that these lesions arise after bronchial formation is complete (weeks 5 to 17) (9).

Like tracheal stenosis, congenital bronchial stenosis, or narrowing of the bronchi, may be intrinsic or due to external compression (25). Intrinsic bronchial stenosis is rare and is usually associated with anomalous cartilage segmentation. Extrinsic bronchial stenosis due to compression is often associated with congenital heart disease. Compression occurs when the pulmonary arteries enlarge in response to pulmonary hypertension, compressing the left upper lobe bronchus. An enlarged left atrium, a bronchogenic cyst, or a teratoma may also compress the left main bronchus.

Congenital bronchomalacia, or dynamic narrowing of the bronchi, is caused by congenital abnormalities or absence of the bronchial cartilage, which lead to collapse, or bronchiectasis, of the affected airway during respiration. Bronchomalacia is a relatively common abnormality of the lower airways and is often associated with tracheomalacia. It may also be caused by extrinsic compression,

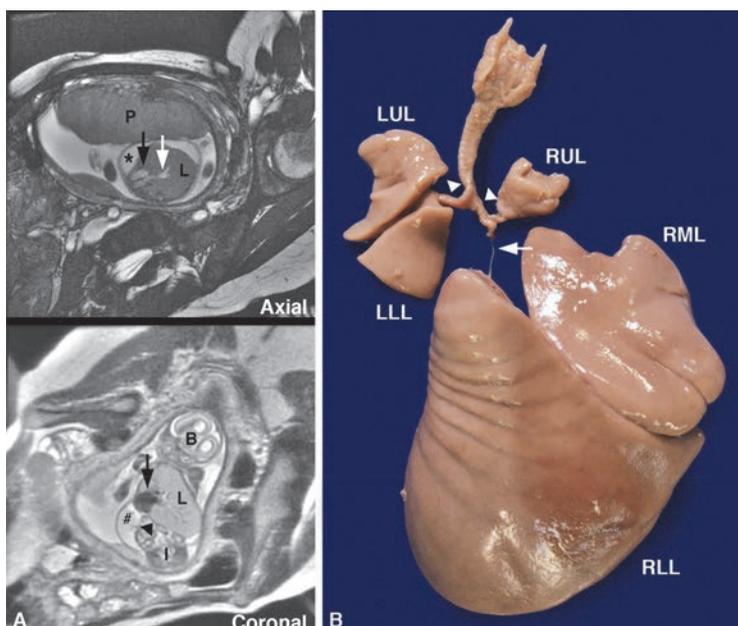


Figure 6-3. Bronchial atresia.

Intrauterine fetal death occurred at 25 weeks gestation after a pregnancy complicated by hydrops fetalis and a fetal MRI showing right bronchial atresia and a large right lung (A, L = lung) with dilated intrapulmonary airways (A, white arrow) associated with a mediastinal shift to the left, displacing the heart (A, black arrows) to about the left chest wall. The diaphragm was inverted (A, arrowhead), and pleural (A, *) and peritoneal (A, #) fluid was noted. The placenta (A, P), brain (A, B) and intestines (A, I) are designated in the fetal MRI for orientation. Autopsy confirmed atresia of the right middle and lower lobe bronchi (B, arrow) with hypertrophy of the right middle (B, RML) and lower lobes (B, RLL) and hypoplasia of the right upper lobe (B, RUL) and left upper (B, LUL) and lower lobes (B, LLL) with patent bronchi (B, arrowheads).

or it may be secondary to infection or to lung/heart-lung transplant. Bronchomalacia is often associated with other anomalies, such as various skeletal dysplasias or diffuse congenital cartilage deficiency (29).

Pulmonary Parenchymal Malformations

Pulmonary Agenesis and Aplasia

Pulmonary agenesis and aplasia represent two forms of arrested lung development with complete absence of lung tissue (52,53). In pulmonary agenesis the absence of lung parenchyma is accompanied by complete absence of the bronchi and vasculature, whereas in pulmonary aplasia a blind-ended rudimentary bronchus is present. Pulmonary agenesis and aplasia differ from pulmonary hypoplasia wherein bronchi, vasculature and distal lung parenchyma are present but incompletely or defectively developed resulting in an overall decrease in lung size.

Pulmonary agenesis is a very rare condition estimated to affect 1 of 15,000 births (52,53). Bilateral pulmonary agenesis is extremely unusual and incompatible with life. Unilateral pulmonary agenesis is more common with the prognosis being variable and best predicted by the severity of the associated anomalies and the presence of genetic abnormalities. Pulmonary agenesis is suspected to result from a disruption of normal lung bud development that begins during the sixth week of gestation. Although the pathogenesis remains unknown, teratogenic insults and defective dorsal aortic arch blood flow in the fourth week of gestation have been proposed as contributing factors (54). The hypothesis that abnormalities in embryonic aortic arch development contribute to the pathogenesis of pulmonary agenesis is based on frequent association of unilateral pulmonary agenesis with ipsilateral malformations of derivatives of the first and second branchial arches and/or radial ray defects (54). Review of 72 cases of pulmonary agenesis associated with other malformation revealed that 82% of cases had malformations of the first and second arch derivatives and/or radial ray defects. Moreover, in all cases, the face and radial ray malformations were ipsilateral to the unilateral pulmonary agenesis, and bilateral facial and/or radial ray anomalies appeared to be indicative of bilateral pulmonary agenesis. Branchial arch and/or radial ray malformations were occasionally on the side of the less-involved lung in bilateral pulmonary agenesis cases, however,

providing evidence that additional etiologic factors are operative in the pathogenesis.

Pulmonary agenesis is frequently associated with other malformations, including cardiovascular, GI, skeletal, GU, limb, and facial anomalies (53,54). Pulmonary agenesis is also a component in several syndromes, including Goldenhar syndrome, Vici syndrome (OMIM #242840), VACTERL syndrome, trisomy 21, and DiGeorge syndrome with microdeletions in 22q11.2 (53). In cases of unilateral pulmonary agenesis, multiple other anomalies are detected in ~75% of cases (53). Pulmonary aplasia can also be associated with aberrant vascular development resulting in a pulmonary artery sling that can result in tracheobronchial compression leading to severe respiratory symptoms (55). The pulmonary arteries are formed by joining of the vascular buds derived from the sixth branchial arches (central pulmonary arteries) to the lung buds derived from the postbranchial vessels (peripheral pulmonary arteries). In the case of right lung aplasia with left pulmonary artery sling, the right vascular bud is believed to connect to the left lung bud because no right lung bud exists. This process implies that in right pulmonary aplasia, the right sixth branchial arch develops initially despite the absence of the right lung bud and persists long enough to connect to the left lung bud (55). Surgical repositioning of the aberrant pulmonary artery can be successful in alleviating respiratory systems (55).

Isolated unilateral pulmonary agenesis that occurs in ~25% of patients has a much improved prognosis compared with those cases associated with other structural abnormalities (53). Compensatory growth of the unaffected lung occurs in individuals with isolated unilateral pulmonary atresia who can live without limitations. Despite concerns related to diminished lung capacity and susceptibility to recurrent pulmonary infections, isolated unilateral pulmonary atresia has been diagnosed incidentally in adults, providing further evidence that unilateral pulmonary agenesis can be associated with adequate respiratory function (56,57).

Pulmonary Dysplasia

These are a group of fatal lung disorders that include congenital acinar dysplasia (also known as acinar dysgenesis or acinar aplasia), congenital alveolar dysplasia, and alveolar capillary dysplasia with or without misalignment of the pulmonary

veins (58–63). Infants with these disorders have persistent pulmonary hypertension and unexplained, severe respiratory distress requiring ventilation and/or ECMO, but deteriorate quickly when support is withdrawn.

These disorders represent a rare form of diffuse interstitial lung disease characterized by uniform developmental impairment of the distal pulmonary airspaces, or acini, resulting in severe pulmonary hypoplasia. Although there is considerable overlap between the clinical and histological features of these disorders, acinar dysplasia is the most severe phenotype, exhibiting an almost complete lack of mature alveoli, with little to no development of the pulmonary acini distal to the bronchioles. The lung lobules are composed of bronchioles lined by ciliated columnar epithelia surrounded by smooth muscle fibers, which terminate directly at the pleura and interlobular septa. Although acinar dysplasia is thought to represent arrest in the early pseudoglandular stage of lung development (8–16 weeks), the bronchiolar

epithelium is well differentiated and representative of the term lung (64). In some cases, a few immature canalicular or saccular structures may be found, but this is rare (59).

In comparison, congenital alveolar dysplasia represents arrest of acinar development in the late canalicular or early saccular stage of lung development (17–24 weeks) with the formation of primitive, somewhat simplified canalicular and/or saccular structures (59,62). The interstitial regions are very wide and composed of loose, primitive mesenchyme with little collagen. Although the capillary bed is extensive, only a few of the capillaries are adjacent to the epithelial surface of the distal airspaces. Thus, development of the alveolar–capillary membrane is impaired, and gas exchange is severely compromised.

Alveolar capillary dysplasia is similar to congenital alveolar dysplasia but exhibits reduced alveolar capillary formation, as well as misalignment of the pulmonary veins (ACD/MPV; Figure 6-4). In general, the capillaries are located

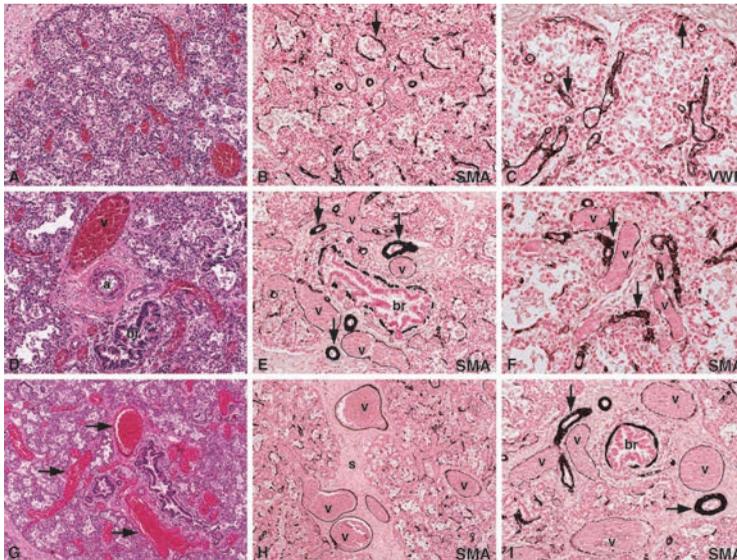


Figure 6-4. Alveolar capillary dysplasia and misalignment of pulmonary veins associated with a *FOXF1* mutation. An infant girl born at term developed progressive cyanosis and respiratory distress several hours after birth, requiring mechanical ventilation and eventually extracorporeal membrane oxygenation (ECMO). After several attempts to discontinue the ECMO, a diagnosis of persistent pulmonary hypertension due to an irreversible, pulmonary lesion was made, and ECMO was discontinued. Histologic examination of the lungs at autopsy revealed diffuse alveolar capillary dysplasia (A) with musculation of the alveolar septa (B, arrow; SMA = alpha smooth muscle actin immunostain) and reduced alveolar capillary formation (C, arrows; VWF = von Willebrand's factor immunostain for endothelial cells), as well as misalignment of the pulmonary veins and arteries (D, br = bronchiole, v = vein, a = artery). In addition, there was muscular hypertrophy of the pulmonary arteries (E, arrows), muscularization of the small pulmonary arterioles (F, arrows), and dilated venules (F, v = dilated venules). Thin-walled, dilated, and congested venous vessels were found to be traveling indiscriminately through the peripheral lobules (G, arrows), as well as in the perilobular septa (H, s = septum, v = veins). In addition, misalignment of the muscular arteries (I, arrows) was observed, many of which were found accompanying the normally positioned pulmonary veins (I, v = veins; br = bronchiole) in the perilobular septa. Original magnifications: 5x (G, H), 10x (A, B, D, E, I), 20x (C, F).

in the interior of thickened alveolar septa instead of in close proximity to the alveolar epithelia (58,60,63). Although the largest pulmonary veins may be located in the interlobular septa, the smaller pulmonary veins are displaced, or “misaligned,” in that they are located adjacent to the pulmonary arteries in the peribronchiolar connective tissue (60). Both the pulmonary veins and the lymphatics are thin walled and dilated, while there is increased medial hypertrophy and hyperplasia of the pulmonary arteries and muscularization of the smaller, peripheral arterioles. One-third of the patients have lymphangiectasis. In addition, there is significant underdevelopment of the pulmonary lobules with reduction and simplification of the distal acinar structures with some alveolar type II cell hyperplasia (63). Recently, three-dimensional reconstruction of lung tissue from patients with ACD/MPV demonstrated that there is a right-to-left vascular shunt linking the systemic and pulmonary circulation, which bypasses the alveolar capillary bed and causes respiratory insufficiency and persistent hypertension (65,66). Patients with ACD/MPV often have additional organ abnormalities, including GI, GU, musculoskeletal, and cardiovascular malformations, as well as disruption of the normal right-left symmetry of intrathoracic or intraabdominal organs (58,67–69). Haploinsufficiency of *FOXF1* (OMIM *601089), a transcription factor important for vascular and alveolar development, causes ACD/MPV. At least 60 distinct mutations and/or genomic deletions in *FOXF1* have been identified in patients with this disorder (70). Most are sporadic, autosomal dominant mutations, although several have been inherited as autosomal recessive disorders with maternal inheritance, consistent with paternal imprinting (71). Inactivation of *Foxf1* in murine endothelial cells inhibited VEGF signaling and decreased expression of endothelial genes critical for vascular development (72).

Congenital Malformations Associated with Lung Growth and Structure

Congenital pulmonary malformations have an estimated incidence of 2–7% (73). The most commonly encountered congenital lung anomalies associated with deficient lung growth and aberrant lung structure include lung agenesis–hypoplasia complex

(pulmonary underdevelopment), bronchogenic cysts, congenital pulmonary airway malformations (CPAM), pulmonary sequestrations (PS) and congenital lobar overinflation (CLO). CPAM and PS have a reported incidence of 50% and 33% of prenatally diagnosed lung lesions, respectively (74). It is widely accepted that congenital lung lesions result from perturbations in lung and airway embryogenesis. Although congenital lung malformations are a heterogeneous group of lesions, there is considerable overlap, and frequently the lesions occur together. Based on these observations, airway obstruction during development has been proposed as a unifying pathogenetic mechanism, with the location of the obstruction within the tracheobronchial tree, completeness of the obstruction, and timing of the developmental insult determining the type of lesion and histopathology (9,75).

In the past, congenital pulmonary malformations were noticed within the first weeks to months of life, but currently these lesions often are diagnosed in utero by ultrasonography and magnetic resonance imaging (MRI) due to advances in technology and implementation of routine investigations at weeks 18–20 of gestation (76–78). Prenatal MRI is highly accurate in defining congenital lung anomalies, with one study showing postnatal confirmation of the prenatal MRI diagnosis in 91% (51/56) of lesions, providing evidence that fetal MRI can provide a specific diagnosis to guide prenatal counseling and patient care (78). Prenatal diagnosis also provides an opportunity to sequentially follow these lesions to better understand their pathophysiological mechanisms. Others warn that those who interpret prenatal imaging should not attempt to make a definitive diagnosis because CPAM, PS, bronchial atresia, and CLO have overlapping features. It has thus been emphasized that only a histologic examination can definitively support a diagnosis, and diagnoses based on imaging alone should be avoided to not complicate interpretation of the literature (79).

Most congenital lung malformations have a favorable prognosis, with a mortality rate of <5% for antenatally detected lesions, and symptoms at birth occurring in 17% of cases (76,79). Poor prognostic factors include mediastinal shift, polyhydramnios and hydrops (76). Timely resection remains the treatment of choice for all symptomatic postnatal lesions; however, much controversy remains around the management of asymptomatic congenital lung malformations, which are usually

discovered by routine fetal ultrasound examination (79,80). Incomplete knowledge surrounding the natural history of congenital lung malformations compromises the ability to define relative risks and benefits of early resection. Arguments for resection of asymptomatic lesions include infection risk, malignant potential, uncertainty in radiographically distinguishing lesions, prevention of pneumothorax, decreasing radiation exposure associated with surveillance, and greater compensatory lung growth early in life. These risks vary among the specific types of congenital lung malformations (80,81). On the basis of risk of infection and malignancy, current recommendations are for resection of bronchogenic cysts, CPAM, and intralobular PS, while asymptomatic CLO and extralobular PS may be observed (80). A large multicenter registry of congenital lung malformations would enhance understanding of the onset, timing, natural history, and prognosis of congenital lung malformations to improve evidence-based approaches to patient diagnosis and management.

Pulmonary Hypoplasia

Pulmonary hypoplasia is defective or incomplete development of the lung resulting in reduced lung size due to decreased numbers or size of acini (Figure 6-5). Lung weight, lung weight/body weight ratios, radial alveolar counts, and lung volume measurements are used to determine the presence of pulmonary hypoplasia (82). Clinically, fetal lung size is determined by two- and three-dimensional ultrasound and MRI (83–85). Primary pulmonary hypoplasia occurs in the absence of an identifiable cause or association and is believed to result from alterations in transcription factor and/or growth factor signaling (86). Retinoic acid signaling deficiencies in animal models results in severe respiratory phenotypes including lung hypoplasia and agenesis (87,88). Retinoic acid also influences perinatal alveolus formation in rodents, which has led to its clinical use for the prevention of bronchopulmonary dysplasia/chronic lung disease of prematurity (89,90). Identification of *NKX2-1* (OMIM *600635) mutations in patients with brain-lung-thyroid syndrome (OMIM #610878) and corresponding phenotypes in *Nkx2-1* deficient murine models, established *NKX2-1* as a critical gene driving lung development. *NKX2-1* encodes the protein thyroid transcription factor-1 (TTF-1).

Expression of TTF-1 is restricted to the developing lung, thyroid, and ventral forebrain, corresponding with the respiratory distress syndrome, congenital hypothyroidism, and benign hereditary chorea phenotypes seen in syndromic patients with heterozygous *NKX2-1* mutations (91–93). Although the pulmonary histopathology is heterogeneous, there are often clear growth abnormalities with alveolar simplification, lobular remodeling, and cyst formation (94). Related phenotypes in *Nkx2-1* deficient mice provide evidence for a causative role for TTF-1 loss in the human syndrome. Heterozygous *Nkx2-1* loss in mice results in neurological and thyroid dysfunctions, whereas homozygous deletion results in complete absence of the thyroid, as well as severe brain defects and lung hypoplasia (92,95). The lungs in *Nkx2-1* null mice consist of bilateral sac-like structures that originate from a short, common tracheoesophageal tube and are lined by primitive epithelial cells (96).

Secondary pulmonary hypoplasia occurs in association with other abnormalities and accounts for >85% of cases (82,97). A wide variety of associated abnormalities have been described, however, and the most frequent associations are with processes that compromise thoracic space or result in oligohydramnios (82). These abnormalities limit fetal breathing movements and lung distension required for normal lung development. Among the most common associated anomalies are congenital diaphragmatic hernia, obstructive uropathy, and renal anomalies, including renal agenesis, renal dysgenesis, and polycystic kidney disease. Thoracic space occupying lesions including CPAM (Figure 6-5D–F), PS, mediastinal masses, and lymphatic malformations can result in pulmonary hypoplasia. Oligohydramnios due to prolonged premature rupture of membranes has also been associated with compromised lung growth. Potter sequence is seen in cases associated with oligohydramnios of any cause and is characterized by sloping forehead, flattened face and nose, receding chin, large ears, broad spade-like hands, and deformations of the limbs secondary to compression within the uterus due to inadequate amniotic fluid. Finally, pulmonary hypoplasia can be seen in association with chromosomal abnormalities (Figure 6-5A–C), including trisomy 13, 18, and 20, and as a component of multiple syndromes including Scimitar (OMIM 608281), Down, Eagle-Barret, and Pena-Ahokeir

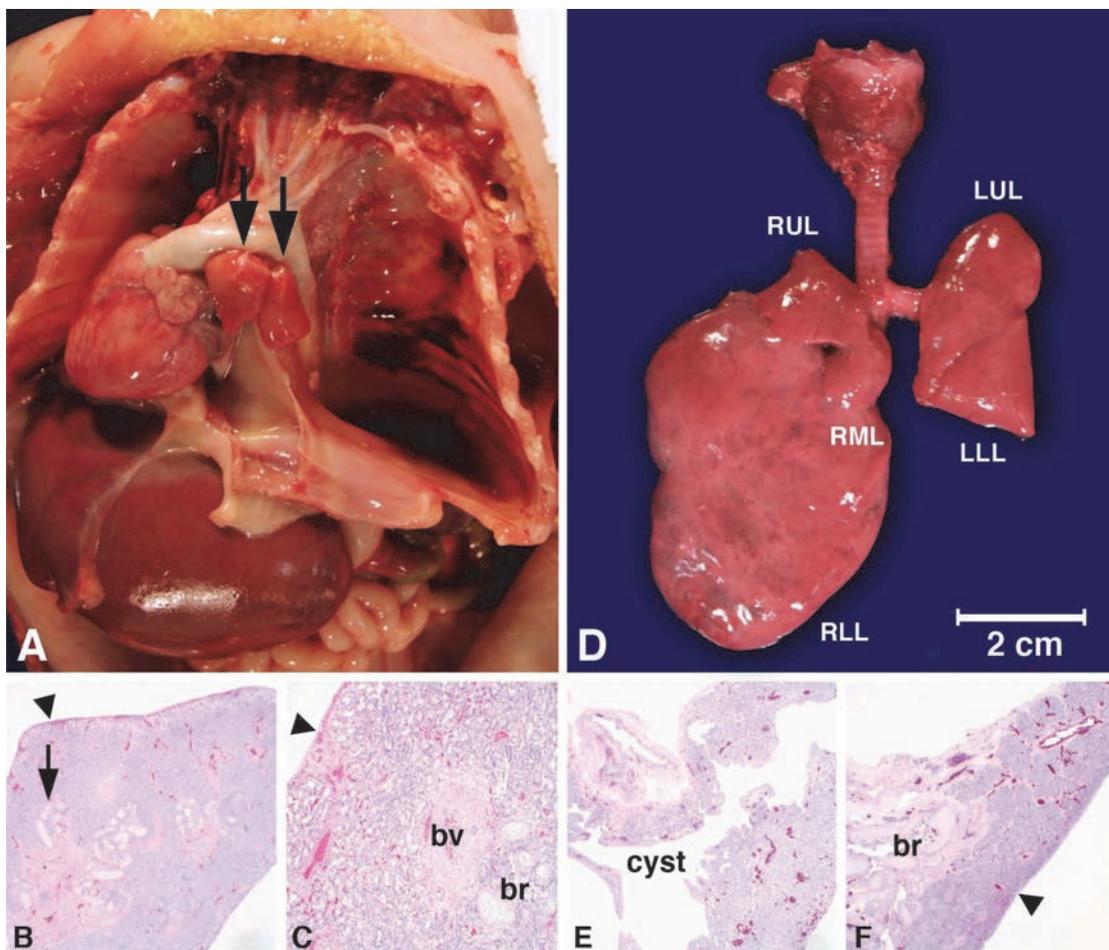


Figure 6-5. Pulmonary hypoplasia. Images from two different patients are shown. Patient 1 (A–C) is an infant born at 31 weeks gestation by Cesarean section after pregnancy complicated by fetal hydrops, ascites, pleural effusions, and an abnormal chromosome karyotype of 46XX with a 13q deletion (13q32.3→13q34) and duplication of ~1.3 Mb from 12q11.1, detected by cytogenetic studies on amniocentesis fluid, microarray studies, and fluorescence in situ hybridization (FISH). Autopsy revealed a hydropic infant with severe bilateral pulmonary hypoplasia (A, arrows) with a combined lung weight = 2.1 grams (normal for 31 weeks gestation = 19.4 ± 6.1 grams), bilateral pleural effusions, ascites, and a small muscular interventricular heart defect. Histologic examination of the lungs revealed development of normal lung structures, including conducting airways (B, arrow and C, br = bronchus) with accompanying arterial vessels (C, bv = blood vessel) and a reduced number of small acini (B–C, arrowheads = pleural surface). Patient 2 (D–F) is a fetus with unilateral, macrocystic, congenital pulmonary airway malformation (CPAM) with a mediastinal shift diagnosed at 22 weeks gestation. Prenatal evaluation also revealed fetal hydrops with congestive heart failure, pleural effusion, ascites, and polyhydramnios. Intermittent variable and late decelerations as well as absent end diastolic blood flow were noted with death in utero occurring at 29 weeks gestation, despite amnioreduction and shunt placement in the right pleural cavity. A torso restricted autopsy revealed ascites, pericardial effusion, and a mediastinal shift with an enlarged right lower lobe (D, RLL) containing a CPAM with multiple large cysts, consistent with CPAM, type 1 (E), and hypoplastic right upper (D, RUL), right middle (D, RML), left upper (D, LUL), and left lower (D, LLL) lobes. Histologic examination of the hypoplastic lobes revealed normally developed conducting airways (F, br = bronchus) with accompanying vascular structures and a reduced number of small acini, resulting in a decreased distance between the hilar bronchus (F, br = bronchus) and the pleural surface (F, arrowhead). Original magnifications: 2x (B, E, F), 10x (C).

syndromes (82). Developmental lung abnormalities, including pulmonary hypoplasia, abnormal pulmonary lobation, and anomalies of laryngeal and tracheal development, are also relatively common in Smith–Lemli–Opitz syndrome (OMIM #270400), an autosomal recessive

malformation syndrome caused by mutations in the *DHCR7* gene (OMIM *602858), which encodes the enzyme 7-dehydrocholesterol reductase that catalyzes the final step in cholesterol biosynthesis (98, 99). *Dhcr7* null mice, a model for the human disease, die within 24 hours of birth with lung

saccular hypoplasia characterized by failure to terminally differentiate alveolar sacs, delayed differentiation of type I alveolar epithelial cells, and an immature vascular network (100). Approximately one-quarter of Smith–Lemli–Opitz syndrome patients also have renal anomalies, including renal hypoplasia or agenesis, which may contribute to the lung hypoplasia (98).

Although pulmonary hypoplasia is often diagnosed prenatally or at birth, lung growth disorders can also present as diffuse lung disease in infancy. In review of lung biopsies from children <2 years of age, lung growth abnormalities were the leading diagnosis, accounting for 25% of cases (101). Pulmonary hypoplasia with prenatal conditions associated with deficient lung growth were present in some cases; however, in 85% of the cases, postnatal growth abnormalities occurred in the setting of prematurity, congenital heart disease, and/or a chromosomal abnormality, Trisomy 21 being the most frequent. The deficient lung development was histologically characterized by variable lobar simplification with subpleurally predominant alveolar enlargement.

Lung biopsy is not generally done for a diagnosis of lung growth abnormality, and infants who come to biopsy typically have diffuse lung disease with pulmonary symptoms and morbidity that are disproportionate to the clinical circumstances (101). The diagnosis of lung growth disorders in this setting is often unsuspected clinically and underrecognized histologically. A history of prematurity or congenital heart disease was highly predictive of lung growth abnormality as the primary histologic finding. Congenital heart diseases with right outflow obstruction are a significant risk factor for prenatally acquired pulmonary hypoplasia, and a decrease in alveolar multiplication may also be acquired postnatally as a direct result of the decrease in pulmonary blood flow (102). Perfusion independent mechanisms are also thought to play a role in the pathogenesis of congenital heart disease–associated pulmonary hypoplasia (102). Patient outcomes are related to the associated conditions, with prematurity being an independent clinical predictor of mortality, and congenital heart disease and pulmonary hypertension being associated with trends toward increased mortality (101). Severity of the growth abnormality, as judged histologically by dramatically increased alveolar size and moderate to severe hypertensive

changes on the biopsy are also associated with increased mortality. Patients with pulmonary hypoplasia associated with congenital diaphragmatic hernias require more pulmonary support initially as compared to patients with associated omphaloceles or congenital lung malformations (103). In addition to lung volumes, disease specific factors such as pulmonary hypertension in congenital diaphragmatic hernia contributed to pulmonary morbidity and overall outcome. Pulmonary hypoplasia can also rarely present in adults as primary unilateral pulmonary hypoplasia without associated anomalies (104–106). Patients may present with wheezing, have recurrent infections, or be asymptomatic with the diagnosis made as an incidental radiographic finding. Unilateral primary pulmonary hypoplasia is typically accompanied by compensatory hypertrophy of the contralateral lung, which likely accounts for the lack of symptoms, delay of diagnosis into adulthood, and favorable outcome.

Congenital Pulmonary Airway Malformations

Congenital pulmonary airway malformations (CPAM; formerly referred to as congenital cystic adenomatoid malformations) are a heterogeneous group of cystic and noncystic lung lesions resulting from aberrant fetal lung development (Figures 6-5 D-F and 6-6). Although CPAMs are rare, with a variable reported incidence between 1 per 8,300 and 1 per 25,000–35,000 births, CPAMs account for 30–40% of all congenital lung diseases and ~95% of all congenital cystic lung diseases (77,78,107). CPAMs are classified into five major types in the Stocker classification system (types 0–4) based on clinical and pathologic features, including cyst size and histologic resemblance to segments of the normal tracheobronchial tree (82). Challenges in applying this classification scheme clinically include overlap among the CPAM types, atypical forms that do not fit well into a specific category, and importantly, indistinguishable features between type 4 CPAM and the cystic neoplasm, pleuropulmonary blastoma (PPB) (9,108). These challenges have led in some instances to the clinical practice of not dividing CPAM into types or limiting categorization of cystic CPAM to large cyst and small cyst types. Despite the challenges, the Stocker classification system is widely used and has

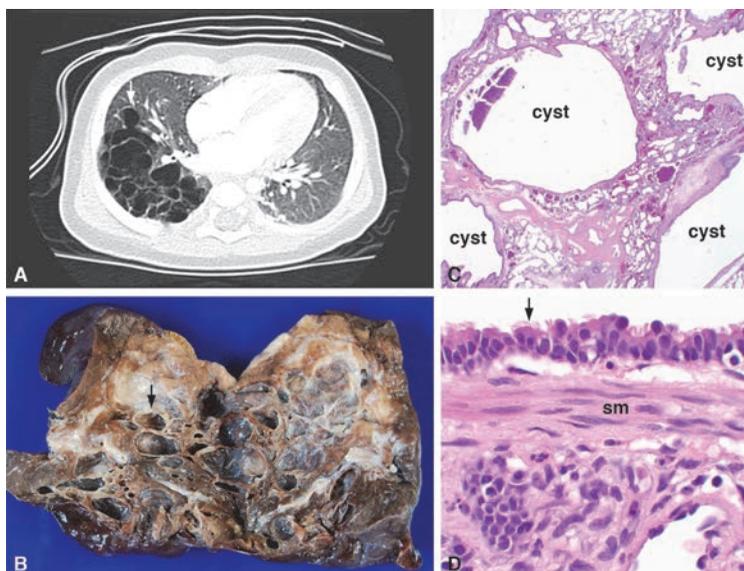


Figure 6-6. Congenital pulmonary airway malformation. A 4-month-old boy was found to have a right lower lobe multicystic lesion by CT imaging (A, arrow) that lacked an aberrant vascular connection to systemic or pulmonary arteries. A lobectomy was performed with the lesion grossly consisting of multiple cysts ranging in size from 1 mm to 1.5 cm in greatest dimension (B, arrow) separated by intervening parenchyma. Histologic examination revealed multiple cysts surrounded by compressed normal parenchyma (C) and lined by ciliated, pseudostratified, columnar epithelium (D, arrow). Smooth muscle bundles (D, sm = smooth muscle) were present in the cyst walls, characteristic of CPAM, type 1. Original magnifications: 2x (C), 100x (D).

utility in delineating several characteristic developmental lung lesions pathologically as well as radiologically.

CPAM type 0, also known as acinar dysplasia or agenesis, is composed of bronchus-like structures with muscle, glands, and cartilage plates separated by prominent mesenchymal tissue. Type 0 CPAMs are rare and largely incompatible with life presenting in term or preterm neonates who are cyanotic at birth and survive only a few hours. Cardiovascular abnormalities and dermal hypoplasia are associated findings (82). Type 1 CPAM, the large cyst type, is the most common subtype accounting for nearly 65% of cases (Figures 6-5 D-F and 6-6) (82). CPAM type 1 is characterized by single or multiple large cysts (3–10 cm in diameter) that resemble bronchi and proximal bronchioles, including segments lined by mucinous epithelium that have been suggested as cells of origin for the mucinous adenocarcinomas rarely reported in these lesions. Type 1 CPAMs are clinically distinct from type 0 in that they primarily present in the first week to month of life but can be seen in older children and adults and are associated with a good prognosis (9,82). Occasionally, these lesions are sufficiently large to result in mediastinal shift and pulmonary hypoplasia (Figure 6-5D–F) (9). Type 2 CPAM, the small cyst type, accounts for 10–15% of cases and is composed of smaller cysts (0.5–2.0 cm in diameter), presenting in the first year of life with a poorer outcome due to the frequent association with other anomalies such

as bilateral renal agenesis, cardiovascular malformations, diaphragmatic hernia, and pulmonary hypoplasia (82). Back-to-back cysts in type 2 CPAMs resemble distal bronchioles and blend with the adjacent parenchyma. Type 3 CPAM occurs infrequently, accounting for 5% of cases, and present exclusively in the first days to month of life with a high mortality rate (82). The lesions present as large, solid, air-containing masses comprised of small cystic spaces (≤ 0.2 cm) that produce a mediastinal shift, often resulting in hypoplasia of the uninvolved lung. Histologically, the lesions are comprised of immature appearing lung that is devoid of bronchi, consisting of bronchiolar-like structures surrounded by alveolar ducts and saccules. CPAM types 1–3 can be distinguished radiologically, with types 1 and 2 being heterogeneous with multiple discrete different-sized cysts and type 3 CPAM being homogeneous and solid, lacking discernible cystic spaces (11, 78). Type 4 CPAMs present as large thin-walled cystic lesions resembling distal acinar structures that are lined by epithelial cells with an alveolar type 1 and/or type II cell phenotype (109). This variant typically presents in the newborn to 4 years of age range and accounts for 10–15% of cases. The morphology, cellular phenotypes, radiologic findings, and clinical presentation of type 4 CPAMs are indistinguishable from cystic PPB and likely represent the same lesion (9,78,110,111). Erroneous designation of cystic PPB as CPAM needs to be avoided given that cystic PPB is a neoplastic

process with potential to progress to an overt sarcoma.

CPAMs usually present as sporadic, nonhereditary lung abnormalities that are associated with other anomalies in 15–20% of cases, particularly in the cases of type 2 lesions (82,107,110). The pathogenesis of CPAM remains unknown. These lesions are thought to result from abnormal branching morphogenesis during lung development. It has been proposed that airway obstruction is the basis for CPAM (9,75,97,112). CPAMs usually communicate with the normal tracheobronchial tree and receive blood supply from pulmonary vessels, which differentiates CPAM from PS, wherein there is systemic blood supply (11,78). Hypothesized mechanisms for CPAM development include failure of appropriate endoderm–mesoderm signaling, imbalance between increased cell proliferation and decreased programmed apoptotic cell death, altered gene expression, and aberrant growth factor signaling during lung morphogenesis (97,113). Experimental studies including genetic modifications in mouse models identify FGF-7, FGF-9, FGF-10, HOXB-5, and SOX-2 as potential molecular mediators of CPAM (97,113–118). Recent studies suggest that distinct patterns of signaling molecule expression may be helpful in distinguishing CPAM from cystic PPB (119). A genetic basis for CPAM type 0 is supported by a tendency for the lesions to recur in families in up to 40% of cases (three of eight families reported in the literature), suggesting an autosomal recessive inheritance pattern (120).

CPAM can be diagnosed antenatally or present as respiratory difficulty and/or infection after birth (97). Indicators of poor prognosis include large lesions, bilateral lung involvement, and hydrops (Figure 6-5D–F) (11,80). The CPAM volume ratio (CVR) has emerged as a useful prognostic tool to identify fetuses at increased risk of developing hydrops (79,80). CVR measures the volume of the lung lesion divided by the head circumference to normalize for gestational age. Prenatal management includes cyst aspiration, pleuroamniotic shunt, and open fetal lung resection (80). Recent reports suggest that maternal steroid therapy may induce regression of cystic lung lesions and thus represent a novel treatment approach (80). Postnatal treatment of symptomatic patients is surgical resection, which generally consists of lobectomy or segmental resection.

Surgical intervention for asymptomatic CPAM remains controversial (11,80). There are multiple reports of adenocarcinoma and adenocarcinoma in situ (formerly bronchioloalveolar carcinoma) arising in CPAM type 1 and PPB occurring in the setting of a CPAM diagnosis (81,110,121). Adenocarcinomas diagnosed in association with CPAM have been shown to have genetic mutations seen in lung cancers occurring outside the setting of CPAM including *K-RAS* (OMIM *190070) and *EGFR* (OMIM *131550) point mutations, and echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (*ALK*, OMIM *105590) rearrangement (122–124). On the basis of risk of malignancy and infection, current recommendations are for resection of asymptomatic as well as symptomatic CPAM (80).

Pleuropulmonary Blastoma

Pleuropulmonary blastoma (PPB) is the most common primary malignant lung neoplasm in childhood (Figure 6-7). PPB presents in three distinct clinicopathologic types that represent tumor progression (125). Type I PPB is characterized by cysts lined by benign-appearing epithelium resting on septa containing undifferentiated mesenchymal cells (Figure 6-7A–G) (108). Overgrowth of the primitive mesenchymal cells results in a combined solid and cystic (type II) or purely solid (type III) PPB with a sarcomatous component that resembles sarcomas occurring in other locations, such as embryonal rhabdomyosarcoma and fibrosarcoma (Figure 6-7H–J). The progression of type I to types II–III PPB is well documented. Importantly, however, not all cystic type I PPBs progress to the more malignant types. Indeed, purely cystic lesions that lack the mesenchymal cell component occur and are subclassified as type 1r or regressed PPBs (108,125). Cystic type I/r PPBs are clinically and radiographically indistinguishable from CPAM (126). Thus, these two lesions must be differentiated pathologically by identifying distinguishing features of type I PPB, including the multilocular architecture, presence of primitive mesenchymal cell collections within the septa, a relatively well-defined border with the adjacent normal lung parenchyma, and the predominantly flattened or alveolar type epithelium lining the cysts (108).

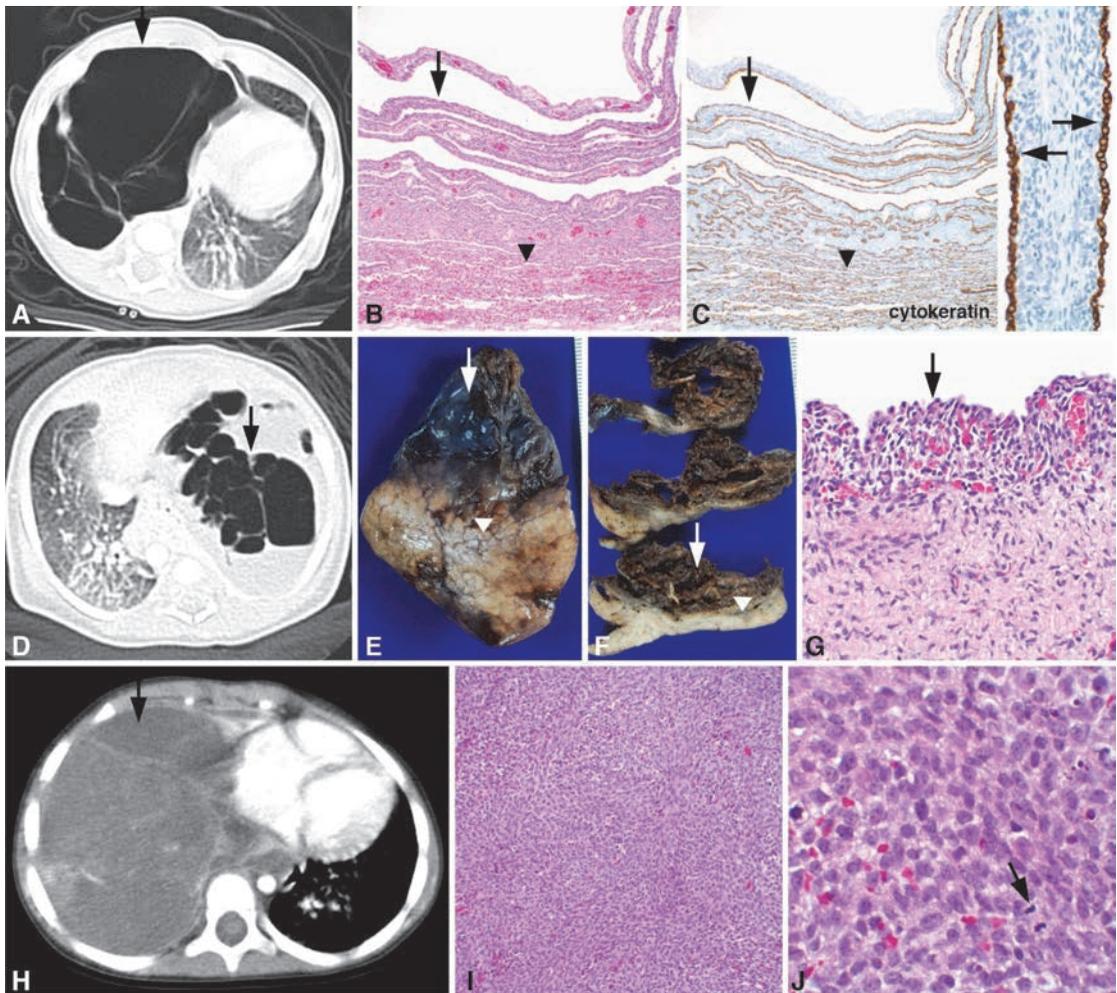


Figure 6-7. Pleuropulmonary blastoma. Images from three different patients are shown, demonstrating the spectrum of pleuropulmonary blastoma (PPB). Patient 1 (A–C) is a 2-month-old girl who was found to have a multicystic right lung lesion by CT scan (A, arrow) that was clinically thought to represent a CPAM, highlighting the overlapping clinical and radiographic features between CPAM and PPB. The lobectomy specimen contained a multilocular cystic lesion (B–C, arrows) with the largest cyst measuring 8 cm in maximum diameter. The cyst walls were thin measuring up to 0.2 cm in thickness, and the internal cyst wall was smooth. Some cysts were air-filled, whereas some of the smaller cysts contained fluid. The lesion was fairly well demarcated from the adjacent normal appearing parenchyma (B–C, below arrowheads). The cysts were lined by cuboidal epithelial cells highlighted by positive immunostaining for the general epithelial cell marker, pancytokeratin (C, arrows). The fibromuscular stroma within the cyst walls contained primitive mesenchymal cells, characteristic of type I PPB. Patient 2 (D–G) is a full-term infant boy delivered by scheduled Cesarean section with a prenatal diagnosis of CPAM detected by fetal MRI. A myelomeningocele and Chiari II malformation were also diagnosed prenatally. High-resolution chromosome analysis revealed a 46XY karyotype with no chromosomal abnormalities. A chest CT scan revealed multiple large rounded lucencies in the left lung with mediastinal shift to the right (D, arrow). The lung lesion was resected on day 15 of life with the lung lobe containing a multiloculated cyst (E–F, arrows) with adjacent uninvolved lung parenchyma (E–F, below arrowheads). Histologic examination of the cysts revealed a benign, cuboidal epithelium (G, arrow) lining the cysts with an underlying cambium layer comprised of a condensation of primitive mesenchymal cells directly subjacent to the epithelium (G, underlying arrow), a characteristic feature that distinguishes type I PPB from CPAM. Patient 3 (H–J) is a 3-year-old girl who was diagnosed with a large solid mass occupying the right chest by CT imaging (H, arrow) that was resected and diagnosed as a type III PPB. Local recurrence occurred 4 years later with microscopic examination of the excision specimen revealing a cellular high-grade sarcoma (I) with histologic features identical to the original tumor, including numerous mitotic figures (J, arrow) indicating a high mitotic rate. No cystic or epithelial component was identified, characteristic of type III PPB. Original magnifications: 10x (B, C), 20x (I), 40x (C inset, G), 100x (J).

PPB was initially recognized as a distinct entity in 1988 (127). Establishment of an International PPB Registry shortly thereafter led to the discovery that PPB was a sentinel tumor of a distinct hereditary syndrome (OMIM #601200) (128). This tumor predisposition syndrome is associated with a broad range of tumors with the most common tumors including PPB, cystic nephroma, ovarian Sertoli-Leydig sex cord-stromal cell tumors, and thyroid multinodular goiter (128–131). In 2009, loss-of-function heterozygous germ line *DICER1* mutations (OMIM *606214) were identified as a genetic cause for this familial syndrome with up to 66% of PPB patients having a mutation (125,132). *DICER1* is required for the generation of mature miRNAs, which are noncoding small RNAs that play a critical role in regulating fundamental processes, including development, cell growth, cell survival, and oncogenesis (133,134). Most carriers of the *DICER1* mutation are unaffected, indicating that tumor risk is modest, and additional events may be required for tumor initiation (131). *DICER1* protein expression was specifically lost in the epithelial component of some type I-II PPBs, suggesting that loss of *DICER1* function in the lung epithelium may predispose to PPB initiation (111,132). Indeed, genetic targeting of *Dicer1* loss to the developing lung epithelium in mice resulted in a cystic PPB phenotype (111). Moreover, studies in mouse models of the disease demonstrated that precise timing of *Dicer1* loss during lung development is critical in determining phenotypic outcomes (111). *DICER1* loss in the developing lung epithelium was sufficient for initiation of cystic PPB but did not result in tumor progression to sarcoma, providing evidence that additional events may be required for PPB progression. Genetic analysis of human PPBs and ovarian Sertoli-Leydig cell tumors identified frequent heterozygous *DICER1* germ line loss-of-function mutations accompanied by somatic mutations in the second *DICER1* allele (135–137). The somatic *DICER1* mutations occurred within the functional ribonuclease RNase IIIb domain of the protein, which is required for generation of mature miRNAs. Loss of the tumor suppressor, p53, was also frequently detected in PPB (136,137). These studies highlight the potential role of RNase IIIb domain *DICER1* mutations and p53 inactivation in PPB pathogenesis.

PPB is diagnosed in children <6 years of age and can arise during fetal development. The clinicobiologic progression from type I to type II and III PPB is reflected in the median age at diagnosis of 8, 35, and 41 months, respectively (125). PPB can also be diagnosed in utero with prenatal diagnosis occurring as early as 23 weeks gestation (125). The vast majority of cases (94%) present in the first 6 years of life, with rare cases presenting in older children and even adults (82). Presenting symptoms include respiratory distress, nonproductive cough, fever, chest pain, and pneumothorax. PPB type I is the strongest predictor of outcome (125). Type I/Ir PPB is associated with a 91% 5-year overall survival, with deaths in this group being due to tumor progression to types II or III. Overall survival rates for type II and type III PPB are 71% and 53%, respectively. Surveillance of *DICER1* mutation carriers may allow early PPB detection in the cystic type I stage resulting in improved outcomes.

Pulmonary Sequestration

Pulmonary sequestrations (PSs) are discrete masses of nonfunctioning bronchopulmonary tissue that lack communication with the normal bronchial tree and receive blood supply from one or more anomalous systemic arteries (Figures 6-8 and 6-9). PSs are classified into extralobular and intralobular types. Extralobular sequestrations (ELs) are anatomically separate from the normal lung with a distinct pleural covering and venous drainage to systemic veins. In contrast, intralobar sequestrations (ILs) are contiguous with the normal lung with a common visceral pleura and venous drainage into the pulmonary veins (Figures 6-8 and 6-9). ILs are more common than ELs, accounting for 75–80% of PSs (11,80,138). In addition to the pathologic differences, ELs and ILs also have differing clinical features (138). ILs rarely produce symptoms before 2 years of age, typically presenting in childhood or adulthood as isolated anomalies with no gender predilection (73,139). In contrast, ELs most commonly present before 6 months of life, with most studies reporting a male:female predominance of 3–4:1. Furthermore, 40–60% of infants with ELs have other associated anomalies (11,73,113,139). Congenital diaphragmatic hernia is the most frequent

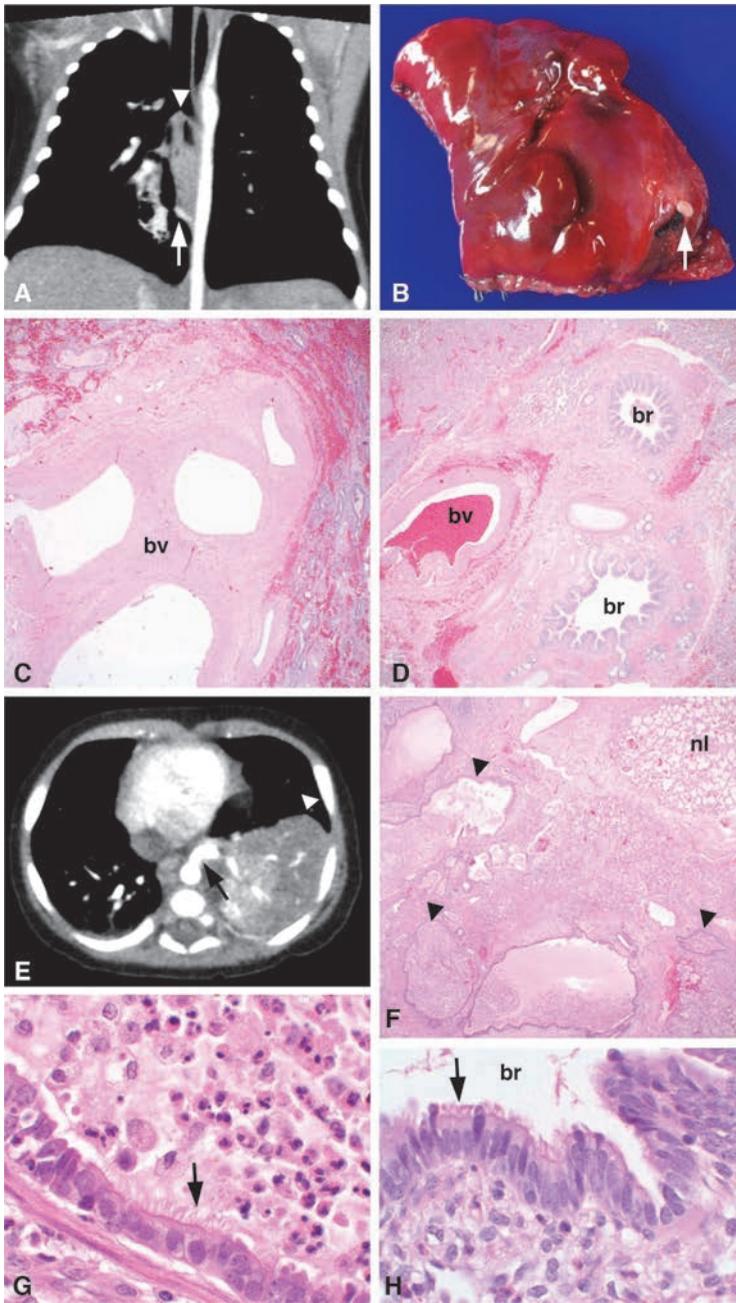


Figure 6-8. Pulmonary sequestration. Images from two different patients are shown. Patient 1 (A–D) is a 55-day-old infant with a prenatal diagnosis of CPAM who had a chest CT scan, which showed a 2.6 x 1.5 x 1.5 cm pulmonary opacity in the medial right lower lobe (A, arrowhead) containing air bronchograms and central lucency. A systemic feeding artery to the pulmonary lesion (A, arrow) was present, consistent with PS. The feeding vessel was ~2 mm in diameter, originating from the anterior aspect of the descending thoracic aorta approximately 3 cm cephalad to the diaphragmatic hiatus to the aorta and coursing posterior to the esophagus to end in the pulmonary lesion. A right lower lobe wedge resection was performed. A vessel unpaired with an airway (B, arrow) was present on the opposite side of the specimen as the normal bronchovascular bundle entering the lung wedge. Histologic examination confirmed the diagnosis of ILS with the isolated feeding vessel consisting of a large elastic artery unpaired with an airway structure (C, bv = blood vessel) that was distinct from the paired bronchus and blood vessel entering the opposite side of the lung wedge (D, br = bronchus, bv = blood vessel). Patient 2 (E–H) is a 3-month-old boy who was found to have a left lower lobe lesion (E, arrowhead) with a large systemic feeder vessel originating from the thoracic aorta (E, arrow), consistent with PS. A left lower lobe lobectomy was performed revealing a feeder vessel entering the specimen ~2 cm from the hilar structures. The lobe was comprised of numerous cysts (F, arrowheads) surrounded by a rim of more normal appearing parenchyma (F, nl = normal), consistent with ILS. The cystic structures were lined by ciliated columnar epithelium (G, arrow) resembling the epithelium (H, arrow) lining normal bronchioles (H, br = bronchiole), characteristic of CPAM, type 2. The dilated airways within the lesion contained acute and chronic inflammatory cells including macrophages and neutrophils admixed with amorphous mucinous material (G, top right), indicative of involvement by an inflammatory/infectious process. Original magnifications: 4x (C, D, F), 100x (G, H).

coexisting anomaly being present in ~16% of cases. Additional congenital lung abnormalities are present in 25% of ELSs, including pulmonary hypoplasia, CPAM, CLO, congenital pulmonary lymphangiectasia, and bronchogenic cysts. Cardiac abnormalities, foregut duplication cysts,

chest wall and vertebral deformities, hindgut duplications, and accessory spleen can also be seen.

The etiology of PS has been actively debated with the precise pathogenesis remaining unknown. Proposed etiologies revolve around

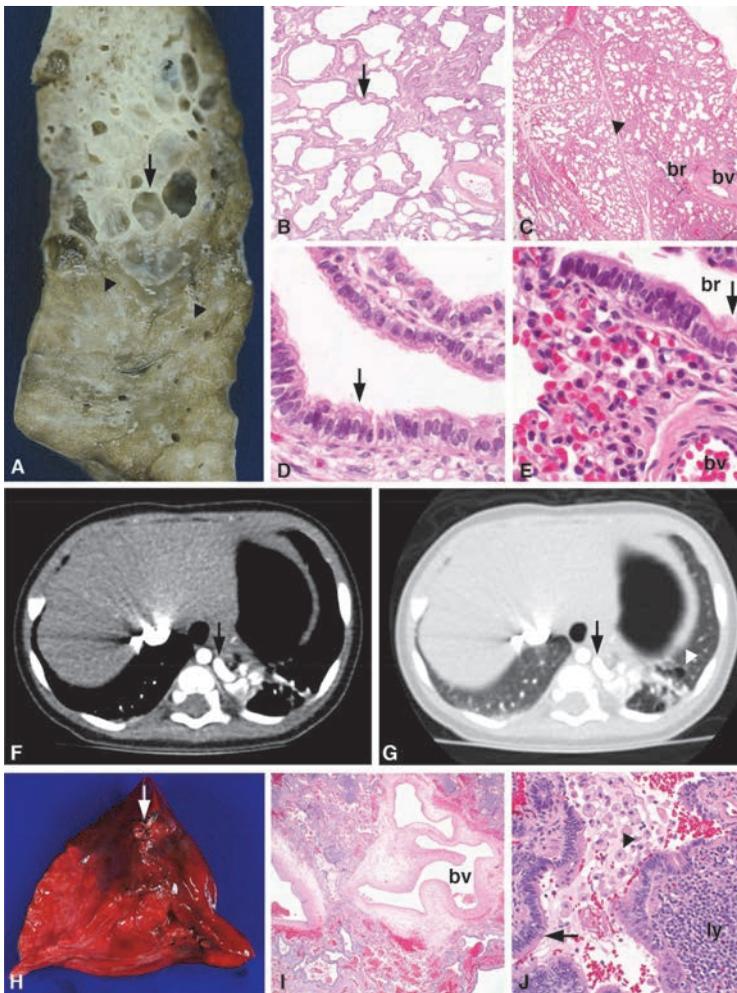


Figure 6-9. Intralobar sequestration with congenital pulmonary airway malformation. Images from two different patients are shown. Patient 1 (A–E) is a 9-day-old infant who was diagnosed with an ILS and had a left lower lobe resection. A solitary feeder vessel measuring 2 mm diameter entered the lung lobe inferior to the hilar bronchus and blood vessels. Gross examination revealed a distinct region within the lung lobe comprised of numerous cystic structures (A, arrow), some containing intraluminal secretions that contrasted with the adjacent more normal appearing lung parenchyma (A, below arrowheads). Histologic examination revealed irregularly shaped, back-to-back cysts (B, arrow) with a paucity of normal alveoli in the portion of the lung lobe supplied by the feeder artery. This histology was in contrast to the more normal-appearing parenchyma, which was comprised of normally developed, paired bronchioles and blood vessels (C, br = bronchiole, bv = blood vessel) and alveoli (C, arrowhead). The cysts comprising the malformation were lined by cuboidal to columnar, ciliated epithelium (D, arrow) and lacked mucus cells and cartilage in the underlying wall, resembling bronchioles in the normally developed lung (E, arrow, br = bronchiole, bv = blood vessel). Together these gross and histologic features support the diagnosis of a hybrid ILS/CPAM type 2 lesion. Patient 2 (F–J) is a 6-month-old boy who had a left lower lobe lesion with a systemic feeder vessel originating from the thoracic aorta (F–G, arrows) by CT imaging, consistent with PS. The lung windows revealed multiple cystic structures within the left lower lobe lesion (G, arrowhead), consistent with CPAM. Left lower lobe lobectomy was performed, and the diagnosis of a hybrid PS/CPAM lesion was pathologically confirmed. A feeder vessel was identified both grossly (H, arrow) and histologically (I, bv = blood vessel). The lung lesion had features of a CPAM, type 2, including multiple cysts measuring up to 0.8 cm in diameter that were surrounded by chronic inflammation (J, ly = lymphocytes) and lined by bronchiolar type epithelium (J, arrow) with intraluminal foamy macrophages (J, arrowhead) and amorphous eosinophilic material. Original magnifications: 4x (B, C, I), 40x (J), 100x (D, E).

the themes of vascular traction, vascular insufficiency, acquired pathology following infection, and foregut maldevelopment (73,113). The most widely accepted theory to best explain the spectrum of PS pathology is a congenital

malformation wherein a supernumerary lung bud forms ventral to the normal primitive foregut. ILS results if the accessory lung bud develops before formation of the pleura, whereas ELS is the outcome if the accessory lung bud develops after

formation of the pleura and the sequestered lung tissue forms its own pleural covering (138). Although the developmental versus acquired nature of ILS was long debated, it now seems clear that most, if not all, of these lesions are truly developmental malformations rather than acquired lesions in the setting of chronic infection (97). The observation that approximately 23% of prenatally detected lung lesions are PS, and the detection of these lesions as early as 16 weeks gestation, provides further evidence that PSs represent a congenital malformation rather than an acquired lesion (11,78). ELSs characteristically have cystic parenchymal maldevelopment with features of small cyst or type 2 CPAM being present in up to 50% of cases (9). The frequent association of PSs with other pulmonary parenchymal abnormalities, including CPAM/PS hybrid lesions (Figures 6-8E-H, and 6-9), supports the concept that multiple congenital lung malformations may have a similar embryologic origin and be part of a malformation sequence rather than representing distinct lesions (9,139). Bronchial atresia has been proposed as the common underlying etiology for congenital bronchopulmonary foregut abnormalities (9,138). This concept is supported by a prospective pathologic review of 47 pulmonary malformations that demonstrated bronchial atresia in all cases of ELS and 82% of ILS (112). Moreover, features of CPAM were present in 91% of both ELS and ILS. Based on these findings, it was proposed that CPAM and PS share the same etiopathogenesis with the spectrum of anatomic manifestations representing aberrant genetic programs and/or other insults that are modified by timing, duration, or completeness of the airway obstruction.

Diagnosis of PS requires a high index of suspicion and visualization of a systemic feeding artery to the lesion. Although newborns with ELS have a broad spectrum of presentations, the majority of patients have feeding difficulties and/or respiratory distress (11,139). Additional manifestations include pneumonia, hemorrhage, hydrops, or congestive heart failure related to the mass effect that can be seen when PS occurs as a hybrid lesion with CPAM or with substantial arteriovenous shunting resulting from the sequestered lobe. ELSs are often discovered on prenatal or neonatal ultrasound or MRI imaging, but may also remain asymptomatic

throughout life or be diagnosed after identification of the associated anomalies (81,138). ELSs rarely become infected because the distinct pleural investment prevents contact with inhaled air (138). In contrast to ELSs, most patients with ILSs present in adolescence or early adulthood with recurrent bacterial pneumonia in the affected lower lobe (138). Common clinical symptoms in adult patients include cough, expectoration, hemoptysis, intermittent fever, and chest pain (140). Compared with pediatric patients, adult PS patients have significantly more respiratory infections, including concurrent Aspergillosis, that more often require lobectomy (140). ILSs comprise 93% of adult cases, likely accounting for the high rate of infection that was not seen in any of the adult patients with ELS (140). In ~15% of cases, ILS is an incidental finding on imaging performed for other reasons (138).

PSs are most readily diagnosed by CT (Figures 6-8 and 6-9) or MRI. PSs are characterized by consolidations, masses, or cystic lesions involving the lower lobes, primarily the left posterior basal segment, that are supplied by anomalous arteries arising from the aorta or other systemic artery (80,138). ELSs are almost always airless due to their separate pleural investment, presenting as homogeneous, sharply defined consolidations medial to the lung or in extrathoracic sites including the mediastinum, embedded in the diaphragm, or in the upper abdomen and peritoneum, where ELSs can mimic neuroblastoma or adrenal hemorrhage (11). ILS has three typical radiologic presentations: a solitary mass, a cystic lesion, or a consolidation.

Definitive PS diagnosis relies on identification of the anomalous artery supplying the lesions. The pedicle containing the vascular structure typically does not contain an accompanying airway. If a bronchus is present within the pedicle, a communication with the digestive system should be highly suspected (9). In 80% of cases, ELSs are supplied by a single artery, arising from the thoracic or abdominal aorta with venous drainage into the azygous system or inferior vena cava (73,138). In 15% of cases, ELSs are supplied by small arterial branches or multiple arteries. ILSs receive arterial supply from the descending thoracic aorta in >90% of cases, with the remaining cases being supplied by multiple sources including the subclavian arteries,

internal thoracic arteries and arteries feeding the chest wall (73). Contrary to ELS, venous drainage in ILS is to the pulmonary veins in 95% of cases with drainage into the azygous system occurring in a minority of cases (138). The diagnostic differential for PS includes acquired systemic artery supply to the lungs in response to chronic inflammation or pulmonary artery obstruction, congenital systemic arterial supply to an otherwise normal lung such as is seen in Scimitar syndrome (which typically involves the right lower lobe rather than the left lower lobe as seen in PS) and primary metastatic tumor (138,139). ILS must also be differentiated from CPAM and CLO with the key differentiating feature being demonstration of a systemic feeding vessel (78,97).

Surgical resection remains the mainstay of treatment for symptomatic PS, with embolization of the feeding vessel being an additional therapeutic consideration. It is recommended that asymptomatic ILS also be resected based on the risk of future infection, hemoptysis, or malignancy (80,138,140). Carcinoma arising in PS is rare, with eight cases reported in association with ILS and a single report of a *BRAF* (OMIM *164757) mutant adenocarcinoma arising in ELS (141). ILS-associated malignancies differ pathologically from the mucinous adenocarcinomas and PPBs associated with CPAM, suggesting potential different pathways of carcinogenesis in the two entities. PS-associated malignancies also occur in older patients (>30 years of age) differing from PPBs in CPAM, which occur in children, and the CPAM-related mucinous adenocarcinomas that are typically identified in younger patients (median age of 20 years).

Treatment of asymptomatic ELS is controversial but can generally be observed with serial monitoring because the lesions may remain asymptomatic throughout life (80,138,140). A conservative treatment approach for asymptomatic ELS is also supported by good outcomes of prenatally diagnosed ELS as well as several studies reporting substantial or complete regression of the lesions on sequential scanning during pregnancy (73,74). A significant number of ELSs are diagnosed prenatally, with PS accounting for 33% of all prenatally diagnosed lung lesions (74). A recent postnatal follow-up study of fetal ELS, however, noted that cases diagnosed with complete regression in utero almost invariably had

ELS persistence in postnatal CT imaging (142). In 18 ELS cases noted to disappear on sequential scanning through pregnancy, postnatal CT demonstrated persistence in 14 cases with the remaining four infants not undergoing postnatal CT. The apparent disappearance sonographically was explained by PS tissue becoming isochogenic with adjacent normal lung parenchyma. Even with persistence of the ELS postnatally, however, antenatally diagnosed PS in the absence of hydrops was associated with an excellent prognosis.

Congenital Lobar Overinflation

Congenital lobar overinflation (CLO; also referred to as congenital lobar emphysema, overinflation syndrome and infantile lobar emphysema) is overdistension or hyperplasia of pulmonary segments or lobes resulting from partial or complete bronchial obstruction (Figure 6-10). The term *overinflation* is used to highlight that this condition is characterized by overdistension of the airways rather than alveolar destruction, as may be implied by the term *emphysema*. The airway obstruction can be caused by bronchial abnormalities, including bronchial atresia, bronchial stenosis, or most commonly, bronchial cartilaginous dysplasia or congenital bronchial cartilage deficiency resulting in bronchial collapse on expiration (82,143). CLO can also present in association with other conditions that lead to extrinsic or intrinsic bronchial obstruction. Causes of extrinsic bronchial obstruction include vascular anomalies such as pulmonary artery slings and anomalous pulmonary venous return as well as mass lesions such as ELS and bronchogenic cysts. Intrinsic obstructive causes include bronchial mucosal folds, bronchial torsion, or luminal obstruction by aspirated meconium, mucus plug, foreign body, or granulation tissue.

CLO has two patterns that have been hypothesized to be related to the timing of bronchial obstruction during lung development and/or the degree of obstruction (144). The classic pattern, seen in nearly 70% of cases, is characterized by uniform overdistension of normally developed alveolar saccules and alveoli. Radiological imaging in these cases shows a hyperlucent, overdistended lobe compressing the uninvolved lobes and producing a mediastinal shift (Figure 6-10A, C) (143). The remaining 30% of cases have a polyalveolar

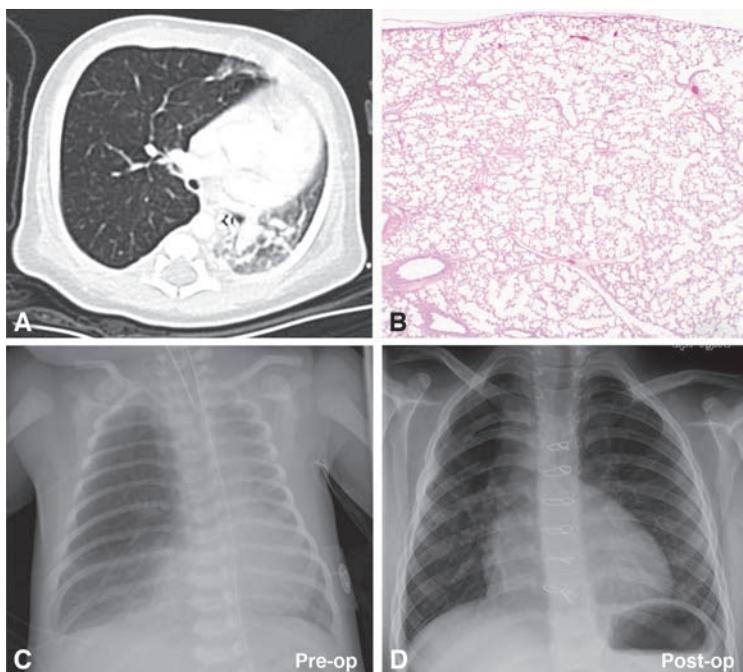


Figure 6-10. Congenital lobar overinflation. A 14-day-old boy with tetralogy of Fallot had marked hyperinflation of the right upper lobe resulting in leftward deviation of the thymus and anterior junction lines as well as compressive atelectasis of the left lung and right middle and lower lobes by CT scan (A). Cardiac anomalies associated with tetralogy of Fallot and a right-sided aortic arch were also present. The right upper lobe was resected and was without gross lesions. Histologic examination revealed normal lung structure with diffuse overinflation of the alveoli (B). Together the radiographic and histologic features are characteristic of CLO. Resolution of the right lung overinflation and left mediastinal shift present in preoperative chest radiographic images (C, Pre-op) was noted in the postoperative chest radiograph taken at ~7.5 years of age (D, Post-op). Original magnification: 2x (B).

pattern with little overdistension of complex acini typically seen in hyperplastic lungs (144). This polyalveolar form may present radiographically as a lobe with normal lucency occupying a disproportionate portion of the hemithorax with mediastinal shift. Polyalveolar lobe is generally believed to be a variant of CLO in which there is an increase in alveolar number associated with enlargement of a lobe, rather than overinflation of a lobe with a normal alveolar number (9). It is hypothesized that “pulmonary hyperplasia” results from relatively complete obstruction of a bronchus in early lung development leading to accelerated lung growth in the final trimester similar to the hyperplasia noted in some infants with laryngeal or tracheal atresia (144).

CLO is a rare condition with a prevalence of 1 per 20,000 to 30,000 deliveries (113). CLO presents in the first week of life in ~50% of cases and within the first 6 months in >80% of cases (82). Although most patients present in the neonatal period, CLO can also be detected in utero and occasionally be diagnosed in children and young adults (11,78,145). Age at the time of diagnosis is inversely related to the severity of respiratory distress (143). Presenting symptoms include dyspnea, tachypnea, cyanosis, infection, wheezing, cough, and hoarseness (143,144). Sudden pneumothorax is a rare presentation. Anomalies

in other organs can be seen in association with CLO in up to 40% of patients, with 70% of these anomalies occurring within the cardiovascular system (82,143). Less commonly, CLO is associated with renal, GI, musculoskeletal, and cutaneous malformations (113). Treatment is based on the severity of respiratory distress. Traditional treatment is lobectomy to ensure compensatory lung growth; however, recent studies indicate that some patients can be safely managed conservatively (143). Long-term follow-up of 30 patients treated with surgery or conservative management revealed good outcomes with all patients having normal oxygen saturation levels and being free of respiratory symptoms, suggesting that patients can benefit from both surgical and conservative management.

Pulmonary Lymphangiectasia

Pulmonary lymphangiectasia (PL; also referred to as congenital pulmonary lymphangiectasia) is a rare disorder characterized by dilated lymphatic vessels with a normal lymphatic distribution within the subpleural region, in interlobular septa, and around bronchovascular bundles. Diagnosis is based on clinical signs together with radiologic imaging and histologic findings, with open lung biopsy considered the diagnostic gold

standard (146). The predominant dilation of nonproliferative lymphatic channels in lymphangiectasis is differentiated from lymphangiomatosis, which is characterized by proliferation of lymphatic channels resulting in an increased number and decreased size of lymphatic spaces with a proliferative spindle cell component. Both conditions preferentially involve lymphatic vessels in a normal lymphangitic distribution, which can lead to challenges in differentiating the two entities clinically and pathologically with limited biopsies.

PL is divided into primary and secondary categories. Primary PL is further subclassified as isolated, generalized, or syndromic based on presentation as an isolated pulmonary lesion, lymphangiectasis involving other tissues in addition to the lung such as bones, viscera and soft tissues, or as a component of a syndrome, respectively (146). Secondary PL occurs in association with obstructive cardiovascular lesions with the most common abnormality being total anomalous pulmonary venous return but also including hypoplastic left heart syndrome, pulmonary vein atresia, congenital mitral stenosis, cor triatriatum, and thoracic duct agenesis (82,146). Cardiovascular anomalies are seen in 60% of PL patients, and renal malformations, generalized lymphangiectasis, and other anomalies are present in another 20% of cases (82,97).

Most PL cases are sporadic with a >2.5:1 male predominance (82,146). The association of PL with numerous syndromes, however, has led to the recommendation that an underlying syndrome should always be considered in PL patients (146). PL has been described in association with chromosomal abnormalities, including Turner, Down, and Phelan McDermid (OMIM #606232) syndromes. PL can also be a component of numerous other syndromes with the RASopathies [Noonan, Cardio-Facio-Cutaneous, and Costello (OMIM #218040) syndromes] being a common association as well as syndromes characterized by lymphedema or generalized lymphatic dysplasia such as Hennekam syndrome. Patients presenting with syndromic PL should be considered for genomic testing. Mutations in genes associated with specific syndromes have been identified in PL patients including *PTPN11* (OMIM *176876) and *SOS1* (OMIM *182530) signaling molecules within the RAS pathway, and *FOXC2* (OMIM *602402) (147–149).

The cause of PL is unknown, and no disease-specific therapies have been developed. Primary congenital PL is believed to be an inherent developmental abnormality of the lymphatic system. Postulated pathogenic mechanisms include a developmental error in which the normal regression of connective tissue elements fails to occur and/or a failure or delay in linkage of isolated lymphatic spaces (97,146). Familial occurrence, although rare, has been described in six affected families suggesting a possible genetic component (146). *VEGFR3* (*FLT4*, OMIM *136352) mutations are described in PL associated with Nonne-Milroy lymphedema syndrome (OMIM #153100), and interestingly, perinatal overexpression of the ligand for this receptor, VEGF-C, was shown to induce PL in a mouse model that phenotypically and histologically resembles the human condition (150). Moreover, VEGFR-3, along with VEGFR-2, was required for the development of lymphangiectasia in the neonatal mice. Together, genetic evaluations of PL patients combined with defining the precise role of the identified molecules in experimental models has great promise for improving our understanding of the molecular mechanisms underlying PL pathogenesis as well as the associated anomalies.

PL is usually a fatal disorder that typically presents in the first hours to days of life as severe respiratory distress resulting from unilateral or bilateral pleural effusions, pulmonary hypoplasia, and surfactant deficiency in combination with prematurity (97,146). The clinical course may be complicated by persistent chylothorax, ventilator dependency, anasarca, arterial hypotension, heart failure, secondary pulmonary hypertension, and progressive respiratory failure. Patients with generalized lymphangiectasis usually have less pulmonary involvement. Neonatal presentation of primary PL limited to the lung was typically considered a uniformly fatal disease, but increased survival has resulted from advances in perinatal care (146). Despite decreased mortality, morbidity may still be high, requiring interdisciplinary long-term follow-up care. PL can also manifest in childhood or even in adult life with a wide spectrum of presentations, including respiratory failure, persistent tachypnea, dyspnea, cough, wheezing, hemoptysis, recurrent airway infections, or as an incidental finding in an asymptomatic patient (146,151). PL can be limited to one or two lobes, which may account for the lack of symptoms and

favorable long-term outcomes in some patients (151). Radiographic findings are not specific, but a reticulonodular interstitial pattern with increased interstitial markings or thickening, likely representing dilated pulmonary lymphatics, add to the diagnostic evaluation especially in the absence of biopsy. Treatment of prenatal fetal chylothorax by thoracentesis, thoracoamniotic shunting, or medical pleurodesis can be performed to prevent severe pulmonary hypoplasia and hydrops. Postnatal treatment of PL is primarily supportive, including mechanical ventilation, surfactant administration, thoracentesis, ECMO, cardio-circulatory support, and total parenteral nutrition (146). Substitution of electrolytes, coagulation factors, and immunoglobulins may also be required due to chylothorax with persistent, refractory chylothorax requiring prolonged conservative and/or surgical interventions.

Summary and Future Directions

Congenital lung malformations are a heterogeneous group of abnormalities resulting from defective foregut specification, branching morphogenesis and cell proliferation, survival, and differentiation. There is considerable overlap among these anomalies, which commonly occur in combination or as hybrid lesions. Clinical diagnosis is challenging given the overlapping features among the lesions, some of which have distinct natural histories and clinical management. Significant advancements in radiologic imaging and routine investigations in utero have resulted in a shift from postnatal to prenatal fetal diagnoses. Continued focus on developing better imaging modalities capable of differentiating among the distinct malformations is needed to combat the current concern that definitive diagnosis cannot be established by prenatal imaging alone but rather relies on histologic examination. Definite prenatal diagnosis will be beneficial in guiding prenatal counseling and clinical intervention. Incomplete knowledge surrounding the natural history of congenital lung malformations compromises the ability to define relative risks and benefits of early interventions. Large multicenter registries would be beneficial to enhance understanding of the onset, timing, natural history, and prognosis of these rare congenital lung malformations to improve evidence-based approaches to patient diagnosis and management.

Prenatal diagnosis provides an opportunity to follow these congenital malformations sequentially to better understand their pathophysiological mechanisms. Overlapping features among the heterogeneous pulmonary malformations suggest common etiologies, but further studies are needed to define the pathogenesis of both distinct and overlapping phenotypes. Proposed unifying mechanisms include airway obstruction and aberrant, embryonic aortic arch development. Failure of appropriate endoderm-mesoderm signaling, deregulated cell proliferation and programmed cell death, altered gene expression, and aberrant growth factor signaling are additional proposed mechanisms. Defining pathogenic mechanisms underlying congenital lung malformations will provide valuable clinically relevant insights into pulmonary development, as well as define mechanisms of disease pathogenesis for the many syndromic conditions and anomalies in other organ systems commonly seen in conjunction with lung malformations.

Genetic analysis of patients with hereditary lung malformations has led to the identification of associated gene mutations. The products of these genes, such as TTF-1, SOX2, FOXF1, and DICER1, are key molecules that control pulmonary development in genetically modified mouse models. Engineering mice with the corresponding human gene mutations results in lung malformations that phenotypically mimic the human disease, establishing a causative relationship. Continued integration of genomic studies in human patients with basic investigational studies defining molecular pathways that control lung development are needed to identify additional molecular markers and causes for the many lung abnormalities with currently unclear pathogenesis. Defining the molecular basis for lung malformations and disease pathogenesis will provide novel approaches to patient diagnosis, surveillance, and management.

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References

- 1 Burri PH. Fetal and postnatal development of the lung. *Annu Rev Physiol.* 1984;46:617–628.
- 2 Burri PH. Structural aspects of prenatal and postnatal development and growth of the lung. In: McDonald JA, ed. *Lung Growth and Development.* New York: Taylor & Francis; 1997:1–36.
- 3 Burri PH. Structural aspects of postnatal lung development – alveolar formation and growth. *Biol Neonate.* 2006;89(4):313–322.
- 4 Langston C, Kida K, Reed M, Thurlbeck WM. Human lung growth in late gestation and in the neonate. *Am Rev Respir Dis.* 1984;129(4):607–613.
- 5 Thurlbeck WM. Postnatal growth and development of the lung. *Am Rev Respir Dis.* 1975;111(6):803–844.
- 6 Zeltner TB, Caduff JH, Gehr P, Pfenninger J, Burri PH. The postnatal development and growth of the human lung. I. Morphometry. *Respir Physiol.* 1987;67(3):247–267.
- 7 Hislop AA, Wigglesworth JS, Desai R. Alveolar development in the human fetus and infant. *Early Hum Dev.* 1986;13(1):1–11.
- 8 Ochs M, Nyengaard JR, Jung A, Knudsen L, Voigt M, Wahlers T, et al. The number of alveoli in the human lung. *Am J Respir Crit Care Med.* 2004;169(1):120–124.
- 9 Langston C. New concepts in the pathology of congenital lung malformations. *Semin Pediatr Surg.* 2003;12(1):17–37.
- 10 Newman B. Congenital bronchopulmonary foregut malformations: concepts and controversies. *Pediatr Radiol.* 2006;36(8):773–791.
- 11 Biyyam DR, Chapman T, Ferguson MR, Deutsch G, Dighe MK. Congenital lung abnormalities: embryologic features, prenatal diagnosis, and postnatal radiologic-pathologic correlation. *Radiographics.* 2010;30(6):1721–1738.
- 12 Shaw-Smith C. Genetic factors in esophageal atresia, tracheo-esophageal fistula and the VACTERL association: roles for FOXF1 and the 16q24.1 FOX transcription factor gene cluster, and review of the literature. *Eur J Med Genet.* 2010;53(1):6–13.
- 13 Skandalakis JE, Gray SW, Ricketts RR. Esophagus. In: Skandalakis JE, Gray SW, eds. *Embryology for Surgeons.* 2nd ed. Baltimore, MD: Williams & Wilkins; 1994:65–112.
- 14 Solomon BD, Bear KA, Kimonis V, de Klein A, Scott DA, Shaw-Smith C, et al. Clinical geneticists' views of VACTERL/VATER association. *Am J Med Genet A.* 2012;158A(12):3087–3100.
- 15 Fausett SR, Klingensmith J. Compartmentalization of the foregut tube: developmental origins of the trachea and esophagus. *Wiley Interdiscip Rev Dev Biol.* 2012;1(2):184–202.
- 16 Skandalakis JE, Gray SW, Symbas PN. The trachea and the lungs. In: Skandalakis JE, Gray SW, eds. *Embryology for Surgeons.* 2nd ed. Baltimore, MD: Williams & Wilkins; 1994:414–450.
- 17 Shaw-Smith C. Oesophageal atresia, tracheo-oesophageal fistula, and the VACTERL association: review of genetics and epidemiology. *J Med Genet.* 2006;43(7):545–554.
- 18 Solomon BD. VACTERL/VATER Association. *Orphanet J Rare Dis.* 2011;6:56.
- 19 Felix JF, Tibboel D, de Klein A. Chromosomal anomalies in the aetiology of oesophageal atresia and tracheo-oesophageal fistula. *Eur J Med Genet.* 2007;50(3):163–175.
- 20 Munzon GB, Martinez-Ferro M. Pediatric tracheal stenosis and vascular rings. *Bulletin of Thoracic Surgery.* 2012;5(II Aralik):207–219.
- 21 Berrocal T, Madrid C, Novo S, Gutierrez J, Arjonilla A, Gomez-Leon N. Congenital anomalies of the tracheobronchial tree, lung, and mediastinum: embryology, radiology, and pathology. *Radiographics.* 2004;24(1):e17.
- 22 de Groot-van der Moeren MD, Haak MC, Lakeman P, Cohen-Overbeek TE, van der Voorn JP, Bretschneider JH, et al. Tracheal agenesis: approach towards this severe diagnosis. Case report and review of the literature. *Eur J Pediatr.* 2012;171(3):425–431.
- 23 Aktogu S, Yuncu G, Halilcolar H, Ermete S, Buduneli T. Bronchogenic cysts: clinicopathological presentation and treatment. *Eur Respir J.* 1996;9(10):2017–2021.
- 24 Garcia-Pena P, Coma A, Enriquez G. Congenital lung malformations: radiological findings and clues for differential diagnosis. *Acta Radiol.* 2013;54(9):1086–1095.
- 25 Giosi D, Bellodi S, Sabatini F, Rossi GA. The lung and the gut: common origins, close links. *Paediatr Respir Rev.* 2006;7 Suppl 1:S235–239.
- 26 Nadeem M, Elnazir B, Grealley P. Congenital pulmonary malformation in children. *Scientifica (Cairo).* 2012;2012:209896.
- 27 Wallis C. Clinical outcomes of congenital lung abnormalities. *Paediatr Respir Rev.* 2000;1(4):328–335.
- 28 Sarper A, Ayten A, Golbasi I, Demircan A, Isin E. Bronchogenic cyst. *Tex Heart Inst J.* 2003;30(2):105–108.
- 29 Gould SJ, Hasleton PS. Congenital Abnormalities. In: Hasleton PS, ed. *Spencer's*

- Pathology of the Lung*. 5th ed. New York: McGraw-Hill, Inc; 1996:57–114.
- 30 Hoffer ME, Tom LW, Wetmore RF, Handler SD, Potsic WP. Congenital tracheal stenosis. The otolaryngologist's perspective. *Arch Otolaryngol Head Neck Surg*. 1994;120(4):449–453.
- 31 Phipps LM, Raymond JA, Angeletti TM. Congenital tracheal stenosis. *Crit Care Nurse*. 2006;26(3):60–69.
- 32 Landing BH, Dixon LG. Congenital malformations and genetic disorders of the respiratory tract (larynx, trachea, bronchi, and lungs). *Am Rev Respir Dis*. 1979;120(1):151–185.
- 33 Chen CP, Lin SP, Su YN, Chien SC, Tsai FJ, Wang W. Craniosynostosis and congenital tracheal anomalies in an infant with Pfeiffer syndrome carrying the W290C FGFR2 mutation. *Genet Couns*. 2008;19(2):165–172.
- 34 Cohen MM, Jr., Kreiborg S. Visceral anomalies in the Apert syndrome. *Am J Med Genet*. 1993;45(6):758–760.
- 35 Gonzales M, Heuertz S, Martinovic J, Delahaye S, Bazin A, Loget P, et al. Vertebral anomalies and cartilaginous tracheal sleeve in three patients with Pfeiffer syndrome carrying the S351C FGFR2 mutation. *Clin Genet*. 2005;68(2):179–181.
- 36 Hockstein NG, McDonald-McGinn D, Zackai E, Bartlett S, Huff DS, Jacobs IN. Tracheal anomalies in Pfeiffer syndrome. *Arch Otolaryngol Head Neck Surg*. 2004;130(11):1298–1302.
- 37 Jones KL, Smith DW. *Smith's recognizable patterns of human malformation*. Philadelphia: Elsevier-Saunders; 2006.
- 38 Kan SH, Elanko N, Johnson D, Cornejo-Roldan L, Cook J, Reich EW, et al. Genomic screening of fibroblast growth-factor receptor 2 reveals a wide spectrum of mutations in patients with syndromic craniosynostosis. *Am J Hum Genet*. 2002;70(2):472–486.
- 39 Lertsburapa K, Schroeder JW, Jr, Sullivan C. Tracheal cartilaginous sleeve in patients with craniosynostosis syndromes: a meta-analysis. *J Pediatr Surg*. 2010;45(7):1438–1444.
- 40 Noorily MR, Farmer DL, Belenky WM, Philippart AI. Congenital tracheal anomalies in the craniosynostosis syndromes. *J Pediatr Surg*. 1999;34(6):1036–1039.
- 41 Scheid SC, Spector AR, Luft JD. Tracheal cartilaginous sleeve in Crouzon syndrome. *Int J Pediatr Otorhinolaryngol*. 2002;65(2):147–152.
- 42 Zackai EH, McDonald-McGinn DM, Stolle C, Huff DS. Craniosynostosis with tracheal sleeve: a patient with Pfeiffer syndrome, tracheal sleeve and additional malformations in whom an FGFR2 mutation was found. *Clin Dysmorphol*. 2003;12(3):209.
- 43 Eswarakumar VP, Horowitz MC, Locklin R, Morriss-Kay GM, Lonai P. A gain-of-function mutation of Fgfr2c demonstrates the roles of this receptor variant in osteogenesis. *Proc Natl Acad Sci U S A*. 2004;101(34):12555–12560.
- 44 Tiozzo C, De Langhe S, Carraro G, Alam DA, Nagy A, Wigfall C, et al. Fibroblast growth factor 10 plays a causative role in the tracheal cartilage defects in a mouse model of Apert syndrome. *Pediatr Res*. 2009;66(4):386–390.
- 45 Wang Y, Xiao R, Yang F, Karim BO, Iacovelli AJ, Cai J, et al. Abnormalities in cartilage and bone development in the Apert syndrome FGFR2(+/-S252W) mouse. *Development*. 2005;132(15):3537–3548.
- 46 Meyerholz DK, Stoltz DA, Namati E, Ramachandran S, Pezzulo AA, Smith AR, et al. Loss of cystic fibrosis transmembrane conductance regulator function produces abnormalities in tracheal development in neonatal pigs and young children. *Am J Respir Crit Care Med*. 2010;182(10):1251–1261.
- 47 DeBoer EM, Swiercz W, Heltshe SL, Anthony MM, Szeffler P, Klein R, et al. Automated CT scan scores of bronchiectasis and air trapping in cystic fibrosis. *Chest*. 2014;145(3):593–603.
- 48 Adam RJ, Michalski AS, Bauer C, Abou Alaiwa MH, Gross TJ, Awadalla MS, et al. Air trapping and airflow obstruction in newborn cystic fibrosis piglets. *Am J Respir Crit Care Med*. 2013;188(12):1434–1441.
- 49 Bonvin E, Le Rouzic P, Bernaudin JF, Cottart CH, Vandebrouck C, Crie A, et al. Congenital tracheal malformation in cystic fibrosis transmembrane conductance regulator-deficient mice. *J Physiol*. 2008;586(13):3231–3243.
- 50 Wallace HL, Southern KW, Connell MG, Wray S, Burdyga T. Abnormal tracheal smooth muscle function in the CF mouse. *Physiol Rep*. 2013;1(6):e00138.
- 51 Carden KA, Boiselle PM, Waltz DA, Ernst A. Tracheomalacia and tracheobronchomalacia in children and adults: an in-depth review. *Chest*. 2005;127(3):984–1005.
- 52 Kayemba-Kay's S, Couvrat-Carcauzon V, Goua V, Podevin G, Marteau M, Sapin E, et al. Unilateral pulmonary agenesis: a report of four cases, two diagnosed antenatally and

- literature review. *Pediatr Pulmonol.* 2014;49(3):E96–102.
- 53 Russell BC, Whitecar P, Nitsche JF. Isolated unilateral pulmonary agenesis and other fetal thoracic anomalies. *Obstet Gynecol Surv.* 2014;69(6):335–345.
- 54 Cunningham ML, Mann N. Pulmonary agenesis: a predictor of ipsilateral malformations. *Am J Med Genet.* 1997;70(4):391–398.
- 55 Pierron C, Sigal-Cinquabre A, Lambert V, Le Bret E. Left pulmonary artery sling with right lung aplasia. *J Pediatr Surg.* 2011;46(11):2190–2194.
- 56 Holstein A, Weber M. An extraordinary finding – accidental diagnosis of complete pulmonary aplasia in a 90-year-old lady. *Age Ageing.* 2009;38(4):487.
- 57 Kwon SH, Oh JH, Sung DW. Incidentally found right pulmonary aplasia in an adult patient: the 64-slice MDCT findings. *J Thorac Imaging.* 2009;24(1):56–58.
- 58 Bishop NB, Stankiewicz P, Steinhorn RH. Alveolar capillary dysplasia. *Am J Respir Crit Care Med.* 2011;184(2):172–179.
- 59 Chow CW, Massie J, Ng J, Mills J, Baker M. Acinar dysplasia of the lungs: variation in the extent of involvement and clinical features. *Pathology.* 2013;45(1):38–43.
- 60 Dishop MK. Paediatric interstitial lung disease: classification and definitions. *Paediatr Respir Rev.* 2011;12(4):230–237.
- 61 Langenstroer M, Carlan SJ, Fanaian N, Attia S. Congenital acinar dysplasia: report of a case and review of literature. *AJP Rep.* 2013;3(1):9–12.
- 62 Langston C, Dishop MK. Diffuse lung disease in infancy: a proposed classification applied to 259 diagnostic biopsies. *Pediatr Dev Pathol.* 2009;12(6):421–437.
- 63 Melly L, Sebire NJ, Malone M, Nicholson AG. Capillary apposition and density in the diagnosis of alveolar capillary dysplasia. *Histopathology.* 2008;53(4):450–457.
- 64 Wert SE, Proffitt S, Kirwin KL, Langston C, Whitsett J. Acinar dysplasia is associated with the absence of TTF-a and HNF3-B expression during human lung development. *Pediatr Res.* 1996;39:355A.
- 65 Galambos C, Sims-Lucas S, Abman SH. Three-dimensional reconstruction identifies misaligned pulmonary veins as intrapulmonary shunt vessels in alveolar capillary dysplasia. *J Pediatr.* 2014;164(1):192–195.
- 66 Galambos C, Sims-Lucas S, Ali N, Gien J, Dishop MK, Abman SH. Intrapulmonary vascular shunt pathways in alveolar capillary dysplasia with misalignment of pulmonary veins. *Thorax.* 2015;70(1):84–85.
- 67 Miranda J, Rocha G, Soares P, Morgado H, Baptista MJ, Azevedo I, et al. A novel mutation in FOXF1 gene associated with alveolar capillary dysplasia with misalignment of pulmonary veins, intestinal malrotation and annular pancreas. *Neonatology.* 2013;103(4):241–245.
- 68 Nguyen L, Riley MM, Sen P, Galambos C. Alveolar capillary dysplasia with misalignment of pulmonary veins with a wide spectrum of extrapulmonary manifestations. *Pathol Int.* 2013;63(10):519–521.
- 69 Stankiewicz P, Sen P, Bhatt SS, Storer M, Xia Z, Bejjani BA, et al. Genomic and genic deletions of the FOX gene cluster on 16q24.1 and inactivating mutations of FOXF1 cause alveolar capillary dysplasia and other malformations. *Am J Hum Genet.* 2009;84(6):780–791.
- 70 Sen P, Dharmadhikari AV, Majewski T, Mohammad MA, Kalin TV, Zabielska J, et al. Comparative analyses of lung transcriptomes in patients with alveolar capillary dysplasia with misalignment of pulmonary veins and in foxf1 heterozygous knockout mice. *PLoS One.* 2014;9(4):e94390.
- 71 Sen P, Yang Y, Navarro C, Silva I, Szafranski P, Kolodziejska KE, et al. Novel FOXF1 mutations in sporadic and familial cases of alveolar capillary dysplasia with misaligned pulmonary veins imply a role for its DNA binding domain. *Hum Mutat.* 2013;34(6):801–811.
- 72 Ren X, Ustiyani V, Pradhan A, Cai Y, Havrilak JA, Bolte CS, et al. FOXF1 transcription factor is required for formation of embryonic vasculature by regulating VEGF signaling in endothelial cells. *Circ Res.* 2014;115(8):709–720.
- 73 Corbett HJ, Humphrey GM. Pulmonary sequestration. *Paediatr Respir Rev.* 2004;5(1):59–68.
- 74 Nunes C, Pereira I, Araujo C, Santo SF, Carvalho RM, Melo A, et al. Fetal bronchopulmonary malformations. *J Matern Fetal Neonatal Med.* 2014:1–5.
- 75 Kunisaki SM, Fauza DO, Nemes LP, Barnewolt CE, Estroff JA, Kozakewich HP, et al. Bronchial atresia: the hidden pathology within a spectrum of prenatally diagnosed lung masses. *J Pediatr Surg.* 2006;41(1):61–65; discussion 61–65.
- 76 Beydon N, Larroquet M, Coulomb A, Jouannic JM, Ducou le Pointe H, Clement A, et al. Comparison between US and MRI in the prenatal assessment of lung

- malformations. *Pediatr Radiol*. 2013;43(6):685–696.
- 77 Kongstad T, Buchvald F, Brenoe J, Petersen BL, Tabor A, Nielsen KG. Radiology, histology and short-term outcome of asymptomatic congenital thoracic malformations. *Acta Paediatr*. 2012;101(2):155–158.
- 78 Pacharn P, Kline-Fath B, Calvo-Garcia M, Linam LE, Rubio EI, Salisbury S, et al. Congenital lung lesions: prenatal MRI and postnatal findings. *Pediatr Radiol*. 2013;43(9):1136–1143.
- 79 Baird R, Puligandla PS, Laberge JM. Congenital lung malformations: informing best practice. *Semin Pediatr Surg*. 2014;23(5):270–277.
- 80 Wall J, Coates A. Prenatal imaging and postnatal presentation, diagnosis and management of congenital lung malformations. *Curr Opin Pediatr*. 2014;26(3):315–319.
- 81 Laberge JM, Bratu I, Flageole H. The management of asymptomatic congenital lung malformations. *Paediatr Respir Rev*. 2004;5 Suppl A:S305–312.
- 82 Stocker JT, Mani H, Husain AN. The respiratory tract. In: Stocker JT, Dehner LP, Husain AN, eds. *Pediatric Pathology*. 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2011:441–515.
- 83 Coleman A, Phithakwatchara N, Shaaban A, Keswani S, Kline-Fath B, Kingma P, et al. Fetal lung growth represented by longitudinal changes in MRI-derived fetal lung volume parameters predicts survival in isolated left-sided congenital diaphragmatic hernia. *Prenat Diagn*. 2015;35(2):160–166.
- 84 de Castro Rezende G, Pereira AK, Araujo Junior E, Reis ZS, Vieira Cabral AC. Prediction of lethal pulmonary hypoplasia among high-risk fetuses via 2D and 3D ultrasonography. *Int J Gynaecol Obstet*. 2013;123(1):42–45.
- 85 Vergani P. Prenatal diagnosis of pulmonary hypoplasia. *Curr Opin Obstet Gynecol*. 2012;24(2):89–94.
- 86 Joshi S, Kotecha S. Lung growth and development. *Early Hum Dev*. 2007;83(12):789–794.
- 87 Chen F, Cao Y, Qian J, Shao F, Niederreither K, Cardoso WV. A retinoic acid-dependent network in the foregut controls formation of the mouse lung primordium. *J Clin Invest*. 2010;120(6):2040–2048.
- 88 Mendelsohn C, Lohnes D, Decimo D, Lufkin T, LeMeur M, Chambon P, et al. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development*. 1994;120(10):2749–2771.
- 89 Darlow BA, Graham PJ. Vitamin A supplementation to prevent mortality and short- and long-term morbidity in very low birthweight infants. *Cochrane Database Syst Rev*. 2011(10):CD000501.
- 90 Massaro GD, Massaro D. Postnatal treatment with retinoic acid increases the number of pulmonary alveoli in rats. *Am J Physiol*. 1996;270(2 Pt 1):L305–310.
- 91 Devriendt K, Vanhole C, Matthijs G, de Zegher F. Deletion of thyroid transcription factor-1 gene in an infant with neonatal thyroid dysfunction and respiratory failure. *N Engl J Med*. 1998;338(18):1317–1318.
- 92 Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, et al. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev*. 1996;10(1):60–69.
- 93 Shetty VB, Kiraly-Borri C, Lamont P, Bikker H, Choong CS. NKX2-1 mutations in brain-lung-thyroid syndrome: a case series of four patients. *J Pediatr Endocrinol Metab*. 2014;27(3–4):373–378.
- 94 Hamvas A, Deterding RR, Wert SE, White FV, Dishop MK, Alfano DN, et al. Heterogeneous pulmonary phenotypes associated with mutations in the thyroid transcription factor gene NKX2-1. *Chest*. 2013;144(3):794–804.
- 95 Pohlentz J, Dumitrescu A, Zundel D, Martine U, Schonberger W, Koo E, et al. Partial deficiency of thyroid transcription factor 1 produces predominantly neurological defects in humans and mice. *J Clin Invest*. 2002;109(4):469–473.
- 96 Minoo P, Su G, Drum H, Bringas P, Kimura S. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(–/–) mouse embryos. *Dev Biol*. 1999;209(1):60–71.
- 97 Gupta K, Das A, Menon P, Kakkar N, Rao KL, Joshi K. Revisiting the histopathologic spectrum of congenital pulmonary developmental disorders. *Fetal Pediatr Pathol*. 2012;31(2):74–86.
- 98 Nowaczyk MJ, Irons MB. Smith-Lemli-Opitz syndrome: phenotype, natural history, and epidemiology. *Am J Med Genet C Semin Med Genet*. 2012;160C(4):250–262.
- 99 Waterham HR, Hennekam RC. Mutational spectrum of Smith-Lemli-Opitz syndrome. *Am J Med Genet C Semin Med Genet*. 2012;160C(4):263–284.
- 100 Yu H, Wessels A, Chen J, Phelps AL, Oatis J, Tint GS, et al. Late gestational lung hypoplasia in a mouse model of the Smith-Lemli-Opitz

- syndrome. *BMC Dev Biol.* 2004;4:1.
- 101 Deutsch GH, Young LR, Detering RR, Fan LL, Dell SD, Bean JA, et al. Diffuse lung disease in young children: application of a novel classification scheme. *Am J Respir Crit Care Med.* 2007;176(11):1120–1128.
- 102 Ruchonnet-Metrailler I, Bessieres B, Bonnet D, Vibhushan S, Delacourt C. Pulmonary hypoplasia associated with congenital heart diseases: a fetal study. *PLoS One.* 2014;9(4):e93557.
- 103 Akinkuotu AC, Sheikh F, Cass DL, Zamora IJ, Lee TC, Cassady CI, et al. Are all pulmonary hypoplasias the same? A comparison of pulmonary outcomes in neonates with congenital diaphragmatic hernia, omphalocele and congenital lung malformation. *J Pediatr Surg.* 2015;50(1):55–59.
- 104 Faruqi S, Varma R, Avery G, Kastelik J. Pulmonary hypoplasia. *Intern Med.* 2011;50(10):1129.
- 105 Georgescu A, Nuta C, Bondari S. 3D imaging in unilateral primary pulmonary hypoplasia in an adult: a case report. *Case Rep Radiol.* 2011;2011:659586.
- 106 Katsenos S, Antonogiannaki EM, Tsintiris K. Unilateral primary lung hypoplasia diagnosed in adulthood. *Respir Care.* 2014;59(4):e47–50.
- 107 Cloutier MM, Schaeffer DA, Hight D. Congenital cystic adenomatoid malformation. *Chest.* 1993;103(3):761–764.
- 108 Hill DA, Jarzembowski JA, Priest JR, Williams G, Schoettler P, Dehner LP. Type I pleuropulmonary blastoma: pathology and biology study of 51 cases from the international pleuropulmonary blastoma registry. *Am J Surg Pathol.* 2008;32(2):282–295.
- 109 Morotti RA, Cangiarella J, Gutierrez MC, Jagirdar J, Askin F, Singh G, et al. Congenital cystic adenomatoid malformation of the lung (CCAM): evaluation of the cellular components. *Hum Pathol.* 1999;30(6):618–625.
- 110 Nasr A, Himidan S, Pastor AC, Taylor G, Kim PC. Is congenital cystic adenomatoid malformation a premalignant lesion for pleuropulmonary blastoma? *J Pediatr Surg.* 2010;45(6):1086–1089.
- 111 Wagh PK, Gardner MA, Ma X, Callahan M, Shannon JM, Wert SE, et al. Cell- and developmental stage-specific Dicer1 ablation in the lung epithelium models cystic pleuropulmonary blastoma. *J Pathol.* 2015;236(1):4–521.
- 112 Riedlinger WF, Vargas SO, Jennings RW, Estroff JA, Barnewolt CE, Lillehei CW, et al. Bronchial atresia is common to extralobar sequestration, intralobar sequestration, congenital cystic adenomatoid malformation, and lobar emphysema. *Pediatr Dev Pathol.* 2006;9(5):361–373.
- 113 Correia-Pinto J, Gonzaga S, Huang Y, Rottier R. Congenital lung lesions – underlying molecular mechanisms. *Semin Pediatr Surg.* 2010;19(3):171–179.
- 114 Gonzaga S, Henriques-Coelho T, Davey M, Zoltick PW, Leite-Moreira AF, Correia-Pinto J, et al. Cystic adenomatoid malformations are induced by localized FGF10 overexpression in fetal rat lung. *Am J Respir Cell Mol Biol.* 2008;39(3):346–355.
- 115 Jancelewicz T, Nobuhara K, Hawgood S. Laser microdissection allows detection of abnormal gene expression in cystic adenomatoid malformation of the lung. *J Pediatr Surg.* 2008;43(6):1044–1051.
- 116 Tichelaar JW, Lu W, Whitsett JA. Conditional expression of fibroblast growth factor-7 in the developing and mature lung. *J Biol Chem.* 2000;275(16):11858–11864.
- 117 Volpe MV, Pham L, Lessin M, Ralston SJ, Bhan I, Cutz E, et al. Expression of Hoxb-5 during human lung development and in congenital lung malformations. *Birth Defects Res A Clin Mol Teratol.* 2003;67(8):550–556.
- 118 Wang X, Wolgemuth DJ, Baxi LV. Overexpression of HOXB5, cyclin D1 and PCNA in congenital cystic adenomatoid malformation. *Fetal Diagn Ther.* 2011;29(4):315–320.
- 119 Lezmi G, Verkarre V, Khen-Dunlop N, Vibhushan S, Hadchouel A, Rambaud C, et al. FGF10 Signaling differences between type I pleuropulmonary blastoma and congenital cystic adenomatoid malformation. *Orphanet J Rare Dis.* 2013;8:130.
- 120 DeBoer EM, Keene S, Winkler AM, Shehata BM. Identical twins with lethal congenital pulmonary airway malformation type 0 (acinar dysplasia): further evidence of familial tendency. *Fetal Pediatr Pathol.* 2012;31(4):217–224.
- 121 MacSweeney F, Papagiannopoulos K, Goldstraw P, Sheppard MN, Corrin B, Nicholson AG. An assessment of the expanded classification of congenital cystic adenomatoid malformations and their relationship to malignant transformation. *Am J Surg Pathol.* 2003;27(8):1139–1146.
- 122 Hasegawa M, Sakai F, Arimura K, Katsura H, Koh E, Sekine Y, et al. EGFR mutation of adenocarcinoma in congenital cystic adenomatoid malformation/congenital pulmonary airway

- malformation: a case report. *Jpn J Clin Oncol.* 2014;44(3):278–281.
- 123 Kim MY, Kang CH, Park SH. Multifocal synchronous mucinous adenocarcinomas arising in congenital pulmonary airway malformation: a case report with molecular study. *Histopathology.* 2014;65(6):926–932.
- 124 Tetsumoto S, Kijima T, Morii E, Goya S, Minami T, Hirata H, et al. Echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) rearrangement in congenital pulmonary airway malformation. *Clin Lung Cancer.* 2013;14(4):457–460.
- 125 Messinger YH, Stewart DR, Priest JR, Williams GM, Harris AK, Schultz KA, et al. Pleuropulmonary blastoma: A report on 350 central pathology-confirmed pleuropulmonary blastoma cases by the International Pleuropulmonary Blastoma Registry. *Cancer.* 2015;121(2):276–285.
- 126 Oliveira C, Himidan S, Pastor AC, Nasr A, Manson D, Taylor G, et al. Discriminating preoperative features of pleuropulmonary blastomas (PPB) from congenital cystic adenomatoid malformations (CCAM): a retrospective, age-matched study. *Eur J Pediatr Surg.* 2011;21(1):2–7.
- 127 Manivel JC, Priest JR, Watterson J, Steiner M, Woods WG, Wick MR, et al. Pleuropulmonary blastoma. The so-called pulmonary blastoma of childhood. *Cancer.* 1988;62(8):1516–1526.
- 128 Priest JR, Watterson J, Strong L, Huff V, Woods WG, Byrd RL, et al. Pleuropulmonary blastoma: a marker for familial disease. *J Pediatr.* 1996;128(2):220–224.
- 129 Foulkes WD, Bahubeshi A, Hamel N, Pasini B, Asioli S, Baynam G, et al. Extending the phenotypes associated with DICER1 mutations. *Hum Mutat.* 2011;32(12):1381–1384.
- 130 Rio Frio T, Bahubeshi A, Kanellopoulou C, Hamel N, Niedziela M, Sabbaghian N, et al. DICER1 mutations in familial multinodular goiter with and without ovarian Sertoli-Leydig cell tumors. *JAMA.* 2011;305(1):68–77.
- 131 Slade I, Bacchelli C, Davies H, Murray A, Abbaszadeh F, Hanks S, et al. DICER1 syndrome: clarifying the diagnosis, clinical features and management implications of a pleiotropic tumour predisposition syndrome. *J Med Genet.* 2011;48(4):273–278.
- 132 Hill DA, Ivanovich J, Priest JR, Gurnett CA, Dehner LP, Desruisseau D, et al. DICER1 mutations in familial pleuropulmonary blastoma. *Science.* 2009;325(5943):965.
- 133 Medina PP, Slack FJ. microRNAs and cancer: an overview. *Cell Cycle.* 2008;7(16):2485–2492.
- 134 Stefani G, Slack FJ. Small non-coding RNAs in animal development. *Nat Rev Mol Cell Biol.* 2008;9(3):219–230.
- 135 Heravi-Moussavi A, Anglesio MS, Cheng SW, Senz J, Yang W, Prentice L, et al. Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. *N Engl J Med.* 2012;366(3):234–242.
- 136 Pugh TJ, Yu W, Yang J, Field AL, Ambrogio L, Carter SL, et al. Exome sequencing of pleuropulmonary blastoma reveals frequent biallelic loss of TP53 and two hits in DICER1 resulting in retention of 5p-derived miRNA hairpin loop sequences. *Oncogene.* 2014;33(45):5295–5302.
- 137 Seki M, Yoshida K, Shiraishi Y, Shimamura T, Sato Y, Nishimura R, et al. Biallelic DICER1 mutations in sporadic pleuropulmonary blastoma. *Cancer Res.* 2014;74(10):2742–2749.
- 138 Walker CM, Wu CC, Gilman MD, Godwin JD, 2nd, Shepard JA, Abbott GF. The imaging spectrum of bronchopulmonary sequestration. *Curr Probl Diagn Radiol.* 2014;43(3):100–114.
- 139 Freedom RM, Yoo SJ, Goo HW, Mikailian H, Anderson RH. The bronchopulmonary foregut malformation complex. *Cardiol Young.* 2006;16(3):229–251.
- 140 Sun X, Xiao Y. Pulmonary sequestration in adult patients: a retrospective study. *Eur J Cardiothorac Surg.* 2015;48(2):279–282.
- 141 Belchis D, Cowan M, Mortman K, Rezvani B. Adenocarcinoma arising in an extralobar sequestration: a case report and review of the literature. *Lung Cancer.* 2014;84(1):92–95.
- 142 Zhang H, Tian J, Chen Z, Ma X, Yu G, Zhang J, et al. Retrospective study of prenatal diagnosed pulmonary sequestration. *Pediatr Surg Int.* 2014;30(1):47–53.
- 143 Ozcelik U, Gocmen A, Kiper N, Dogru D, Dilber E, Yalcin EG. Congenital lobar emphysema: evaluation and long-term follow-up of thirty cases at a single center. *Pediatr Pulmonol.* 2003;35(5):384–391.
- 144 Mani H, Suarez E, Stocker JT. The morphologic spectrum of infantile lobar emphysema: a study of 33 cases. *Paediatr Respir Rev.* 2004;5 Suppl A: S313–320.
- 145 Seo T, Ando H, Kaneko K, Ono Y, Tainaka T, Sumida W, et al. Two cases of prenatally diagnosed congenital lobar emphysema caused by lobar bronchial atresia. *J Pediatr Surg.* 2006;41(11):e17–20.

- 146 Reiterer F, Grossauer K, Morris N, Uhrig S, Resch B. Congenital pulmonary lymphangiectasis. *Paediatr Respir Rev.* 2014;15(3): 275–280.
- 147 de Bruyn G, Casaer A, Devolder K, Van Acker G, Logghe H, Devriendt K, et al. Hydrops fetalis and pulmonary lymphangiectasia due to FOXC2 mutation: an autosomal dominant hereditary lymphedema syndrome with variable expression. *Eur J Pediatr.* 2012;171(3):447–450.
- 148 Fabretto A, Kutsche K, Harmsen MB, Demarini S, Gasparini P, Fertz MC, et al. Two cases of Noonan syndrome with severe respiratory and gastrointestinal involvement and the SOS1 mutation F623I. *Eur J Med Genet.* 2010;53(5):322–324.
- 149 Mathur D, Somashekar S, Navarrete C, Rodriguez MM. Twin infant with lymphatic dysplasia diagnosed with Noonan syndrome by molecular genetic testing. *Fetal Pediatr Pathol.* 2014;33(4): 253–257.
- 150 Yao LC, Testini C, Tvorogov D, Anisimov A, Vargas SO, Baluk P, et al. Pulmonary lymphangiectasia resulting from vascular endothelial growth factor-C overexpression during a critical period. *Circ Res.* 2014;114(5): 806–822.
- 151 Boland JM, Tazelaar HD, Colby TV, Leslie KO, Hartman TE, Yi ES. Diffuse pulmonary lymphatic disease presenting as interstitial lung disease in adulthood: report of 3 cases. *Am J Surg Pathol.* 2012;36(10): 1548–1554.

Lung Structure at Preterm and Term Birth

Jason C. Woods and Johannes C. Schittny

Abstract

When lung development is not interrupted by premature birth and unaffected by genetic or environmental disturbances, all components develop with complex control to form a functional organ with a predictable timeline during fetal development. In this chapter we describe the relationship between morphological development and function in both physiological and pathological conditions in human lung development. Tree-like growth of the lung begins during the first few weeks postconception, with the embryonic stage characterized by branching morphogenesis in both the airways and blood vessels, separately in the left and right lung buds, which appear near day 26 postcoitus (p.c.). Branching continues through the embryonic stage, with proliferation of mesenchymal and epithelial cells and apoptosis near branch points and in the areas of new formation. The pseudoglandular stage (weeks 5–17 p.c.) is characterized by accelerated cellular proliferation and airway and vascular branching, with epithelial differentiation in proximal and distal airways. Further epithelial differentiation, angiogenesis of the parenchymal capillary network, and the first formation of the air–blood barrier characterize the canalicular stage (16–26 weeks p.c.), just before the completion of branching morphogenesis (saccular stage, weeks 24–38 p.c.) and the start of alveolarization (week 36 through adolescence).

Keywords:

Structure, differentiation, alveolarization, branching, diffusion front

Overview

A functional lung has a high surface area for gas exchange with a thin blood–gas barrier, a surfactant system facilitating inflation of the lung and efficient blood–gas exchange, and a conductive airway tree. In addition, these airway and gas-exchange structures are served by a vascular system mirroring the airways to facilitate arterial supply of oxygen and venous return of carbon dioxide. When development is not interrupted by premature birth and unaffected by genetic or environmental disturbances, all components develop with complex control to form a functional organ with a predictable timeline during fetal development. We will describe the relationship between morphological development and function in both physiological and pathological conditions in human lung development.

Structural Perspective on Lung Function

The combination of a very thin tissue barrier and a very large air–blood contact surface, together with the ability to move large volumes of gas in and out of the lungs within a few seconds, is an

amazing evolutionary achievement necessary for adequate gas exchange. Up until the moment of the first breath *ex utero*, fetal gas exchange occurs through the placenta, and immediately after birth, the lung must deal with assault from the outside environment, not least from gaseous oxygen and particulates, and provide adequate gas exchange for survival. The lungs continue to develop well beyond birth. The available surface area for gas exchange continues to grow for what is hypothesized to be many years after birth by continued alveolarization in the normal lung.

Congenital pulmonary abnormalities largely result from aberrant protein expression resulting in structural vascular abnormalities often associated with congenital heart disease (1), metabolic enzyme activity, or ion transport defects and are often progressive (2). Pulmonary abnormalities that arise as a result of preterm birth are typically due to underdeveloped alveolar and/or vascular structures and have immediate detrimental effects at birth. Preterm birth is essentially the arrest of late-lung development, manifesting as lung malformation. The extent to which the lung is malformed depends on the gestational age at which birth occurs and lifesaving interventions (including high oxygen, mechanical ventilation, and

surfactant), and the resulting bronchopulmonary dysplasia (BPD) can range from transient respiratory deficits to lifelong effects (3).

Anatomic development of the structures that provide for convective flow of gas to and from lung acini and development of the gas-exchange structures within those acini are both essential to proper physiological function at birth. Recent research has shown that many of the cell types in the pulmonary system can regenerate, thus effecting repair and restoring near-normal function of damaged tissue. However, the success of this built-in repair mechanism depends on the cause of injury (4). This chapter describes normal lung development of both the conducting and respiratory airways throughout gestation, for the reader to understand normal and abnormal lung physiology at premature and mature birth and beyond.

The Pulmonary Airway Tree: Conduction and Diffusion, the Diffusion Front, and Scaling

The lung is an impressive organ because it provides new oxygen at each breath to an internal

pulmonary surface area the size of a tennis court ($\sim 130 \text{ m}^2$ for a healthy adult near functional residual capacity). The relationship between bronchial tissue and the arteriovenous structures required for efficient gaseous exchange is intimate and inseparable. However, although there are certain similarities between the structure of the vascular and bronchial trees, there are also some important differences that largely relate to fluid flow differences between gases and liquids. Figure 7-1 depicts the branching structure of airways and illustrates the “convection zone” (smooth-walled airways where gas flow is pressure driven) down to the terminal bronchiole and the “respiratory zone,” where gas exchange occurs and diffusive gas transport is faster than pressure-driven flow, at physiologic pressures. The “diffusion front,” or the boundary between these two zones, can vary depending on exertion – moving slightly proximal during quiet rest and more distal during exercise and rapid breathing (5). Abnormalities in airways at any level can significantly alter air flow and subsequent gas exchange. Small changes in conducting airways, such as wall remodeling or temporary increases in wall

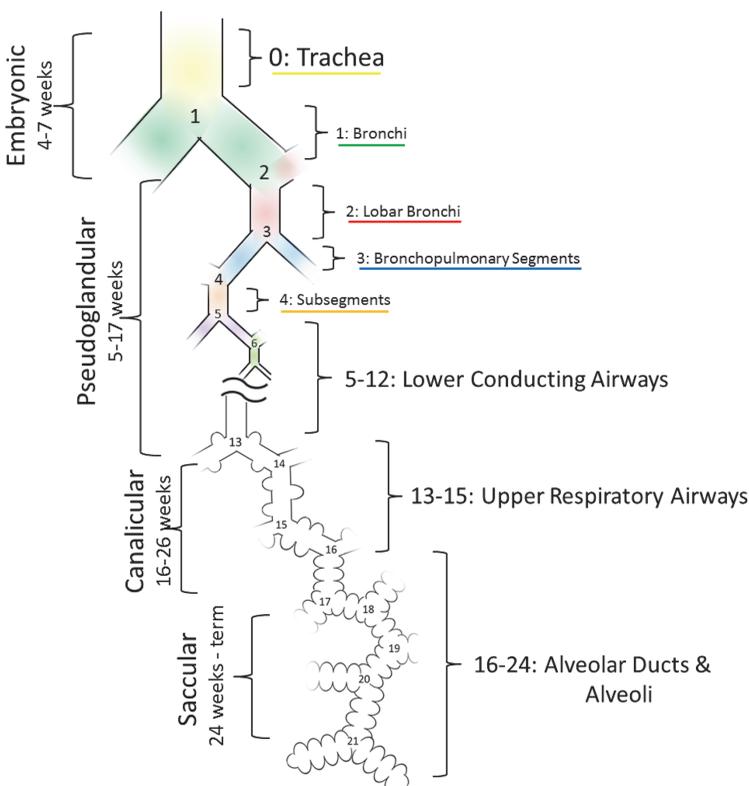


Figure 7-1. Airway branching with gestational age. The stages of lung development and the development of the bronchial tree. On average an airway of a human lung ends after approximately 23 generations in an alveolar sacculle; however, due to the shape of the lung, a range of 18 to 30 generations has been observed for lung pathways of varying lengths.

thickness, can significantly affect the downstream flow and may alter the laminar/turbulent balance of the normal airway tree (6). Similarly, alterations to acinar airways (in the respiratory zone), such as alveolar-wall simplification or thickening, can significantly affect either the available gas-exchange surface area or increase the diffusion time to the gas-blood barrier (7).

The Pulmonary Vascular Tree

Bronchial circulatory structures are also highly specialized, conforming to function (8). Elliott and Reid (9, 10) categorized pulmonary vessels into conventional and supernumerary; conventional vessels are located adjacent to the bronchial tree, and their branches and supernumerary vessels supply the nearest acinar structures. Supernumerary vessels are found throughout the pulmonary tree, but tend to be more numerous toward the periphery of lung tissue. Developmentally, the conventional and supernumerary vessels are established in the same temporal space as the pulmonary structures they accompany/supply, and pre-acinar pulmonary vessel anatomy is complete at midgestation. Vascular development after 20 weeks consists largely of changes in vessel wall thickness, cellular constituents, and muscle formation. At birth, pulmonary arterial walls quickly thin (by around 50% as smooth muscle cells reduce their overlap), and the medial thickness continues to decrease over the first 3 months of life, with little physiological change thereafter. The rate of arterial formation increases for 2 months after birth, and then slows to match the rate of alveolar formation. These anatomical changes reflect functional changes throughout late gestation and birth/perinatal development. Pulmonary circulation in the fetus has relatively

high vascular resistance and low blood flow (11), which changes at birth and the first inspiration when the foramen ovale and ductus arteriosus close, shunting blood to the lungs (12). Higher PO_2 in the pulmonary artery is sensed by the endothelium, and higher shear stress acts to switch the function of these vessels in the breathing infant (13).

Pre- and Postnatal Lung Development

Embryonic Stage (Weeks 4–7 p.c., 6–9 Weeks PMA)

Tree-like growth of the lung begins during the first few weeks postconception; the embryonic stage is characterized by branching morphogenesis of both the airways and blood vessels, arising from a mixture of largely undifferentiated cells that will later segment themselves into the smooth muscle, blood vessels, lymphatic cells, and epithelium. It was once thought that both lungs formed from a single anlage; however, we now know that each of the two lungs is formed by an independent anlage (14,15). The two lung buds first appear at day 26 p.c. (postcoitum, days after conception)/5 weeks PMA (postmenstrual age), having emerged from the ventral wall of the primitive foregut endoderm at approximately 3 weeks p.c./5 weeks PMA (Figure 7-2). The lung buds then grow into the surrounding mesoderm just anterior and parallel to the initial formation of the esophagus. On week 4 p.c./6 weeks PMA, when the embryo is still only a few millimeters long, the trachea (formed from the fusion of the lung buds) and larynx form just above the lung anlage. Between days 33 and 41 p.c. (week 7 PMA) the second bifurcation occurs, forming

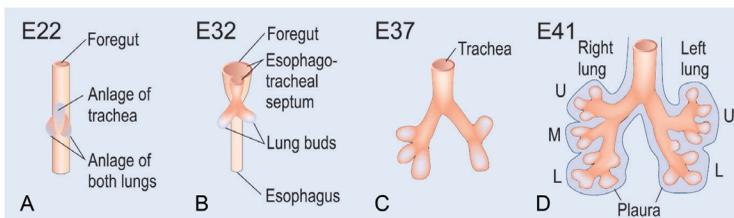


Figure 7-2. Early human lung development. At day 26 postcoitum (p.c.) the anlage of the two lungs forms on both sides lateral of the anlage of the trachea by outpouchings of the foregut (A) (15). The prospective trachea forms by a distal-to-proximal segregation from the foregut. At day 32 the lung anlage gives rise to the future main bronchi (B). The lobar bronchi are formed due to continued branching at day 37 (C). Later at day 41 the segmental bronchi are following (D). After the formation of the pleura, organogenesis is completed (D). U, upper lobe; m, middle lobe; l, lower lobe (from Schittny *entwiclung - von der anlage zur adulten lung*).

what will eventually be the lobes (two left and three right) of each of the lungs. A third round of branching occurs at week 8 PMA (41–44 days p.c.), with approximately 8–9 and 10 segmental branches on the left and right respectively, which establishes the bronchopulmonary segments of the mature lung.

Significant proliferation of mesenchymal and epithelial cells occurs during this stage, with apoptosis near branch points and in the areas of new formation. The mesenchyme will later differentiate into progenitor cells, which will give rise to blood vessels, smooth muscle, cartilage, lymphatics, and connective tissue. At the embryonic stage, the epithelial cells are simple and undifferentiated, forming a pseudostratified columnar epithelium. The mesenchyme remains loosely constructed to allow mass flow of proteins necessary for development. The basement membrane, however, is intact and patent, which is an important control mechanism. Although the primitive trachea and esophagus are completely separated, each lack cartilage, smooth muscle, and nerves. By the end of this stage, the primitive vasculature has been established with pulmonary arteries branching from the embryonic sixth aortic arch and veins emerging from the left atrium of the developing heart.

Clinical Aspects of the Embryonic Stage

Perhaps unsurprisingly, abnormalities in this stage of lung development are critical in postnatal mortality and morbidity of the fetus and are often related to lung bud development, trachea/esophagus separation, proximal airway, and initial lobe formation. Some structural abnormalities such as pulmonary valve stenosis, pulmonary agenesis, or aplasia generally are considered nonsurvivable, whereas others may cause significant pulmonary morbidity. Tracheoesophageal fistula, which encompasses a variety of permutations of abnormal connections between the trachea and esophagus, is a common congenital abnormality associated with this stage of lung development, often accompanied by esophageal atresia, a defect whereby the upper and lower segments of the esophagus do not connect. Postsurgical survival in full-term infants is high, but in low-birth-weight infants, tracheoesophageal fistula is often accompanied by other congenital malformations and a concomitantly higher risk for poor outcomes (16).

Pseudoglandular Stage (Weeks 5–17 p.c., 7–19 Weeks PMA)

By week 6 p.c./week 8 PMA of development, the growing lungs can be distinguished as two separate organs, assuming a glandular appearance consisting of multiple, branching epithelial tubules surrounded by extensive regions of mesenchyme. Early in this stage of lung development, cellular proliferation increases, accelerating airway and vascular branching. By week 17 p.c./week 19 PMA, the conducting airways including the terminal bronchioles are formed – the upper lobes having 12–17 airway branching generations, 18–23 generations in the middle lobes, and 14–23 generations in the lower lobes.

At the beginning of the pseudoglandular stage, the respiratory bronchioles are lined with pseudostratified columnar epithelium, supported by a basement membrane scaffold with adjacent mesenchyme, all surrounded by an extracellular matrix rich in the macromolecules needed for cellular differentiation, migration, adhesion, and proliferation (Figure 7–3A, D). The respiratory epithelium differentiates, forming tall columnar epithelial cells in the proximal airways and cuboidal epithelium in the distal acinar tubules. Further differentiation forms ciliated and mucous cells in the trachea and proximal airways. Supportive cartilage appears in the trachea by week 10 p.c. The mesenchymal cells differentiate, forming myofibroblasts by week 7 PMA, thus laying the foundation for the developing smooth muscle layer to form around the tubules. By the end of the pseudoglandular stage (week 17 p.c.), cartilage extends to the segmental bronchi and smooth muscle extends to the alveolar ducts.

Cell growth and differentiation drive the development of the pulmonary arteries and veins, and by the end of this stage, all pre-acinar vasculature is formed from endothelial tubes. In the distal branches of the terminal bronchioles, acinar tubules and buds establishing the primitive acinus are also formed by the end of the pseudoglandular stage (17).

Clinical Aspects of the Pseudoglandular Stage

In this stage, spontaneous, peristaltic contractions of the airway smooth muscle are observed. There is firm evidence to support that a maintained pressure differential between the airway lumen and surrounding tissue is essential for normal

lung development. It is postulated that these contractions contribute to fetal lung development by mechanoreceptor-mediated growth factor production (18) and also function to prevent uncontrolled distension of the airways as lung fluid is secreted into the lumen (18).

It is also in this stage (around 10 weeks p.c.) that fetal breathing movements can be discerned (19). Recent *in vitro* studies have investigated the signaling pathways linking fetal breathing movements and lung development. These movements stretch and move fluid into and out of the lungs. Mechanical stretch upregulates the release of serotonin via mechanosensitive channels (20), which promotes differentiation of epithelial cells. Stretch also increases epithelial cell proliferation (21) and stimulates secretion of lung surfactant lipids from type II epithelial cells (22).

Developmental abnormalities during the pseudoglandular stage are generally related to airway and vascular branching and growth. Abnormal branching can result in pulmonary hypoplasia or sequestration. Absence or abnormal weakening of supportive cartilage is associated with tracheo- or bronchomalacia, which, respectively, can cause trachea or airway collapse during respiration. Importantly, during this stage of development, the pleuroperitoneal membrane closes. Failure of the membrane to close completely often results in the herniation of the developing abdominal organs into the chest cavity. This congenital diaphragmatic hernia (CDH) often leads to pulmonary hypoplasia and pulmonary hypertension, and although the underlying cause of CDH remains unknown, there is evidence to implicate genetic mutations to the transcriptional and growth factors related to lung development (23).

Canalicular Stage (Weeks 16–26 p.c., 18–27 Weeks PMA)

The canalicular stage is characterized by the differentiation of the epithelial cells, angiogenesis of the parenchymal capillary network, and the first formation of the air–blood barrier. Some of the most distal generations of airways (future alveolar ducts) still remain to be formed at this stage (17).

“Canalization” of the Lung Parenchyma.

Before the first air–blood barriers can be formed the mesenchymal capillaries must be proximal to the epithelial cell lining of the future airways. Two steps are necessary for this process: (1) During the

pseudoglandular stage the mesenchymal capillaries form a loose three-dimensional network. A high density of capillaries is achieved by an enhanced angiogenesis (Figure 7-3 A→B). The respiratory airways (airways distal of the purely conducting airways) start to grow in length and width; their shape changes, and they are also called “canaliculi.” This canalization of the respiratory airways and capillaries contributes to the nomenclature for this stage. (2) Due to increasing volume of the future airways, the mesenchyme condenses; as a result the epithelium of the future airways comes into close contact with the mesenchymal capillary network (Figure 7-3 A→B). Programmed cell death (apoptosis) occurs during condensation of the mesenchymal tissue. The involvement of apoptosis is consistent with a reduction of mesenchymal cells during this stage.

Epithelial Differentiation – Formation of the Air–Blood Barrier

During the canalicular stage, the differentiation of the parenchymal epithelium becomes morphologically visible, even if the cell fate is decided much earlier (15). The cuboidal glycogen-rich epithelium of the future gas-exchange region differentiates into the type I and type II alveolar epithelial cell (Figure 7-3 E→F). Type II alveolar epithelial cells are the progenitors of type I epithelial cells (24). Therefore, it is not surprising that the future alveolar epithelial cells contain few, small, lamellated bodies before a differentiation into type I and type II cell becomes morphologically visible (25).

The type I cells spread out and form thin sheet-like extensions covering most of the inner surface of the future gas-exchange region (Figure 7-4). At locations where the type I alveolar epithelial cells make close contact with the endothelium of the capillaries, an air–blood barrier forms (Figs. 7-3 b, e→f; 7-4 a). The tissue of the air–blood barrier is reduced to a sheet-like extension of the type I epithelial cell and the thin part of the endothelial cell. Both cell types are separated by their basement membranes, which are fused to one common basement membrane possessing one central lamina densa and two lamina lucida facing the epithelial or endothelial cells, respectively (Figures 7-3F, 7-4A, B). Currently, it is poorly understood how the air–blood barrier forms; it is likely that mesodermally derived endothelium interacts with endodermally derived epithelium (26). This hypothesis is

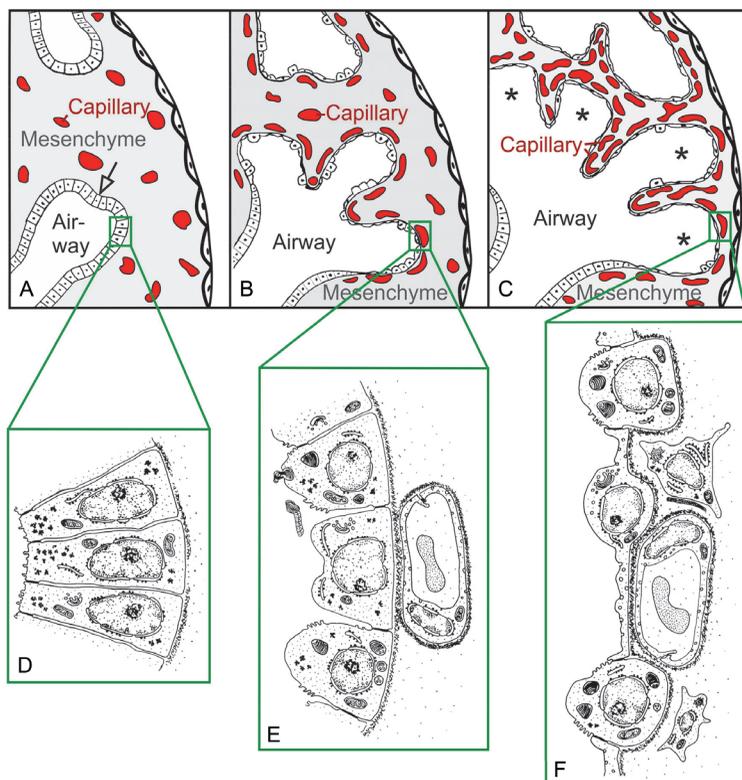


Figure 7-3. Morphological development of the lung parenchyma during the pseudoglandular, canalicular, and saccular stages. The epithelial tubules branch constantly during the pseudoglandular stage and penetrate into the surrounding mesenchyme (open arrow, branching point, **(A)**). The mesenchyme contains a loose capillary network (**A**). The epithelium itself is tall and columnar (**D**). The canalicular stage (**B**) is characterized (1) by a differentiation of the tall columnar epithelial cells into prospective lining and secretory cells (type I and type II epithelial cells, **E and F**), (2) by a widening of the future airways (**B**), (3) by a multiplication of the capillaries and their first close contacts to the epithelium (**B**), and (4) by the formation of first air–blood barriers (**E–F**). Throughout the saccular stage (**C**) the mesenchyme further condenses, resulting in the formation of the thick immature inter-airway septa, which contain a double-layered capillary network – one layer on either side of the septum. The widened terminal ends of the bronchial tree are recognized as saccules (asterisks). (a–c, modified from Caduff and coworkers *Anat. Rec.* 1986;216:154–164); d–e from Burri and Weibel *Ultrastructure and morphometry of the developing lung*.

supported by a phenotype of a transgenic mouse, where the sequence coding for the nitrogen-binding site of laminin (γ 1III4, within the laminin- γ 1-chain) was selectively deleted. The basement membranes of the air–blood barrier were disrupted or missing in large part in these mice. Due to a failure of pulmonary gas exchange the mice died at birth (27).

During the canalicular stage, type II epithelial cells start to produce surfactant, which is morphologically visible by the first accumulation of lamellar bodies. In most species surfactant appears late in gestation (at about 80–90% of the total duration of gestational period). In humans small amounts of surfactant are already present at weeks 22–24 p. c./weeks 24–26 PMA (60% gestation) (28), which facilitate survival of very prematurely born babies. Before the lung fully matures, surfactant appears to be more abundant in apical than in basal lung regions (29). This observation may explain clinical findings that in some prematurely born babies, hyaline membrane disease is more pronounced in basal than in apical lung regions.

An acinus is a small tree of gas-exchanging airways, which is fed by the most distal purely

conducting airways. Boyden (30) observed the birth of the acinus during the canalicular stage; however, the airways are already formed during the pseudoglandular stage (17). Due to the aforementioned differentiation of the epithelia, a distinction of conducting and gas-exchanging airways becomes feasible during the canalicular stage, and therefore the acini may now be recognized.

Clinical Aspects of the Canalicular Stage

The canalicular stage may be the most critical stage for very immature, prematurely born infants. After the formation of the first air–blood barrier and the start of a minimal production of surfactant, some pulmonary gas exchange becomes possible. At the end of the canalicular stage, survival is feasible – but only with the specialized support of a neonatal intensive care unit. Survival itself is strongly dependent on the amount of functional gas-exchange surface area, which is determined by the amount of formed air–blood barrier and by the amount of available surfactant. Even if the surface area of the air–blood barrier is sufficient, only limited areas of the lung will be inflated if surfactant is not available in adequate amounts.

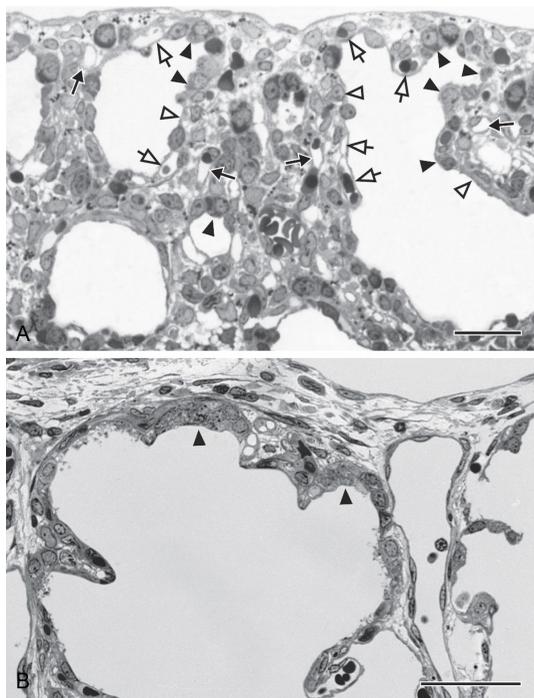


Figure 7-4. Formation of the air–blood barrier. During the early canalicular stage (A, rat lung) the epithelium of the terminal airways is still cuboidal and glycogen-rich (closed arrow head). Already a bit more proximal, the epithelium begins to flatten out (open arrow head) and to form the first thin air–blood barriers. During the latter process capillaries, which are located inside the mesenchyme (closed arrow), “move” toward the epithelium (open arrow). In human lung (B, postnatal day 26) remnants of the cuboidal epithelium (closed arrow head) are still present at the uttermost periphery of the gas-exchange region – even if alveolarization started already (B). This demonstrates the large overlap between different phases of lung development, especially if peripheral and central parts are compared. In addition, it illustrates the central-to-peripheral progression of lung development. Light microscopical images, bar, 50 μm .

Alveolar capillary dysplasia (ACD, also congenital alveolar dysplasia) is a very rare congenital disease that is a malformation or absence of the pulmonary blood vessels, in particular of the alveolar capillaries. In principle, the blood vessels develop in parallel to the airways, and therefore, the etiology of ACD is already present at the pseudoglandular stage. The malformation becomes more pronounced in the canalicular stage because the surface area of the air–blood barrier is greatly reduced, concomitant with a greatly reduced capillary mesenchymal network. In addition to a reduction of pulmonary gas-exchange capacity, ACD causes persistent pulmonary hypertension.

Saccular Stage (Weeks 24–38 p.c., 26–40 Weeks PMA)

The saccular stage represents an intermediate stage between completion of branching morphogenesis and the start of the alveolarization. While the most distal generations of airways have likely been already laid down by the end of the canalicular stage, the addition of a very few terminal airways may still occur during the beginning of the saccular stage in some species (31). The name “saccular stage” describes the typical appearance of peripheral airway clusters forming widened airspaces called saccules or terminal sacs.

Expansion of the Gas-Exchange Area

Airspace generations distal to the terminal bronchioles are widening and lengthening during this stage and are forming the required space for subsequent alveolarization. At the terminal end of the bronchial tree, sacculi form (Figure 7.3D, F); these sacculi are smooth walled, separated by septa, and covered with type I and II pneumocytes. The primary septum contains two networks of capillaries for the adjacent sacculi, as well as some elastic and collagen fibers. At this stage, the interstitial space is still cell-rich and has an important regulatory and developmental role for the epithelium above (32). By term birth, all generations of the conducting and respiratory branches have been generated. The sacculi are thin, smooth-walled sacks and correspond to the later alveolar sacculi. At the end of this phase, the interstitial fibroblasts begin with the production of extracellular material in the interductal and intersaccular space.

Time of Birth. The maturity of the lung at birth of different species correlates with the physical activity of the newborns (Table 7-1). If high activity is required at birth, the lungs are near fully matured and capable of high amounts of gas exchange. For example, in precocial animals like sheep (33) and guinea pigs (34), alveolarization starts well before birth, and at term, their lungs appear nearly mature. Alveolarization of altricial species, such as rats (35), mice (36), and humans (37), starts only shortly before birth (humans) or after birth (mice and rats). At the extreme end of this range, one of the most immature lungs at birth is the lung of the marsupial quokka wallaby (*Setonix brachyurus*), which is born in the canalicular stage (38).

Table 7-1. Stages of lung development and their time scale. Because the stages are defined mainly by morphological criteria, their beginning and end do not represent sharp borders. In addition, stages may overlap (in particular the alveolar stages and the stage of microvascular maturation), and regional differences are also common – especially between central and peripheral regions. Nutrition and litter size influence the exact timing of development (28, 47, 69–72). Monkey, Rhesus monkey; E = embryonic day (days postcoitum); n.d., not determined; P, postnatal day; * weeks postcoitum; ** unpublished observation of JCS (based on Schittny and Burri 2008 (14)).

Period	Stage	Duration	Characteristics
Embryonic	Embryonic	Rabbit: n.d.–E18 Sheep: E17–E30 Mouse: E9,5–E12 Rat: E11–E13 Monkey n.d.–E55 Human: E26–E49 (4–7 weeks*)	Start of organogenesis; formation of major airways
Fetal	Pseudoglandular	Rabbit: E18–E24 Sheep: E30–E85 Mouse: E12–E16.5 Rat: E13–E18.5 Monkey E55–E85 Human: E35–E119 (5–17 weeks*)	Formation of bronchial tree and large parts of prospective respiratory parenchyma; birth of the acinus
	Canalicular	Rabbit: E23–E27 Sheep: E80–E120 Mouse: E16.5–E17.5 Rat: E18.5–E20 Monkey E75–E115 Human: E112–E182 (16–26 weeks*)	Completion of branching morphogenesis = formation of last generations of airways; epithelial differentiation; first air– blood barrier; appearance of surfactant
	Saccular or terminal sac	Rabbit: E27–E30 Sheep: E110–E140 Mouse: E17.5–P4 Rat: E21–P4 Monkey E105–term Human: E168–E266 (24–38 weeks*)	Expansion of (future) airspace
Postnatal	Alveolarization, first phase/ classical alveolarization	Rabbit: E30–term (E31) Sheep: E120–term (E145) Mouse: P4–P21 Rat: P4–P21 Monkey E125–< P180** Human: E252 (36 weeks* preterm)–3 years	Alveolarization by formation of secondary septa (septation); alveolar septa are still immature; they are containing a double-layered capillary network
	Alveolarization, second phase/continued alveolarization	Rabbit: term (E31)–n.d. Sheep: term (E145)–n.d. Mouse: P14– young adulthood (~P36) Rat: P14–young adulthood (~P60) Monkey < P180**–young adulthood (7–8 years) Human: 2 years–young adulthood (17–21 years)	Alveolarization by formation of secondary septa (septation), but now lifting off mature alveolar septa containing a single-layered capillary network.

Table 7-1. (cont.)

Period	Stage	Duration	Characteristics
	Microvascular maturation	Rabbit: n.d. Sheep: n.d. Mouse: P14–P21 Rat: P14–P21 Monkey n.d.–< P180** Human: 0–3 years	Remodeling and maturation of interalveolar septa and of the capillary bed (the double-layered capillary network is transformed to a single-layered network).

Regardless of how mature the lung may be at term, alveolarization continues at least as long as the lungs are growing – until young adulthood in most species (14).

Clinical Aspects of the Saccular Stage

At this stage, imbalances in the pressure differential between intraluminal airway and extraluminal spaces can have lasting detrimental effects on lung development. Fetuses exposed to oligohydramnios have an 80% higher risk of respiratory failure than their unexposed counterparts (39). Oligohydramnios is associated with pulmonary hypoplasia and is often part of a pleiotropic manifestation of autosomal recessive polycystic kidney disease (40).

BPD (bronchopulmonary dysplasia, a chronic lung disease of infancy) is the most common cause of pulmonary morbidity in premature infants and is associated with long-term morbidities (41). BPD is a complication of respiratory distress syndrome of premature birth and is most often seen in very low- or extremely low- birth-weight infants who required ventilation, antenatal steroids, and surfactant therapy for survival. Infants with BPD typically have fewer, larger alveoli (and therefore decreased gas-exchange surface area) and insufficient vascularization to the alveoli (and therefore less-efficient gas exchange) than their term counterparts. A number of factors can contribute to BPD, including maternal drug use or smoking, extent of prematurity, sepsis, and overly aggressive ventilation. Recent studies postulate a role for stem cell depletion in the etiology of BPD, and stem cell therapy has proven beneficial in experimental models (42). Children who were preterm have a significant airflow limitation during the first years of life, and their lung function can deteriorate in their first year (43).

Alveolarization (Week 36–Young Adulthood)

The classical model of alveolarization comprises a phase of classical (bulk) alveolarization (Figure 7-5) followed by the phase of microvascular maturation (maturation of the alveolar septa, Figure 7-6) (35). It was previously postulated (35) that the formation of new alveoli ceased after the maturation of the alveolar septa. Recently, this model was modified to include a phase of continued alveolarization because it was recognized that new alveoli are forming at least until young adulthood (14). However, the rate of increase in the number of alveoli is lower during continued alveolarization than during classical alveolarization. This biphasic behavior of alveolarization has been observed in humans, rats, and mice (44).

Classical Alveolarization (Week 36–3 or More Years)

Alveoli begin to form at around 36 weeks, and by birth there are up to 50 million alveoli (45), while in the adult human lung, there are over 300 million alveoli. Taken together, this requires that the majority of alveolarization takes place postnatally (46). Independent of the species and the stage of lung development at birth, the switch from classical to continued alveolarization occurs simultaneously with microvascular maturation. Therefore, the animal data are a good representation for the understanding of human lung development (31). Immature sacculi (with a double capillary layer as described earlier) mature by secondary alveolarization (Figure 7-7). A septal layer forms from the primary sacculi, concomitant with microvascularization and apoptosis in the interstitial layer (Figures 7-7 and 7-8), to form mature alveoli with one capillary layer and a thin

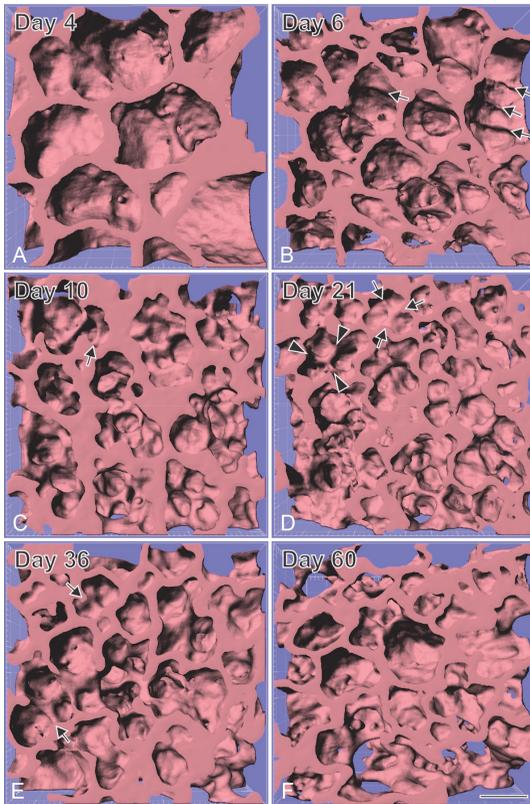


Figure 7-5. Visualization of Alveolarization. 3-D visualizations of terminal airspaces throughout alveolarization of the rat lung. At postnatal day 4 (A) large sacculi are formed by branching morphogenesis. Between days 6 and 36 (A–E), many low-rising septa are observed, which are viewed as newly forming septa (arrows). While between days 4 and 21 (A–D), the size of the terminal airspaces decreases, an increase of the airspaces was observed between days 21 and 60 (E and F). Rat lungs were imaged by synchrotron radiation-based X-ray tomographic microscopy after a classical embedding for electron microscopy. Bar, 50 μm at the surface of the sample only because the image represents a perspective view. From Schittny, Mund *Am. J. Respir. Crit. Care Med.* 2008;177:A317; Schittny *entw. lungung – von der anlage zur adulten lungung*.

Caption for Figure 7-6. (cont.) Comparison between classical and late alveolarization. During classic alveolarization new septa are lifted off immature septa containing a double-layered capillary network (red, A–C) consisting of two sheets of capillaries. In this process one layer (sheet) folds up, while the opposing layer stays constant. During the phase of microvascular maturation the double-capillary network fuses to a single-layered sheet-like capillary network. Now, following the mechanism of late alveolarization, new alveolar septa are still formed by an upfolding of the capillary layer (red, D–F), even if the alveolar surface opposing the upfolding is now missing its capillaries (D). This gap is immediately closed by angiogenesis (yellow arrow in E and F). In both modes of alveolarization, a sheet-like capillary layer folds up (B and E) to form a double-layered capillary network inside the newly formed septum (C and F). Regardless how and when a new septum is formed, it will mature shortly after by a fusion of the double-layered capillary network Schittny, Mund. *Am. J. Respir. Crit. Care Med.* 2008;177:A317

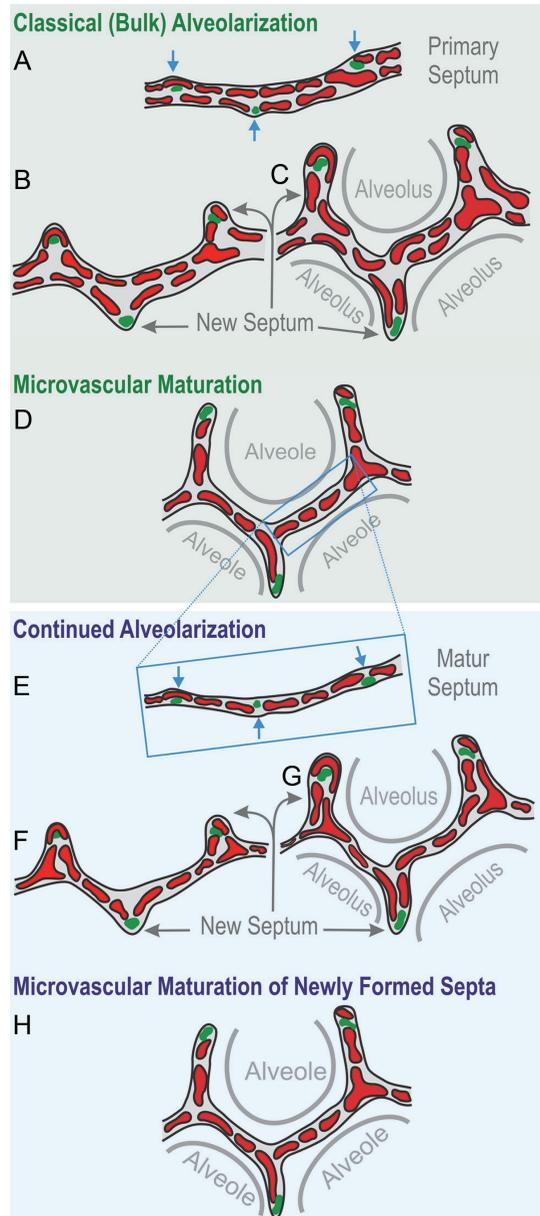


Figure 7-6. Schematic drawing of classical alveolarization. A wall of a sacculi represents a primary septum. It contains a double-layered capillary network, where every layer of the capillary layer appears as a perforated sheet. At sites where new septa (or secondary septa) will be formed (blue arrows, A) smooth muscle cell precursors, elastic fibers and collagen fibrils (green spots) accumulate. Secondary septa are formed by upfolding (blue arrows) of one of the two capillary layers (red, B). As a result, newly formed secondary septa (gray arrows) subdivide preexisting airspaces, and new alveoli are born (C). At this stage, all septa are immature, showing two capillary layers (= primitive septa). During the phase of microvascular maturation the double-layered capillary network fuses to a single-layered capillary network. The resulting mature septa are optimized for highly efficient gas exchange and a low need of material to build them.

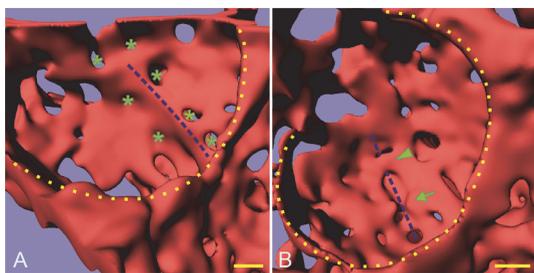


Figure 7-7. Late alveolarization. Vascular Mercox[®] casts of 21-day-old rat lungs are visualized in 3-D after imaging using synchrotron radiation-based X-ray tomographic microscopy. The images show the lumen of the capillaries, which is identical to the inner surface of the capillaries. An upfolding of a single-layered capillary network (blue dashed lines in A) was observed inside the cavity of alveoli. These kinds of upfoldings are indicative for the formation of new septa. The 3-D visualization permitted a look at the backside of the same septum (B). At the site of the folding, a local duplication of the existing capillary network was detected (covering of the blue dashed line in B). Although in this example half of the duplication is already formed (arrowhead), the second half is most likely just forming by sprouting angiogenesis (arrow in B). In addition, (forming) tissue posts inside the capillary network (holes in the vascular cast, green asterisk) are indicative for intussusceptive angiogenesis (67). The growth of the capillary network is necessary to allow the upfolding. The yellow dotted lines label the entrances of the alveoli. Bar, 10 μ m (the magnification varies inside the image due to the foreshortened view). From Schittny, Mund, Stamanoni. *Am. J. Physiol Lung Cell Mol. Physiol.* 2008;294(2):L246–L254.

blood–gas interface (47). Elastin fibers also play an important role in the shaping of the alveoli. At sites where myofibroblasts, elastic fibers, and collagen fibrils are accumulating, new septa are lifted off preexisting ones. These new septa start as a shallow upfolding in a preexisting airspace and rise to mature height during alveolarization (Figures 7-5 to 7-7). This process takes place in the terminal ends of the airways (sacculi) as well as in the alveolar ducts.

Microvascular Maturation (0 Years–3 Years)

Concomitant with classical alveolarization and apoptosis of interstitial cells, the capillary layer surrounding the alveoli develop from a double layer to a single layer, which facilitates efficient and maximal gas exchange. Capillaries fuse to form a single lumen, and there is enhancement of mature area growth along with suppression of immature tissue profile growth (Figure 7-8) (47). These adaptations appear to be regulated by many complex interacting signaling processes, which have, so far, largely been identified by gene expression array profiles (48).

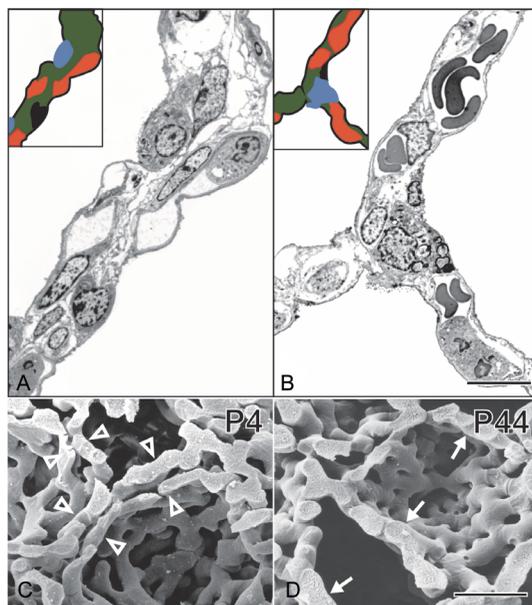


Figure 7-8. Maturation of the alveolar septa/microvascular maturation. In immature septa each alveolar surface is served by its own sheet like capillary layer. The two capillary layers are separated by a central sheet of interstitial tissue. (A) Human lung, postnatal day 26, electron micrography; schematic drawing: red, capillaries; green, interstitial tissue; black, alveolar epithelial cell type I; blue, alveolar epithelial cell type II. Upon maturation the connective tissue layer (green) condenses and thins out so that the two capillary layers merge. In the resulting septum, the connective tissue skeleton of the alveolar septum is interwoven with a now single-layered capillary network. (B) Adult human, lung electron micrography; with similar, schematic drawing). Scanning electron microscopic images of vascular cast (Mercox[®]) of rat lungs show a double-layered capillary network in immature septa at day 4 (C, open arrowhead) and a single-layered capillary network in mature alveolar septa at day 44 (D, arrow). Bar, 25 μ m. From Schittny *entwicklung - von der anlage zur adulten lunge*.

Continued Alveolarization (2 Years–Young Adulthood)

The questions of how much continued alveolarization and to what age alveolarization continues are still not well understood. The concept of classical and continued alveolarization predicts that a remodeling and repair of the lung parenchyma is in principle possible at any age. Experimental models support this hypothesis. (1) In small laboratory animals starvation causes a reduction of the number of alveoli. After refeeding alveolar numbers increased to normal (49, 50). This result is consistent with nutritional emphysema in humans (51). (2) While a treatment with glucocorticoids during classical alveolarization causes a

reduction of alveolarization (52), this phenotype can be rescued by a treatment with retinoic acid during continued alveolarization (53). These treatments appear to act multifunctionally, for example, a neonatal dexamethasone treatment alters peaks of cell proliferation and cell death (54), as well as the expression of cyclin-dependent kinase inhibitors p21 and p27 (55) and of the extracellular matrix proteins tenascin-C and elastin (56).

Unfortunately, such treatment of early premature babies can injure the pulmonary structures and may also induce BPD. High oxygen, mechanical ventilation, antenatal corticosteroids, and surfactant replacement on one hand are necessary for the survival. On the other hand, these treatments affect the lung in the middle of its development (canalicular to saccular stage). At this time point only the foundation for future alveolarization is present, and as a result the further development of the conducting and the gas-exchanging airways may be altered (57). BPD-induced alteration of the conducting airways, however, may be as important a phenomenon as the alteration of the lung parenchyma, as chronic airflow obstruction can persist into adulthood (57).

The recent finding that alveolarization continues at least until young adulthood shows not only the potential of a therapy of structural lung disease in newborn or adults (see earlier), but also the risk that drugs that alter lung development may cause unwanted side effects. For example, treatment of asthma with glucocorticoids or of severe acne with retinoids may alter lung structure in juveniles. Recently it was shown by ^3He diffusion magnetic resonance imaging (MRI) in humans that at least the alveolar structures may be rescued back to normal (58). These methods use inhaled hyperpolarized ^3He gas to provide imaging contrast via MRI and are very sensitive to acinar-airspace dimensions and alveolar number (59,60).

Clinical Aspects of Continued Alveolarization

The mature human lung contains all the materials and genetic instructions necessary to form new alveoli. Slow, peripheral septation has been observed in children and cannot be excluded in adults. Supporting this hypothesis are the

accounts of remarkable lung function recovery after tissue resection (61) and late recovery of lungs thought to be permanently impaired by BPD (41). There is additional evidence to support continued alveolarization from ^3He gas diffusion MRI. Compensatory lung growth has been observed in adult humans and rodents, suggesting that there remains some potential for neoalveolarization in adulthood (62, 63). Human studies of ^3He gas diffusion MRI in healthy pediatric volunteers suggest that both alveolar enlargement and continued neoalveolarization are part of normal aging (64,65).

Conclusion

Although recent studies, particularly those using proteomic and gene array methodologies, have shed new light on many aspects of physiological and pathological lung development, it is clear that we still require additional knowledge on precise structure–function relationships throughout lung development and control mechanisms involved in human lung formation. Neoalveolarization, for example, seems to occur well into childhood, but we do not understand precisely at what age this slows or stops and whether adult neoalveolarization arises from the same control mechanisms. Noninvasive imaging methods, combined with sensitive proteomics, are likely to help complete our understanding of lung structural development in the future. Although X-ray methods will continue to be an extraordinary resource for ex vivo and animal studies, the future of in vivo methods is likely to include more MRI and noninvasive or less-ionizing methods for quantification of alveolar structure and maturation. Hyperpolarized-gas MRI, for example, allow quantification of alveolar size/structure and regional gas exchange properties and are likely to provide new information on alveolarization and vascularization throughout childhood. Low-dose X-ray computed tomography and ultrashort echo time (UTE) MRI will contribute to our understanding of regional structure and function in both the clinic and research. Combined with new proteomic methods that can quantify small changes in metabolic pathways over time, we are poised to make significant translational progress in facilitating alveolarization and vascularization in the coming decades.

References

- 1 Healy F, Hanna BD, Zinman R. Pulmonary complications of congenital heart disease. *Paediatr. Respir. Rev.* 2012;13(1):10–15.
- 2 Keeler AM, Flotte TR. Cell and gene therapy for genetic diseases: inherited disorders affecting the lung and those mimicking sudden infant death syndrome. *Hum. Gen. Ther.* 2012;23(6):548–556.
- 3 Madurga A, Mizíková I, Ruiz-Camp J, et al. Recent advances in late lung development and the pathogenesis of bronchopulmonary dysplasia. *Am J. Physiol. Lung Cell Mol. Physiol.* 2013;305(12):L893–905.
- 4 Herriges M, Morrisey EE. Lung development: orchestrating the generation and regeneration of a complex organ. *Development.* 2014;141(3):502–513.
- 5 Verbanck S, Paiva M. Gas mixing in the airways and airspaces. *Compr. Physiol.* 2011;1(2):809–813.
- 6 Aysola R, de Lange EE, Castro M, et al. Demonstration of the heterogeneous distribution of asthma in the lungs using CT and hyperpolarized helium-3 MRI. *J. Magn. Res. Imaging.* 2010;32(6):1379–1387.
- 7 Merritt TA, Deming DD, Boynton BR. The “new” bronchopulmonary dysplasia: challenges and commentary. *Semin. Fetal Neonatal Med.* 2009;14(6):345–357.
- 8 Gao Y, Raj JU. Regulation of the pulmonary circulation in the fetus and newborn. *Physiol. Rev.* 2010;90(4):1291–1335.
- 9 Elliott FM, Reid LM. Some new facts about the pulmonary artery and its branching pattern. *Clin. Radiol.* 1965;16:193–198.
- 10 Reid LM. Structural remodelling of the pulmonary vasculature by environmental change and disease. In: Wagner W, Weir E, eds. *The Pulmonary Circulation and Gas Exchange*. New York, NY: Futura; 1994:77–110.
- 11 Hislop A, Reid LM. Intrapulmonary arterial development during fetal life-branching pattern and structure. *J. Anat.* 1972;113:35–48.
- 12 Rudolph AM. Aortopulmonary transposition in the fetus: speculation on pathophysiology and therapy. *Pediatr. Res.* 2007;61:375–380.
- 13 Hislop A, Reid LM. Fetal and childhood development of the intrapulmonary veins in man-branching pattern and structure. *Thorax.* 1973;28:313–319.
- 14 Schittny JC, Burri PH. Development and growth of the lung. In: Fishman AP, et al. eds. *Fishman's pulmonary diseases and disorders*. New York, NY: McGraw-Hill; 2008:91–114.
- 15 Cardoso WV, Lu J. Regulation of early lung morphogenesis: questions, facts and controversies. *Development.* 2006;133(9):1611–1624.
- 16 Kovesi T, Rubin S. Long-term complications of congenital esophageal atresia and/or tracheoesophageal fistula. *Chest.* 2004;126(3):915–925.
- 17 Kitaoka H, Burri PH, Weibel ER. Development of the human fetal airway tree: analysis of the numerical density of airway endtips. *Anat. Rec.* 1996;244(2):207–213.
- 18 Sparrow MP, Warwick SP, Mitchell HW. Fetal airway motor tone in prenatal lung development of the pig. *Eur. Resp. J.* 1994;7:1416–1424.
- 19 Koos BJ, Rajae A. Fetal breathing movements and changes at birth. *Adv. Exp. Med. Biol.* 2014;814:89–101.
- 20 Pan J, Copland I, Post M, et al. Mechanical stretch-induced serotonin release from pulmonary neuroendocrine cells: implications for lung development. *Am. J. Resp. Cell Mol. Biol.* 2006;290:L185–193.
- 21 Liu M, Tanswell AK, Post M. Mechanical force-induced signal transduction in lung cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 1999;277:L667–683.
- 22 Scott JE, Yang SY, Stanik E, et al. Influence of strain on [3H]thymidine incorporation, surfactant-related phospholipid synthesis, and cAMP levels in fetal type II alveolar cells. *Am. J. Resp. Cell Mol. Biol.* 1993;8:258–265.
- 23 Harting MT, Lally KP. The Congenital Diaphragmatic Hernia Study Group registry update. *Semin. Fetal Neonatal Med.* 2014;19(6):370–375.
- 24 Bachofen M, Weibel ER. Alterations of the gas exchange apparatus in adult respiratory insufficiency associated with septicemia. *Am. Rev. Respir. Dis.* 1977;116(4):589–615.
- 25 Mercurio AR, Rhodin JA. An electron microscopic study on the type I pneumocyte in the cat: differentiation. *Am. J. Anat.* 1976;146(3):255–271.
- 26 Schittny JC, Paulsson M, Vallan C, et al. Protein cross-linking mediated by tissue transglutaminase correlates with the maturation of extracellular matrices during lung development. *Am. J. Physiol. Lung Cell Mol. Physiol.* 1997;17(3):334–343.
- 27 Willem M, Miosge N, Halfter W, et al. Specific ablation of the nidogen-binding site in the laminin gamma1 chain interferes with kidney and lung development. *Development.* 2002;129(11):2711–2722.
- 28 Burri PH. Lung development and pulmonary angiogenesis. In: Gaultier C, Bourbon J, Post M, eds. *Lung Disease*. New

- York, NY: Oxford University Press; 1999:122–151.
- 29 Howatt WF, Avery ME, Humphreys PW, et al. Factors affecting pulmonary surface properties in the foetal lamb. *Clin. Sci.* 1965;29(2): 239–248.
- 30 Boyden EA. The structure of the pulmonary acinus in a child of 6 years and 8 months. *Am. J. Anat.* 1971;132(3):275–299.
- 31 Schittny JC, Mund SI A re-examination of the maturation of the alveolar septa revealed that microvascular maturation takes place in parallel to alveolarization. *Am. J. Respir. Crit. Care Med.* 2008;177:A317 (ATS poster, manuscript in preparation).
- 32 Rannels SR, Rannels DE. The type-II Pneumocyte as a model of lung cell interaction with the extracellular matrix. *J. Mol. Cell. Cardiol.* 1989;21 (Suppl. 1):151–159.
- 33 Alcorn DG, Adamson TM, Maloney JE, et al. A morphologic and morphometric analysis of fetal lung development in the sheep. *Anat. Rec.* 1981;201(4):655–657.
- 34 Sosenko IR, Frank L. Guinea pig lung development: antioxidant enzymes and premature survival in high O₂. *Am. J. Physiol.* 1987;252(4 pt 2):R693–698.
- 35 Burri PH, Dbaly J, Weibel ER. The postnatal growth of the rat lung. *Anat. Rec.* 1974;178 (4):711–730.
- 36 Amy RW, Bowes D, Burri PH, et al. Postnatal growth of the mouse lung. *J. Anat.* 1977; 124(pt 1):131–151.
- 37 Zeltner TB, Caduff JH, Gehr P, et al. The postnatal development and growth of the human lung. I. Morphometry. *Respir. Physiol.* 1987;67(3): 247–267.
- 38 Makanya AN, Sparrow MP, Warui CN, et al. Morphological analysis of the postnatally developing marsupial lung: The quokka wallaby. *Anat. Rec.* 2001; 262(3):253–265.
- 39 Chien LN, Chiou HY, Wang CW, et al. Oligohydramnios increases the risk of respiratory hospitalization in childhood: a population-based study. *Pediatr. Res.* 2014;75(4): 576–581.
- 40 Hartung EA, Guay-Woodford LM, Autosomal recessive polycystic kidney disease: a hepatorenal fibrocystic disorder with pleiotropic effects. *Pediatrics.* 2014; 134(3):833–845.
- 41 Carraro S, Filippone M, Da Dalt L, et al. Bronchopulmonary dysplasia: the earliest and perhaps the longest lasting obstructive lung disease in humans. *Early Hum. Dev.* 2013;89(Suppl 3): 3–5.
- 42 O'Reilly M, Thébaud B. The promise of stem cells in bronchopulmonary dysplasia. *Semin. Perinatol.* 2013;37(2): 79–84.
- 43 Hofhuis W, Huysman MW, van der Wiel EC, et al. Worsening of V_{max}FRC in infants with chronic lung disease in the first year of life. a more favorable outcome after high-frequency oscillation ventilation. *Am. J. Respir. Crit. Care Med.* 2002;166(12):1539–1543.
- 44 Tschanz SA, Salm LA, Roth-Kleiner M, et al. Rat lungs show a biphasic formation of new alveoli during postnatal development. *J. Appl. Physiol.* 2014. 117(1):89–95.
- 45 Langston C, Kida K, Reed M, et al. Human lung growth in late gestation and in the neonate. *Am. Rev. Respir. Dis.* 1984. 129:607–613.
- 46 Burri PH. Structural aspects of postnatal lung development – alveolar formation and growth. *Biol. Neonate.* 2006;89(4):313–322.
- 47 Schittny JC, Djonov V, Fine A, et al. Programmed cell death contributes to postnatal lung development. *Am. J. Respir. Cell Mol. Biol.* 1998;18(6):786–793.
- 48 Wolff J-C. Molecular mediators of alveolarization. In: *Faculties of Veterinary Medicine and Medicine.* Justus Liebig University Giessen: Giessen; 2010.
- 49 Kalenga M, Tschanz SA, Burri PH. Protein deficiency and the growing rat lung. II. Morphometric analysis and morphology. *Pediatr. Res.* 1995;37(6):789–795.
- 50 Kalenga M, Tschanz SA, Burri PH. Protein deficiency and the growing rat lung. I. Nutritional findings and related lung volumes. *Pediatr. Res.* 1995; 37(6):783–788.
- 51 Coxson HO, Chan IH, Mayo JR, et al. Early emphysema in patients with anorexia nervosa. *Am. J. Respir. Crit. Care Med.* 2004;170(7):748–752.
- 52 Massaro D, Massaro GD. Dexamethasone accelerates postnatal alveolar wall thinning and alters wall composition. *Am. J. Physiol.* 1986;251: R218–R224.
- 53 Massaro GD, Massaro D. Retinoic acid treatment partially rescues failed septation in rats and in mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2000;278(5):L955–L960.
- 54 Luyet C, Burri PH, Schittny, JC. Suppression of cell proliferation and programmed cell death by dexamethasone during postnatal lung development. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2002;282(3):L477–L483.

- 55 Corroyer S, Schittny JC, Djonov V, et al. Impairment of rat postnatal lung alveolar development by glucocorticoids: involvement of the p21CIP1 and p27KIP1 cyclin-dependent kinase inhibitors. *Pediatr. Res.* 2002; 51(2):169–176.
- 56 Roth-Kleiner M, Berger TM, Gremlich S, et al. Neonatal steroids induce a down-regulation of tenascin-C and elastin and cause a deceleration of the first phase and an acceleration of the second phase of lung alveolarization. *Histochem. Cell Biol.* 2014;141(1):75–84.
- 57 El Mazloum D, Moschino L, Bozzetto S, et al. Chronic lung disease of prematurity: long-term respiratory outcome. *Neonatology.* 2014;105(4):352–356.
- 58 Narayanan M, Beardsmore CS, Owers-Bradley J, et al. Catch-up alveolarization in ex-preterm children: evidence from (3)He magnetic resonance. *Am. J. Respir. Crit. Care Med.* 2013;187(10):1104–1109.
- 59 Yablonskiy DA, Sukstanskii AL, Woods JC, et al. Quantification of lung microstructure with hyperpolarized 3He diffusion MRI. *J. Appl. Physiol.* 2009;107(4):1258–1265.
- 60 Woods JC, Choong CK, Yablonskiy DA, et al. Hyperpolarized 3He diffusion MRI and histology in pulmonary emphysema. *Magn. Reson. Med.* 2006;56(6):1293–1300.
- 61 Bates DV, Macklem PT, Christie RV. *Respiratory Function in Disease; An Introduction to the Integrated Study of the Lung.* 2nd ed. Philadelphia, PA: Saunders; 2004.
- 62 Butler JP, Loring SH, Patz S, et al. Evidence for adult lung growth in humans. *N. Engl. J. Med.* 2012;367(3):244–247.
- 63 Wang W, Nguyen NM, Guo J, et al. Longitudinal, noninvasive monitoring of compensatory lung growth in mice after pneumonectomy via 3He and 1H magnetic resonance imaging. *Am. J. Resp. Cell Mol. Biol.* 2013;49(5):697–703.
- 64 Altes TA, Mata J, De Lange EE, et al. Assessment of lung development using hyperpolarized helium-3 diffusion MR imaging. *J. Magn. Res. Imaging.* 2006;24(6):1277–1283.
- 65 Narayanan M, Owers-Bradley J, Beardsmore CS, et al. Alveolarization continues during childhood and adolescence; new evidence from helium-3 magnetic resonance. *Am. J. Resp. Crit. Care Med.* 2012;185(2):186–191.
- 66 Schittny JC. Strukturelle entwicklung – von der anlage zur adulten lunge. In: von Mutius E, et al., eds. *Pädiatrische Pneumologie.* Berlin: Springer; 2014.
- 67 Caduff JH, Fischer LC, Burri PH. Scanning electron microscopic study of the developing microvasculature in the postnatal rat lung. *Anat. Rec.* 1986;216:154–164.
- 68 Burri PH, Weibel ER. Ultrastructure and morphometry of the developing lung. In: Hodson WA, ed. *Development of the Lung.* New York and Basel: Marcel Dekker;1977;215–268.
- 69 Schittny JC, Mund SI, Stampanoni M. Evidence and structural mechanism for late lung alveolarization. *Am. J. Physiol Lung Cell Mol. Physiol.* 2008;294(2):L246–L254.
- 70 Miettinen PJ, Warburton D, Bu D, et al. Impaired lung branching morphogenesis in the absence of functional EGF receptor. *Dev. Biol.* 1997;186(2):224–236.
- 71 Bryden MM, Evans H, Binns W. Embryology of the sheep. 3. The respiratory system, mesenteries and celom in the fourteen to thirty-four day embryo. *Anat. Rec.* 1973;175(4):725–735.
- 72 Ten Have-Opbroek AAW. Lung development in the mouse embryo. *Exp. Lung Res.* 1991;17:111–130.

Surfactant During Lung Development

Timothy E. Weaver, Lawrence M. Nogee, and Alan H. Jobe

Abstract

Surfactant is a unique lipid and protein substance made by type II cells in the lung that provides inflation stability, decreases the work of breathing, and has components with innate host defense properties. Surfactant is normally synthesized and secreted into the airspaces of the fetal lung as term approaches, but it can be induced earlier in gestation by fetal exposures to corticosteroids or inflammation. The surfactant deficiency associated with preterm birth can cause severe respiratory failure termed respiratory distress syndrome (RDS), a frequently lethal disease before the availability of clinical surfactants to treat infants. Surfactant components each have complex metabolic characteristics in the premature and mature lung. Term infants can have severe surfactant dysfunction because of rare mutations that disrupt surfactant protein synthesis or processing. The research resulting in the understanding of surfactant metabolism and function and subsequent treatment of RDS is a highlight of progress from science to cure strategies in pulmonary medicine.

Keywords:

Surfactant lipids, surfactant proteins, pressure-volume curve, oxygenation, respiratory distress syndrome, antenatal corticosteroids, induced lung maturation, surfactant protein-B deficiency, ABCA3 deficiency

An obligate requirement for the survival of the air breathing newborn is the development and maturation of the gas exchange potential of the lungs. Lung structural development provides the matrix on which a unique substance – pulmonary surfactant – can stabilize a very large surface area of epithelium juxtaposed to an extensive capillary bed to permit gas exchange. The essential properties of surfactant are its biophysical characteristics to form a stable surface film with the capacity to dynamically regulate surface tensions of the air–fluid interface to very low values. The fetal lung develops the cellular machinery from midgestation to ensure adequate surfactant function at term. This Chapter is the story of the cell biology that supports the synthesis, storage, and secretion of surfactant; its function; and genetic abnormalities that disrupt surfactant metabolism. The focus is on normal development during late gestation, the normal transition to air breathing, and abnormalities that disrupt surfactant function in the newborn period. The discussion includes quite old information about surfactant and recent cell biology and genetics that reveal the complexities of how surfactant function is maintained in the airspaces.

A Brief History

A brief history of the discovery of surfactant and its importance to lung development documents the remarkable progress in the field since Pattle and Clements independently reported a substance that lowered surface tensions of an air–water interface in edema fluid and lung extracts (1). Avery and Mead (2) in 1959 demonstrated that premature infants who died of respiratory distress syndrome (RDS) had less surfactant in saline extracts of their lungs than infants who died of other causes. Subsequently, measurements of surfactant components that were secreted from the fetal lung into amniotic fluid were developed into clinical tests for fetal lung maturation. Examples were measuring biophysical properties of surfactant in amniotic fluid (3) with the shake test and biochemical measurements of the lecithin to sphingomyelin ratio (4), and percent phosphatidylglycerol in amniotic fluid in 1976 (5). Concurrently, Liggins and Howie (6) reported in 1972 that maternal treatments with corticosteroids matured the fetal lung and decreased RDS and death in preterm infants, primarily by increasing surfactant in the fetal lung. Continuous positive airway pressure (CPAP) was the first effective therapy for RDS that

stabilized surfactant function by helping the infant to recruit and maintain a functional residual capacity (7). Despite a lack of understanding of the importance of surfactant proteins to surfactant function, Enhorning and Robertson (8) in the 1970s demonstrated in animal models that surfactant treatments given via the trachea could strikingly improve the lung function of preterm animals. The successful treatment of infants with surfactant was first reported in 1980 by Fujiwara and colleagues (9). Simultaneously, numerous research groups were describing the developmental biology of surfactant and established the essential contributions of the surfactant-specific proteins to this remarkable substance (10). Surfactant treatments were approved by the FDA for the treatment of RDS in 1990 and are now standard of care. Surfactant treatment, together with improvements in the general care of the preterm infant, is the major success story in neonatology. Today infants that are not otherwise compromised should no longer die of RDS. The biology behind this clinical success is surprisingly complex.

Surfactant Lipids

Surfactants used for compositional analyses are recovered from lungs by bronchoalveolar lavage, and intracellular surfactant is isolated from subcellular fractions. Surfactant from all mammalian species is about 70% to 80% phospholipids, about 8% protein, and about 8% neutral lipids, primarily cholesterol and triglyceride (Figure 8-1). About 50% of the phospholipid species are saturated phosphatidylcholines, meaning that the fatty acids

esterified to 1 and 2 positions of the glycerol-phosphocholine backbone are predominantly 16-carbon palmitic acids. Some of the saturated phosphatidylcholine has 14-carbon myristic acid in the 2 position. Most other phosphatidylcholine species in surfactant have a fatty acid with one double bond in the 2 position of the molecule. Saturated phosphatidylcholine is the principal surface-active lipid component of surfactant and can be used as a relatively specific probe for surfactant metabolism. The acidic phospholipid, phosphatidylglycerol, is unique to surfactant and is present in small amounts that vary between 4% and 15% of the phospholipids in different species (5). Surfactant phospholipids from the immature fetus or newborn contain relatively large amounts of phosphatidylinositol, which then decrease as phosphatidylglycerol appears with fetal lung maturity (Table 8-1).

Surfactant Proteins

Four proteins that are relatively specific for surfactant are recovered with bronchoalveolar lavage fluid. These proteins have been widely referred to as Surfactant Protein-A (SP-A), SP-B, SP-C, and SP-D. The human genes encoding these proteins are *SFTPA*, *SFTPB*, *SFTPC*, and *SFTPD*, respectively. Insights into the function of these proteins have come primarily from gene-targeted mice and from in vitro assays of surface properties. The four proteins associate with surfactant lipids to different degrees: SP-A and SP-D are primarily water-soluble collectins (collagen-lectin proteins), whereas SP-B and

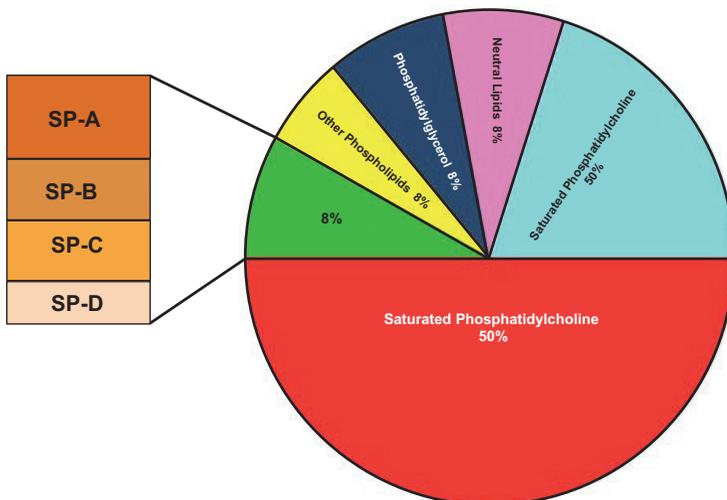


Figure 8-1. Composition of surfactant from bronchoalveolar lavage. Representative values for surfactant from adult mammalian species. The primary surface active components are the saturated phosphatidylcholine and the hydrophobic proteins SP-B and SP-C.

SP-C are remarkably hydrophobic small peptides that are exclusively associated with surfactant phospholipids (11).

Surfactant Protein-A

SP-A monomer is a water-soluble 24-kDa protein that is heavily glycosylated to yield a protein of about 36 kDa. SP-A monomers contain a collagenous domain that assembles into a collagen-like triple helix to form a trimer; six trimeric subunits associate to form a multimeric protein with a molecular mass of about 650 kDa (12)(Figure 8-2).

Table 8-1. Changes in surfactant composition and function with lung maturation.

Surfactant Components	Change
Saturated Phosphatidylcholine	Increases in ratio of saturated to total phosphatidylcholine
Phosphatidylinositol	Decreases
Phosphatidylglycerol	Increases
Surfactant Proteins	Increase
Surfactant Function	
Ability to lower surface tension	Improves
Sensitivity to inactivation	Decreases

SP-A associates primarily with saturated phosphatidylcholine and is associated with tubular myelin, a unique square lattice membrane structure that may be involved in host defense. Surfactant from mice that completely lack SP-A does not form tubular myelin, but the mice have normal lung function (13). The absence of SP-A in mice also does not disrupt the secretion, clearance, or catabolism of surfactant lipids.

The major function of SP-A is as an innate host defense protein and regulator of inflammation in the lung (14). SP-A binds to gram-positive and gram-negative bacteria, viruses, and fungi primarily through its carbohydrate recognition domain (CRD). SP-A facilitates phagocytosis of pathogens by macrophages and may directly kill some microbes via membrane permeabilization. SP-A can inhibit inflammation induced by lipopolysaccharide (LPS), peptidoglycan, or zymosan by binding to cell surface receptors (e.g. CD14 and TLR2) for these pathogen-derived molecules. Binding of SP-A to SIRP α also inhibits pro-inflammatory signaling. SP-A can also evoke a context-dependent pro-inflammatory response by binding to rough LPS (via the CRD) and CD14 (via the neck region) or to calreticulin/CD91 on the surface of macrophages via the collagen-like domain. SP-A levels are low in surfactant from preterm lungs and increase as the type II cell numbers increase and mature. The fetal lung increases expression of SP-A in response to

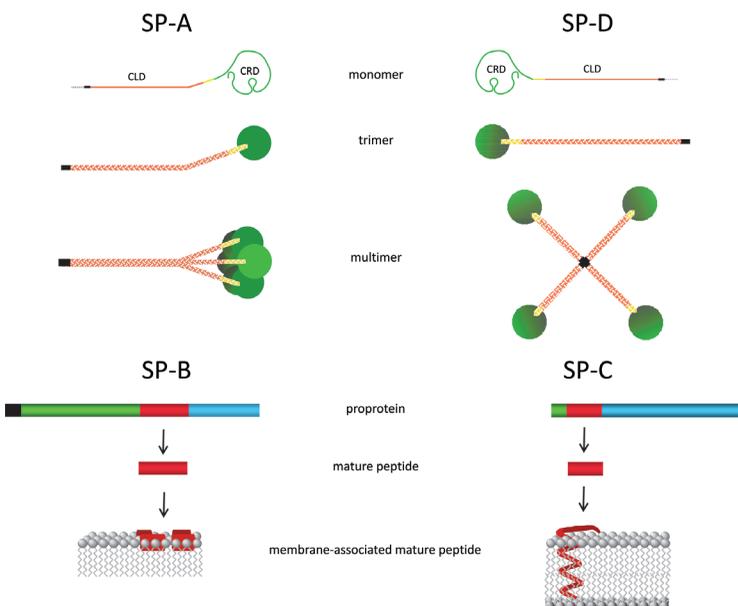


Figure 8-2. Surfactant Protein Structures SP-A and SP-D are collectins that each contain a collagen-like domain (CLD) and a carbohydrate recognition domain (CRD). The CLD facilitates formation of an SP-A octadecamer and an SP-D dodecamer. The dashed line represents the signal peptide, the black line is the NH₂-terminal domain, and the yellow line is the neck region. SP-B and SP-C are synthesized as propeptides that are proteolytically processed to very hydrophobic mature peptides that associate with surfactant membranes: Mature SP-B forms amphipathic helices that associate with the surface of the membrane, whereas mature SP-C forms a metastable α helix that spans the membrane bilayer. Black represents the signal peptide, green is the NH₂-terminal propeptide domain, red is the mature peptide, and blue is the COOH-terminal peptide domain.

corticosteroid or chorioamnionitis in animal models (15).

SP-D

SP-D is a collectin that shares structure and innate host defense functions with SP-A (12). The 43 kDa monomer also forms trimers through its collagen-like domain. Twelve monomers associate to form a multimeric protein composed of four trimeric subunits. SP-D expression in the lung is restricted primarily to type II epithelial and Clara cells, but it is widely expressed by other epithelial cells in the body. SP-D is present in lower amounts in bronchoalveolar lavage than SP-A and is primarily recovered in the water-soluble fraction. As for SP-A, SP-D binds to pathogen-associated molecular patterns (PAMPs) through its CRD; facilitates uptake of bacteria, viruses, and fungal pathogens by macrophages; and may directly kill some pathogens by membrane permeabilization (11). Although SP-D does not appear to interact with intracellular surfactant synthesis, storage and secretion pathways, it does bind to phosphatidylinositol. Absence of SP-D in mouse models is associated with altered surfactant metabolism and a greatly increased alveolar surfactant pool size. SP-D deficient mice also have progressive emphysema related to increased oxidant stress associated with altered macrophage function (16). Addition of SP-D to surfactant used to treat preterm sheep decreases ventilator-mediated inflammation (17). SP-D expression is increased by antenatal corticosteroids and by fetal exposure to inflammation in animal models. Exposure to both corticosteroids and inflammation further increases expression (15).

SP-B

SP-B is a hydrophobic peptide of 79 amino acids that is cleaved from a precursor protein of approximately 40 kDa prior to association with surfactant lipids in type II epithelial cells (18). Surfactant lipids and proteins (SP-B and SP-C) are stored as concentric bilayer membranes (lamellae) in specialized secretory organelles (lamellar bodies) prior to secretion into the alveolar airspaces. The ability of SP-B to both lyse and fuse bilayer membranes is critical for organization of surfactant membranes within lamellar bodies and for transition of newly secreted membranes to a surface active film at the alveolar air-liquid interface. SP-B is absolutely essential for

lung function as knockout mice have normal lung structure at birth, but cannot initiate air breathing because of a lack of functional surfactant (19). In the absence of SP-B, type II cells fail to form lamellar bodies or process SP-C. Thus, SP-B is required for both the synthesis and assembly of surfactant in type II cells as well as for its function in the alveolar compartment. SP-B comprises only about 2% of surfactant, and when this amount is decreased in adult mice, the animals develop lung injury and respiratory failure (20). Production of large amounts of appropriately folded active synthetic SP-B peptide has not been achieved, but shorter peptide mimetics have been developed for use in synthetic surfactant preparations with some success. As with the other surfactant components, SP-B expression and amounts increase with advancing gestational age and increase with antenatal corticosteroid or fetal exposure to inflammation (15).

SP-C

SP-C is the other hydrophobic protein in surfactant that comprises up to 4% of surfactant. Organic solvent extracts of surfactant recover both SP-B and SP-C with lipids. Like SP-B, SP-C is synthesized as a precursor protein that is processed to an extremely hydrophobic 35 amino acid (4 kDa) protein that associates with lipids in lamellar bodies (18). The mRNA for SP-C appears in the epithelium of the developing airways from early gestation. With advancing lung maturation, SP-C expression is restricted to type II epithelial cells. The amino acid sequence and cellular localization of SP-C are remarkably similar across mammalian species consistent with an important, but poorly understood, function. Although mice that lack SP-C have normal lung structure and surfactant function at birth (21), these animals develop strain-dependent, progressive interstitial lung disease (ILD) and emphysema as they age. Although not critical for survival, SP-C contributes to surface film stability by interacting with SP-B and lipids, thus contributing to the unique surface properties of surfactant. SP-C expression increases in the fetal lung as type II cell numbers and maturation increase.

Metabolism of Surfactant Synthesis and Secretion

Type II cells and macrophages are the cell types responsible for the major pathways involved in

surfactant metabolism (Figure 8-3). The synthesis and secretion of surfactant is a complex sequence of biochemical synthetic events that results in the release by exocytosis of lamellar bodies to the alveolus (18). The basic pathways for the synthesis of phospholipids are common to all mammalian cells. Specific enzymes use glucose, phosphate, and fatty acids as substrates for phospholipid synthesis. Surfactant lipids are synthesized primarily in the endoplasmic reticulum (ER) and transported to lamellar bodies, the storage organelle for surfactant. An important component of the pathway involved in the intraorganelle transfer of newly synthesized

surfactant lipids is ABCA3, a phospholipid import protein located in the limiting membrane of the lamellar body. The orientation of ABCA3 in the membrane and the absence of typical lamellar bodies in ABCA3 null mice strongly suggest that surfactant phospholipids are transported directly from the ER to the lamellar body, via as yet unidentified lipid transfer proteins located in the cytosol of type II cells. Candidate phosphatidylcholine transfer proteins and other potential lipid importers have been identified in the lamellar body proteome, but the involvement of these proteins in surfactant biosynthesis has yet to be evaluated (22). In the adult lung,

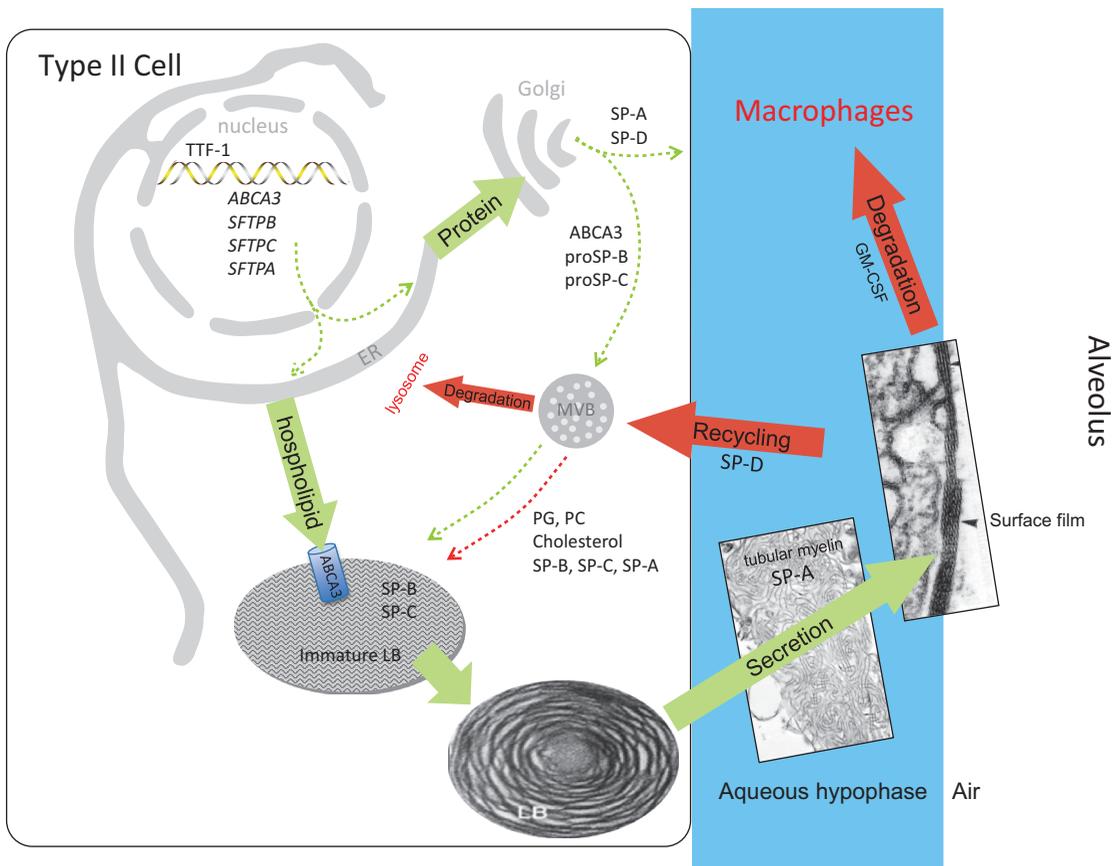


Figure 8-3. Surfactant metabolism biosynthesis of surfactant likely involves distinct pathways for surfactant proteins and lipids (green arrows). Newly synthesized SP-B and SP-C are trafficked from the ER to lamellar bodies (LB) via the Golgi and multivesicular body (MVB), whereas SP-A and SP-D are constitutively secreted by the type II epithelial cells (dashed green arrows). In contrast, surfactant phospholipids are likely directly transported from the ER to specified lipid importers (ABCA3) in the lamellar body-limiting membrane (solid green arrow). Surfactant proteins and lipids are assembled into bilayer membranes that are secreted into the alveolar airspace, where they form a surface film at the air-liquid interface. Cyclical expansion and compression of the bioactive film results in incorporation and loss (red arrows) of lipids/proteins from the multilayered surface film. Surfactant components removed from the film are degraded in alveolar macrophages or are taken up by the type II epithelial cell for recycling (dashed red arrow) or degradation in the lysosome (solid red arrow). The MVB plays a key role in the integration of surfactant synthesis, recycling, and degradation pathways. Green arrows indicate biosynthetic pathways; red arrows indicate degradation and recycling pathways.

surfactant secretion is presumed to be regulated by the microenvironment of the alveolus. Secretion can be stimulated by beta-agonists, purinergic agonists, or hyperventilation. In the fetus, surfactant is released into fetal lung fluid in increasing amounts as gestation advances. Surfactant is secreted into fetal lung fluid and is carried out of the lung with fetal breathing and subsequently swallowed or diluted in amniotic fluid. Thus, amniotic fluid can be used to evaluate the development of the surfactant system.

Alveolar Cycle of Surfactant

After exocytosis of lamellar bodies by type II cells, surfactant proceeds through a series of form transitions that define its metabolic and functional roles. The exocytosed lamellar bodies “unravel” into the elegant “tubular myelin” structure with SP-A at the corners of the lattice (Figure 8-3). Tubular myelin requires at least SP-A, SP-B, and the phospholipids for its unique structure. Tubular myelin and other loose, less-structured surfactant lipoprotein membrane arrays are the pool in the fluid hypophase that supplies surfactant for the surface film within the alveolus and small airways. Compression of this film squeezes out unsaturated lipids and some protein components of surfactant with concentration of saturated phosphatidylcholine in the surface film. New surfactant enters the surface film from tubular myelin and the loose lipid arrays, and “used” surfactant leaves as small vesicles, which then are cleared from the airspaces. The major differences in composition between the surface-active surfactant structures and the small vesicular forms are that the small forms contain SP-D but little SP-A, SP-B, or SP-C (23). The small vesicles of used surfactant are much less surface active and seem to be the major catabolic form of the lipids that are taken up by type II cells and by alveolar macrophages for recycling or catabolism.

Alveolar macrophages are the sentinel immune cells of the lung. These cells are in the airspaces directly in contact with the alveolar hypophase and surfactant. Fetal monocytes seed the developing lung and undergo granulocyte-macrophage colony-stimulating factor (GM-CSF) mediated differentiation to alveolar macrophages shortly after birth (24). Once differentiated, alveolar macrophages have a relatively long life span under normal conditions. Important functions of alveolar

macrophages include immune surveillance, phagocytosis of invading microorganisms, antigen presentation, interactions with adaptive immune cells, and surfactant homeostasis. Fetuses have very few alveolar macrophages. In mice, primitive macrophages can be detected in the lung interstitium from early gestation, while in other species including nonhuman primates and sheep, few macrophages are found in the fetal lung (25). Fetal exposure to inflammation and lung injury can mature lung monocytes into macrophages and stimulate their migration into the fetal alveolar spaces (26).

The number and maturity of type II epithelial cells increase in the fetal human after about 20 weeks gestation. Immature type II cells with large intracellular lakes of glycogen mature with the disappearance of glycogen and the appearance of more and larger lamellar bodies. Just preceding and following birth, lamellar bodies are secreted to yield an alveolar pool that is primarily lamellar bodies and tubular myelin (27). This surfactant then begins to function with aeration of the lung. As the newborn goes through neonatal transition, the percentage of surface-active forms falls, and the small vesicular forms increase. At equilibrium approximately 50% of the surfactant in the airspaces is in a surface-active form, and 50% is in the inactive vesicular form. Conversion from surface active to inactive surfactant forms occurs more rapidly in the preterm, presumably because there is a lower pool size and less-effective surfactant with lower amounts of the surfactant proteins. Pulmonary edema can further accelerate the process, with the net result being a depletion of the surface-active fraction of surfactant despite normal surfactant pool sizes.

Surfactant Turnover

The Adult Lung

In the adult human, the amount of surfactant in the airspaces recoverable by bronchoalveolar lavage is about 5 mg/kg body weight (28). There are no reliable measurements of turnover of the surfactant components in the normal adult human. In animal models (primarily the rabbit) the airspace pool size of surfactant is about 16.5 mg/kg, and the lamellar body pool size is about 24 mg/kg (Figure 8-4). The kinetics of secretion were measured with radiolabeled precursors of surfactant components. Clearance kinetics were

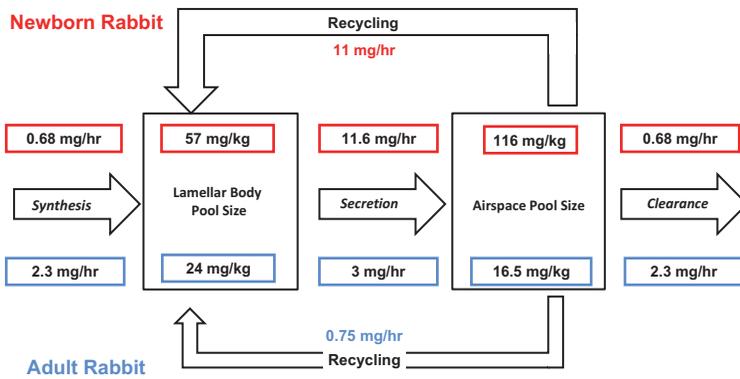


Figure 8-4. Estimates of pool sizes and flux rates of surfactant in adult rabbits (blue) and in 3-day-old newborn rabbits (red). All values were measured as saturated phosphatidylcholine and have been converted to mg/kg total surfactant (1 μ mol saturated phosphatidylcholine is equivalent to about 1.5 mg total surfactant). The 3-day rabbits weighed about 65 g. The values are from modeling studies using radiolabeled precursors of saturated phosphatidylcholine by Jacobs and colleagues(29–31).

measured using radiolabeled surfactant components given into the airspaces (29–31). The lag from synthesis to peak accumulation of surfactant lipids in the airspace is about 15 hours. Once secreted, the half-life of the subsequent linear loss from airspace is about 10 hours. The curves have been modeled to demonstrate that the surfactant lipids SP-B and SP-C from the airspace appear in lamellar bodies for resecretion with an efficiency of about 25% (31). The residual surfactant lost from the airspaces is catabolized by alveolar macrophages. Some surfactant moves from the alveoli into small airways, but large amounts of surfactant are not lost up the airways. This elaborate steady-state control of surfactant pool sizes and therefore function in the adult lung can be disrupted by injury to type II cells and by a block in surfactant catabolism by alveolar macrophages from a lack of GM-CSF signaling resulting in alveolar proteinosis (32).

The Newborn Lung

Surfactant pool sizes and turnover times are quite different in preterm and term newborns (Figure 8-4). Following the observation of Avery and Mead that saline extracts of the lungs of infants with RDS had high minimum surface tensions (2), decreased alveolar and tissue surfactant pools were demonstrated in preterm animals. Increasing surfactant pool sizes correlated with improving compliances, although other factors such as structural maturation also influence lung function (33). Premature infants who died with RDS without mechanical ventilation in the 1960s had surfactant pool sizes that were less than about 5 mg/kg of body weight. Preterm lambs with RDS can be managed with respiratory support if their surfactant pool sizes exceed about 4 mg/kg (34). Of note, the quantity of

surfactant recovered from the airspaces of infants with RDS is about the same as the amount of surfactant found in the alveoli of healthy adult animals or humans (28). Nevertheless, much less surfactant is recovered from preterms than healthy term animals that have surfactant pool sizes of about 100 mg/kg of body weight (35). The large amount of surfactant in amniotic fluid in the human at term indirectly indicates that the term human fetal lung also has large pool sizes. The fetal lung at term has more surfactant in lamellar bodies and the fetal lung fluid than at any time during life as a biological adaptation to ensure neonatal transition to air breathing. Surfactant function is concentration dependent, and the high amounts of surfactant in the fetal lung facilitate film formation and the establishment of surface forces that promote fluid clearance. The high surfactant pool sizes present at term birth progressively decrease to normal values for the adult animal by about 7 days in the rabbit. There is no information for the time of transition of surfactant pool sizes in the human.

Preterm infants that develop RDS often have a “honeymoon” period of relatively normal lung function that can last for several hours prior to progressive respiratory failure. Their small surfactant pool sizes are sufficient for initial transition to air breathing, but that surfactant has decreased function relative to surfactant from mature infants and is more sensitive to inactivation. Part of the problem may be that following preterm birth the surfactant stores in the type II cells are depleted, limiting the potential to quickly increase alveolar surfactant pool sizes. The increase in the pool size of alveolar surfactant after preterm birth has been measured in ventilated preterm monkeys recovering from RDS (36). The surfactant pool size increased toward the

100 mg/kg value measured in term monkeys within 3 to 4 days. Similarly, the concentration of saturated phosphatidylcholine in airway aspirates from infants with RDS increased over a 4- to 5-day period to become comparable to values for normal or surfactant-treated infants (37). This slow increase in pool size is consistent with a clinical course of RDS of about 5 days without surfactant treatment.

The only surfactant pool that can be sampled from the newborn human is that which can be recovered by a tracheal aspirate procedure, which severely limits the analyses that are possible. In term newborn rabbits turnover studies using radiolabeled precursors demonstrated that synthesis and clearance was about 10-fold less in the newborns than the adults (29) (Figure 8-4), but the high lamellar body and airspace pools were maintained by a recycling rate for saturated phosphatidylcholine of >90%. The phospholipids were recycled as intact molecules without degradation and resynthesis.

Metabolic measurements have been made in preterm infants with the material recovered in tracheal aspirates using stable isotopes given by intravascular injection to measure endogenous synthesis and secretion (38,39). Following the intravascular administration of labeled glucose or palmitic acid precursors, rapid incorporation into surfactant phosphatidylcholine is followed by long time delays for the movement of surfactant components from the ER to lamellar bodies for secretion. In infants with RDS, glucose-labeled phosphatidylcholine was detected in the airway samples after about 20 hours, and peak enrichment of the stable isotope in the airspaces occurred at about 70 hrs (38). Therefore, delays between synthesis and secretion and the interval to peak airway accumulation of endogenously synthesized surfactant lipid is very long in the preterm human.

The slow secretion and alveolar accumulation of surfactant are balanced in the term and preterm lung by slow catabolism and clearance. Trace amounts of radiolabeled surfactant phospholipid mixed with treatment doses of surfactant and given into the airspaces to infants with RDS had half-life values of several days (39). Surfactant phosphatidylcholine labeled with intravascular glucose had a half-life after peak secretion of about 80 hours for infants with RDS on conventional or high-frequency oscillation (40). Fractional synthetic rates also were similar at 4.5%

per day. The half-life of surfactant phosphatidylcholine was 62 hours in term infants, but infants with pneumonia had much shorter half-life for surfactant phosphatidylcholine of about 30 hours.

Less is known about the metabolism of the surfactant proteins in the preterm lung. In animal models, SP-A, SP-B, and SP-C seem to have alveolar clearance kinetics that are similar to saturated phosphatidylcholine. These proteins also seem to be recycled to some degree from the airspace back into lamellar bodies for resecretion with surfactant (35). In the ventilated preterm human, SP-B was labeled using leucine and its secretion and clearance from airway samples measured (41). The estimate of catabolic rate was about one alveolar pool equivalent per day with a half-life of about 16 hours. The relationships between the metabolism of surfactant proteins and lipids remain to be studied in the human and during development. The presumed function of the recycling pathways is to reassemble the components to regenerate biophysically active surfactant.

Surfactant Physiology in the Lung

The gas exchange surfaces of alveoli are complex polygonal shapes that are interdependent in that their structures are determined by the shapes and elasticity of neighboring alveoli and airways. The forces acting on the pulmonary microstructure are chest wall elasticity, lung tissue elasticity, and surface tensions of the air-fluid interfaces in the small airways and alveoli. At static equilibrium, a surfactant film will reduce surface tension from the value of 72 mN/m for water to about 23 mN/m. Surface area compressions of about 25% will decrease surface tension to close to 0. In the normal lung, the surface area changes of the alveolar surface with tidal breathing are not large. Nevertheless, surface forces balance the inflation of the lung across the approximately 500 million alveoli and their connecting small airways in all lung regions. As noted earlier, the problem for the term fetus transitioning to air breathing is to move fluid from the airspaces while establishing alveolar expansion. Surfactant is critical for this process together with active Na⁺ clearance. The infant makes high negative-pressure breaths that move fluid down the airway tree. The surface film is quickly formed on the expanding air-fluid interface to retain air as a

functional residual capacity. The fluid moves into the lung interstitium to be cleared from the lung over hours. Surfactant also facilitates both clearance and the maintenance of patency of the small airways.

The effects of surfactant on the preterm surfactant-deficient lung are demonstrated by pressure-volume relationships during quasi-static inflation and deflation. The preterm surfactant-deficient lung does not begin to inflate until pressures exceed 20 cm H₂O (42) (Figure 8-5A). Multiple airways connect to distal saccules/alveoli with different radii. The pressure needed to open a lung unit (airway plus distal structures) is related to the radius of curvature and surface tension of the meniscus of fluid in the airspace leading to each lung unit. The units with larger radii and lower surface tensions will “pop” open first because, with partial expansion, the radius increases and the forces needed to finish opening the unit decrease. The movement of an air–fluid interface with high surface tensions in the airways causes very high shear forces that can disrupt the airway epithelium (43). With surfactant treatment, the fluid menisci in the airways have lower surface tensions that decrease the opening pressure from about 25 to 15 cm H₂O in this example. The subsequent inflation is more

uniform as more units open at lower pressures, resulting in less epithelial injury and less overdistention of the open units.

A particularly important effect of surfactant on the surfactant-deficient lung is the increase in maximal volume at maximal pressure. In this example, maximal volume at 35 cm H₂O is increased about 2.5-fold with surfactant treatment. Pressures above 35 cm H₂O in control lungs result in lung rupture with little further volume accumulation. The opening pressures of many distal lung units in the surfactant-deficient lung exceed 35 cm H₂O, and an attempt to inflate the lung to full volume with higher pressure will rupture the preterm lung. This volume increase with surfactant treatment improves gas exchange because it is primarily distal lung gas volume. Another important effect of surfactant is lung stabilization on deflation. The surfactant-deficient lung collapses at low transpulmonary pressures, whereas the surfactant-treated lung retains about 40% of the gas volume on deflation to 5 cm H₂O, which is the static equivalent of functional residual capacity.

Surfactant lipid composition and the surfactant proteins SP-B and SP-C strikingly change the behavior of surfactant in the airspaces. For example, natural sheep surfactant can be

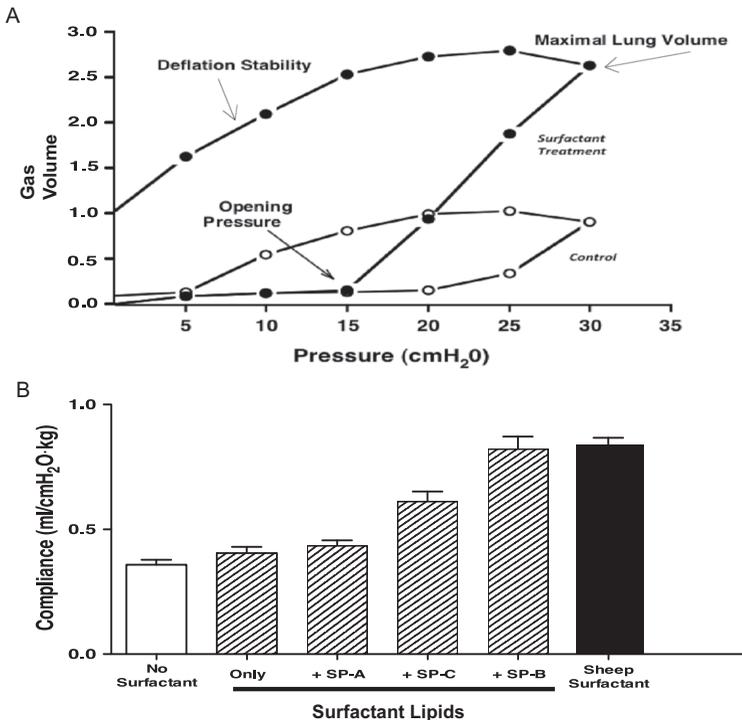


Figure 8-5. Surfactant effects on the preterm lung. (A) Surfactant treatment of the preterm rabbit lung greatly increases lung gas volumes by the combined effects of decreasing opening pressure, increasing maximal lung volume, and increasing deflation stability. Lung gas volumes have been normalized to 1 for the maximal volume at 30 cmH₂O pressure for the control lung. Data derived from (42). (B) Natural sheep surfactant was fractionated into a lipid-only fraction and each of the sheep surfactant proteins. Surfactants were reconstructed using lipids only and lipids plus each of the surfactant proteins. Preterm rabbits were treated with the surfactants and ventilated with similar tidal volumes, and compliances were measured. Sheep surfactant greatly increased compliance relative to the control value in untreated rabbits. Data derived from Rider, Ikegami, Whitset, Hull, Absalom, Jobe. *Am Rev Respir Dis.* 1993;147:669–676.

fractionated into its lipid components and SP-A, S-B, and SP-C for reconstruction experiments to demonstrate physiologic responses of each component in the ventilated preterm rabbit lung (44). In the example in Figure 8-5B, treatment of the surfactant-deficient preterm lung with the surfactant lipids does not improve lung compliance relative to untreated rabbits. Addition of SP-A has minimal effects, while addition of only SP-C improves the pressure volume relationships substantially. However, SP-B plus the surfactant lipids is equivalent to the natural surfactant. The deficit in function of a surfactant with only SP-C can be overcome by adding positive end expiratory pressure during ventilation of the preterm rabbits (45). The physiologic effects of surfactant on the lung are striking and can be demonstrated by giving surfactant to the surfactant deficient lung or removing surfactant from the normal lung. The changes in lung mechanics are accompanied by large changes in gas exchange and lung injury with ventilation of the surfactant-deficient lung.

Surfactant Inactivation

Surfactant function is easily disrupted by multiple factors that frequently occur in the preterm lung (Table 8-2). Once the preterm lung has released surfactant stores at delivery, the surfactant will be depleted with time by the normal conversion of the pool from the tubular myelin and loose surfactant arrays into the catabolic liposomes unless there is active replacement. The generation of new secretion capacity from lamellar bodies from de novo synthesis and recycling is a slow process, and efficient recycling depends on normal lamellar body and alveolar pool sizes. The preterm with just enough surfactant at birth to transition to air breathing may become functionally deficient over hours. Further, the surfactant of the preterm lung has lower amounts of saturated to total phosphatidylcholine and lower amounts of the surfactant proteins (46). The multiple proteins, lipids, and other factors that can interfere with film formation listed in Table 8-2 will be more inhibitory for surfactant from the immature lung than mature surfactant that contains more saturated phosphatidylcholine, more SP-B and SP-C, and particularly more SP-A. Finally, surfactant lipids can function as a thromboplastin and promote

Table 8-2. Surfactant Inactivation – Causes and Interfering Substances

Increased conversion from surface active to inactive forms in airspaces
Proteinaceous pulmonary edema (proteases?)
Low surfactant protein content
Removal of surfactant from airspace pool
Clots and hyaline membranes
Inhibition of surface adsorptions and film stability
Proteins
Edema fluid
Plasma components – albumin, fibrinogen, hemoglobin
Lipids
Cell membrane
Cholesterol
Other inhibitors
Meconium
Bilirubin
Oxidizing agents
Amino acids

clotting of plasma that leaks into the airspaces. The surfactant deficient preterm lung is easily injured by spontaneous or mechanical ventilation with increased permeability of the epithelium, resulting in proteinaceous pulmonary edema (46). Plasma components will clot to form hyaline membranes that can trap surfactant, removing it from the functional pool. Multiple substances interfere with film formation by competing with surfactant for the air–water interface. These inactivation phenomena are concentration dependent, as high concentrations of normal surfactant can form stable surface films in the presence of plasma or inhibitors, while surface film formation by low surfactant concentrations in the hypophase are easily disrupted (47). Thus, the preterm lung is at substantial disadvantages because the immature surfactant with functional deficits is likely to be present in small amounts. The term lung also can have surfactant inactivation by meconium and the inflammatory products from pneumonia.

Surfactant Treatment

The treatment responses to surfactant illustrate the effects of surfactant on the developing lung. It must be emphasized that the development of surfactant treatment has transformed the care of preterm infants (48). The reasons that surfactant treatments are so effective go beyond simply acutely improving surface tensions and thus the physiology of the preterm lung. The major reason that surfactant treatments cause persistent clinical responses is that the metabolic characteristics of surfactant phospholipids and proteins in the preterm are favorable (35). Alveolar and tissue pool sizes are small, and the rate of accumulation is slow. Treatment acutely increases both the alveolar and tissue pools because the exogenously administered saturated phosphatidylcholine is taken up by type II cells and processed for resecretion. The surfactants used clinically are not equivalent in composition or function to native surfactant in the mature lung. Furthermore, airway instillation does not achieve an ideal distribution of surfactant. However, within hours following surfactant treatment of preterm animals, the surfactant recovered by alveolar wash has improved function. Therefore, the preterm lung, if uninjured, can rapidly transform surfactants used for treatment with poor function to a better surfactant (47). Also of benefit is the slow catabolic rate of surfactant, with the result being that the surfactant used for treatment remains in the lungs, is recycled, and is not rapidly degraded. The surfactant used for treatment becomes substrate for the endogenous recycling pathways to increase overall surfactant quantities. Treatment doses of surfactant do not feedback-inhibit the endogenous synthesis of saturated phosphatidylcholine or the surfactant proteins (49). No adverse metabolic consequences of surfactant treatment on the endogenous metabolism of surfactant or other lung functions have been identified.

The static mechanics of the preterm lung are strikingly improved by surfactant treatments (Figure 8-5A). The dynamic lung mechanics also are altered by surfactant treatments (35). The time constant for deflation increases, resulting in less-rapid lung emptying. The clinical correlate is that a surfactant treatment can increase the functional residual capacity of infants with RDS by two mechanisms: the improved deflation stability and the longer expiratory time constant. The

consistent initial response of infants with RDS to surfactant treatments is a rapid improvement in oxygenation, whereas improvements in PCO_2 , compliance, and therefore ventilatory support variables tend to change more gradually. The improved oxygenation without changes in ventilation results from the acute increase in lung volumes following surfactant treatments. In experimental animals, these acute physiological responses are accompanied by much more uniform aeration of the preterm lung at the anatomic level, a decreased lung permeability, and less indicators of lung injury with mechanical ventilation (50,51).

Clinical Lung Maturation

RDS and Induced Lung Maturation

At term the total amount of surfactant present in the human airways plus lamellar body pools probably exceeds that in the adult by about 10-fold on a body weight basis. Lamellar bodies first appear within type II cells by 20 to 24 weeks' gestation in the human fetus, and the amount of saturated phosphatidylcholine in lung tissue progressively increases to term. Lung maturity as defined clinically by the absence of RDS in the human fetus is generally present after 36 weeks of normal gestation, but infants born at 24 weeks can have "mature lungs" based on their ability to exchange oxygen and CO_2 . Therefore, a 12-week window of "early maturation" is possible for the human, in part because the surfactant synthetic and storage machinery can be induced in the human early in gestation.

The clinical syndrome linked to inadequate amounts of surfactant is RDS, but the identification of surfactant deficiency is problematic clinically. There are no tests to measure surfactant amount in the airspaces, and some preterm infants will have sufficient surfactant, but that surfactant may be inhibited by edema or inflammation associated with pneumonia, for example.

From the epidemiologic perspective, the incidence of RDS increases as gestational age decreases. However, incidence of RDS is not easily defined because of variable definitions and the effect of different clinical care strategies on the diagnosis of RDS. For example, from 1997 to 2002 the NICHD Neonatal Research Network defined RDS as the need for oxygen and some ventilatory

support, plus a compatible chest roentgenogram. The Network reported the incidence of RDS for infants less than 1 kg as 63% (52). The definition was changed for 2003 to 2007 to the use of supplemental oxygen for >6 hr and 95% of similar infants than had a diagnosis of RDS (53). In contrast, only about 50% of infants with birth gestations less than 28 weeks that are initially supported with CPAP have sufficient RDS to receive surfactant (54). If the use of surfactant is a surrogate for significant surfactant deficiency, then many very early gestation infants did not have severe RDS. Biologically, this indicates that induced lung maturation is very frequent. This spontaneous early lung maturation in the human fetus is believed to result from stress-induced maturation events that can be maternal, placental, or fetal in origin. Surprisingly, the fetal stress that must accompany fetal growth restriction or pre-eclampsia, does not consistently induce early lung maturation.

A changing epidemiology of RDS results in part from the more frequent clinical use of antenatal glucocorticoids and changes in obstetric practice that delay preterm delivery, presumably allowing the lung to mature. Numerous clinical trials have documented that maternal corticosteroid treatments decrease the incidence of RDS by about 50%, and those infants with RDS tend to have less severe disease (55). Chronic infection and fetal exposure to inflammation and histologic chorioamnionitis is frequent in pregnancies with preterm labor between 22 and 30 weeks gestation and also is associated with a decreased incidence of RDS (56). In experimental models, fetal pro-inflammatory exposures induce striking increases in surfactant and improvements in postnatal lung function without increasing fetal cortisol levels (57). Therefore, fetal exposure to inflammation may have the short-term benefit of increasing surfactant and decreasing RDS.

Fetal plasma cortisol increases as the fetus approaches term and is associated with the large increase in surfactant at term. Corticotropin-releasing hormone-deficient mice and mice with ablation of the glucocorticoid receptor have inadequate surfactant to survive following term birth (58,59). Therefore, endogenous cortisol is essential for normal lung maturation. The very early gestation human fetal lung also is responsive to corticosteroids. Explants of human lung at 14 to 20 weeks gestational age differentiate in organ

culture in the absence of hormonal stimuli, and corticosteroids and thyroid hormones accelerate maturation (60).

The responses of the fetal lung to corticosteroids are multiple and impact many different systems that will influence the clinical outcome (61). Biochemical markers of maturation include glycogen loss from type II cells, increased fatty acid synthesis, increased beta receptors, and increased choline incorporation into surfactant phosphatidylcholine. In vivo, animals demonstrate improved lung function and survival. Corticosteroid treatment also decreases the tendency of the preterm lung to develop pulmonary edema (50). Although the primary effect of corticosteroids on the fetal lung is generally considered to be induction of surfactant synthesis, effects on enzymes in the synthetic pathways for surfactant have not been consistently demonstrated, and surfactant pool sizes do not increase until more than 4 days after maternal glucocorticoid treatments in sheep (61). Corticosteroids induce lung structural maturation by decreasing the amount of mesenchyme and increasing the surface area for gas exchange as is reflected by increased lung volumes within 12 to 24 hrs in fetal sheep (62). In preterm animal models, corticosteroid treatment changes the dose-response curve for surfactant treatments such that less surfactant is needed to achieve larger clinical responses (63). Because of increased lung volume, corticosteroid-treated fetuses also have improved responses to postnatal surfactant (64). There are additive or synergistic effects between the corticosteroid-exposed lungs and surfactant treatments in animal models.

Antenatal corticosteroid treatment is now the standard of practice for pregnancies at risk of preterm delivery (55). This therapy is effective and safe, although there is not long-term follow-up for infants born before 28 weeks' gestation. Repetitive courses of antenatal glucocorticoids have been given at 7- to 10-day intervals, a practice based on the suggestion that the fetal benefit was lost after this interval (61). Maternal glucocorticoid treatments at 7-day intervals in sheep cause fetal growth restriction but augment lung maturation. Randomized trials in women at risk for preterm delivery demonstrate modest benefit, but there is some concern about longer-term outcomes, especially for infants exposed to 4 or more antenatal courses of antenatal corticosteroids (65). There is presently insufficient information

for a strong recommendation about the use of repeated corticosteroid treatments.

The only other lung maturation strategy that has been extensively evaluated clinically is the combination of corticosteroids and thyrotropin-releasing hormone (TRH). Thyroid axis hormones induce lung maturation and can act synergistically with corticosteroids *in vitro*. Thyroid hormones do not cross the human placenta efficiently, but the tripeptide TRH crosses to the fetal circulation and increases fetal thyroid hormone levels. Unfortunately, when evaluated in large randomized, controlled trials, TRH demonstrated no benefit, and possible risks were identified (66).

Tests for Fetal Lung Maturation

Tests for fetal lung maturation depend on amniotic fluid composition reflecting the status of surfactant in the fetal lung. The lung secretes fetal lung fluid at a rate of about 4mL/kg/hr. The flow of fluid out of the lung is episodic and balanced between swallowing and loss of fetal lung fluid to the amniotic cavity. Tests of lung maturation depend on the flow of fetal lung fluid containing surfactant into the amniotic fluid being sufficient to change amniotic fluid composition in a timely manner relative to the state of fetal lung maturation. Tests of lung maturation were developed to identify fetuses with early lung maturation that thus could be delivered without the risk of developing RDS. The lecithin–sphingomyelin (L-S) ratio introduced by Gluck and associates in 1971 remains the standard against which other tests are compared (4). The results are expressed as the ratio of a lecithin (phosphatidylcholine) fraction, that is cold acetone precipitated to enrich for saturated phosphatidylcholine, to sphingomyelin. Sphingomyelin is a membrane lipid and is a nonspecific component of amniotic fluid not related to lung maturation. The sphingomyelin content of amniotic fluid decreases from about 32 weeks gestational age to term, whereas the lecithin content, a large part of which is from the fetal lung, increases.

Each of the surfactant lipids and SPs has a unique developmental profile as surfactant composition changes with development (5). Phosphatidylglycerol normally appears in amniotic fluid at about 35 weeks gestation and indicates lung maturation. Phosphatidylglycerol is present in appreciable amounts only in lung tissue and surfactant.

Therefore, phosphatidylglycerol can be measured in amniotic fluid contaminated with blood or meconium. Numerous other tests for lung maturity have been developed. The TDx-FLM assay of the ratio of amniotic fluid surfactant to albumin seems to be equivalent to the L-S ratio for the prediction of RDS. A currently popular test is a measurement of lamellar body numbers in amniotic fluid (67). Tests of fetal lung maturity are less frequently used in clinical practice than in the past because virtually all women at risk of preterm delivery are treated with antenatal corticosteroids, and the clinical decision to deliver a preterm seldom includes considerations of fetal lung maturation because surfactant treatments are now routine.

Genetic Causes of Surfactant Abnormalities

Four single gene disorders affecting surfactant production are currently known to cause newborn lung disease, as well as lung disease in older children and adults. Specific features of these disorders are summarized in Table 8-3 and discussed in detail in the following sections.

Surfactant Protein-B (SP-B) Deficiency

Loss of function mutations on both alleles of the gene encoding SP-B (*SFTPB*) cause severe neonatal lung disease in humans and lethal neonatal respiratory failure in genetically engineered mice (68–70). SP-B deficient human newborns are usually born at full-term gestation, but have the clinical and radiographic features of RDS in prematurely born infants. Unlike premature infants with RDS who respond favorably to supportive care and surfactant treatments, SP-B deficient infants have progressive disease that is usually fatal within the first 3 months of life. Rarely affected children may survive for longer periods due to mutations that allow for some SP-B production (71,72). Experiments with genetically engineered mice in which the SP-B gene was able to be turned off indicated that an SP-B level of 20% to 30% of control values was needed to maintain normal lung function (73).

Over 40 different *SFTPB* mutations have been reported. The most commonly observed is a frameshift mutation precluding any SP-B production that has accounted for approximately

Table 8-3.

Gene (Locus)	<i>SFTPB</i>	<i>ABCA3</i>	<i>SFTPC</i>	<i>NKX2-1</i>
Protein	SP-B	ABCA3	SP-C	TTF-1
Chromosomal Location	2p11.2	16p13.3	8p21.3	14q13.3
Most frequent mutation	c.397delinsGAA p.Pro133Glu/s*95 (previously referred to as 121ins2)	c.875 A>T (p.E292V or p.Glu292Val)	c.218 T>C (p.I73T or p.Ile73Thr)	None recognized
Inheritance	Recessive	Recessive	Dominant or Sporadic	Sporadic or dominant
Onset	Neonatal	Neonatal to childhood	Infancy > Adult > Neonatal	Often neonatal with associated hypothyroidism
Clinical Presentation	Neonatal RDS	Neonatal RDS Childhood ILD	Childhood ILD Adult ILD Pulmonary Fibrosis Neonatal RDS	Neonatal RDS childhoodILD Recurrent infections
Extrapulmonary Manifestations	None	None	None	Hypothyroidism Neurological - Hypotonia; movement disorder: chorea, ataxia,
Course/prognosis	Early death without transplant	Variable: early death to chronic lung disease	Highly variable; May be severe in infancy but improve to asymptomatic for decades	Variable: early death due to RDS/ILD to variably severe chronic lung disease.

SP: Surfactant Protein
 ABCA3: ATP Binding Cassette, member A3
 TTF-1: Thyroid Transcription Factor 1
 RDS: Respiratory Distress Syndrome
 ILD: Interstitial Lung Disease

two-thirds of the mutant alleles identified to date (70,74). The relative prevalence of this mutation reflects a common ancestral background or “founder” effect of Northern European origin (75). Population-based studies of its frequency have yielded different results depending on the population studied, with carrier rates between 1 in ~600 to 1 in 1000 (76). Combining these data with the relative contribution of this single mutation to disease yields a predicted incidence of SP-B deficiency of less than 1 in one million live births.

The lack of mature SP-B, which is critical for proper surfactant function, is the primary cause of lung disease. Additional changes in surfactant metabolism including markedly reduced amounts of phosphatidylcholine and phosphatidylglycerol in lung fluid, contribute to the severity of the lung disease (74,77). The processing of proSP-C is also impaired and leads to the accumulation and secretion of a partially processed intermediate with retained amino-terminal proSP-C epitopes,

with mature SP-C production also likely reduced (78). Thus multiple abnormalities result in secretion of surfactant that is ineffective in lowering surface tension. The impaired processing of SP-C and reductions in surfactant lipids are likely due to disrupted lamellar body formation, the intracellular storage organelle in type II cells for surfactant lipids and proteins, and the cellular compartment where the final processing steps of proSP-C and proSP-B occur. Collectively these observations support an intracellular role for SP-B (or proSP-B) function along with its role in facilitating surface tension reduction at the alveolar air–liquid interface.

ABCA3 Deficiency

The adenosine triphosphate (ATP) binding cassette family of transporters use energy from the hydrolysis of ATP to move substances across biological membranes, and mutations in their genes often underlie human disease (79). Member A3 of

this family (ABCA3) is localized to the limiting membrane of lamellar bodies, and term human newborns with bi-allelic loss-of-function mutations in the ABCA3 gene (*ABCA3*) develop an RDS-like syndrome (80). Genetically engineered mice unable to produce ABCA3 do not inflate their lungs at birth and die from respiratory failure (81–83). The phospholipid profiles from the lungs of such animals had marked reductions in surfactant phosphatidylcholine and phosphatidylglycerol (83). Analysis of lung fluid from infants who had lung transplantation and were subsequently found to have ABCA3 mutations also demonstrated marked reductions in these same phospholipids compared to infants transplanted for other disorders.(77) Finally, ultrastructural examination of the lung tissue of ABCA3-deficient newborns and animals demonstrates an absence of normally formed lamellar bodies, with numerous small dense bodies with eccentrically placed electron-dense cores giving them a “fried-egg” appearance (80). Collectively these observations support a model where ABCA3 is a key transporter of lipids critical for surfactant function into lamellar bodies.

Over 250 disease-causing mutations scattered throughout the ABCA3 gene (*ABCA3*) have been recognized along with large deletions spanning one or more exons. Although many infants with ABCA3 mutations have a severe phenotype similar to that of SP-B-deficient infants, others have relatively milder disease and present with findings of diffuse or ILD later in childhood (84,85). Not all have a history of neonatal lung disease, and asymptomatic children with ABCA3 mutations and adult-onset disease have also been recognized (86,87). Thus the pulmonary phenotypic spectrum of ABCA3 deficiency is much broader than that of SP-B deficiency. Genotype appears to be an important determinant of the onset and severity of lung disease, with mutations on both alleles that are predicted to preclude any ABCA3 protein expression and function uniformly associated with neonatal-onset disease and death or need for lung transplantation within the first year of life (85).

The pathophysiology of lung disease due to ABCA3 mutations is incompletely understood. The severe RDS observed in newborns with complete loss-of-function mutations can be attributed to a lack of functional surfactant, but the mechanisms whereby some children do not exhibit

signs of surfactant deficiency in the neonatal period yet develop lung disease later in life are unclear. A small number of ABCA3 mutations have been studied in vitro, and missense mutations may cause inappropriate trafficking of the mutant protein, impaired ability to hydrolyze ATP, and/or impaired transport of lipid (88–90). Such mutations may allow for enough surfactant production to prevent neonatal RDS. Alternatively, ABCA3 that is misrouted to the apical surface of the type II cell may allow for secretion of sufficient amounts of surfactant lipids and proteins to prevent neonatal RDS; this hypothesis has not been experimentally tested. The mechanisms leading to ILD later in life are even less well understood. Chronically perturbed surfactant metabolism and stoichiometry resulting from decreased or abnormal ABCA3 function may injure alveolar type II cells, leading to eventual inflammation, tissue injury, and fibrosis. Finally, as ABCA3 expression is developmentally regulated, heterozygosity for an ABCA3 mutation may increase the risk for RDS in prematurely born infants due to the reduced function from one allele (91).

The precise incidence and prevalence of ABCA3 deficiency are unknown. In population-based studies, the carrier rate of the ABCA3 p.Glu292Val mutation, which has accounted for < 10% of identified mutations, has ranged from 1 in 80 (1.3%) to 1 in 125 (76,92). A case-control study of late-preterm infants with and without RDS found a carrier frequency of 3.7% in the European population and 1.5% in the African descent population (91). Databases derived from massive parallel sequencing projects (Exome Variant Server, www.evs.gs.washington.edu/EVS; 1000 genomes, www.1000genomes.org) list multiple ABCA3 coding variants. While many of these are likely not disease-causing, variants known and highly likely to be associated with disease are listed. Thus carrier frequency for any functional ABCA3 mutation may be as high as 1 in 35 individuals, which would translate to a predicted disease incidence as high as 1 in 4,500 births.

SP-C Dysfunction

Mutations in the SP-C gene (*SFTPC*) result in lung disease that is highly variable in its onset and severity, ranging from severe neonatal RDS to adult-onset ILD and pulmonary fibrosis, with

some individuals with *SFTPC* mutations asymptomatic into their fifth or sixth decade of life (68,93,94). Disease may result from a mutation on just one *SFTPC* allele, either sporadically due to de novo mutations or inherited in an autosomal dominant pattern. All reported disease-causing *SFTPC* mutations are predicted to alter the coding sequence of proSP-C, and lung disease due to *SFTPC* mutations is thought to result from the production of an abnormal protein rather than the lack of production from one allele (haploinsufficiency). One *SFTPC* mutation that results in the substitution of threonine for isoleucine in codon 73 (p.Ile73Thr) has been identified in multiple unrelated individuals and has accounted for 25%–50% of the reported cases to date (95). The mutation has been associated with both sporadic as well as familial disease and has been found in individuals with different ethnic backgrounds and on different *SFTPC* haplotypes, indicating that recurrent mutation at this location in this gene may occur. Other mutations are scattered throughout the gene, but many are located in the region encoding the last ~100 amino acids of proSP-C, a domain with homology to a group of proteins associated with familial dementias and malignancy (BRICHOS domain) (96,97).

The pathophysiology of lung disease due to *SFTPC* mutations is complex and may depend on the specific mutation. Mutations in the BRICHOS domain often result in an unstable proprotein that is misfolded and targeted for degradation in the ER (96,97). Depending on the amount of abnormal protein produced and capacity of the cell to handle it, the misfolded protein may aggregate, and the unfolded protein response (UPR) activated, with subsequent caspase activation, cell death due to apoptosis and inflammation (97). Self-association of normal and mutated proSP-C in the secretory pathway may result in degradation of the protein derived from the normal allele, leading to deficiency of mature SP-C in a dominant negative mechanism. Expression of misfolded proSP-C may also result in cellular stress that can be exacerbated by additional injury. Cells in culture that were stably transfected with a known disease-causing *SFTPC* mutation did not exhibit toxicity at baseline, but died when subsequently infected with respiratory syncytial virus (98). In addition, transgenic mice expressing a different mutant form of SP-C exposed to bleomycin developed more prominent

inflammatory and fibrotic responses than in their wild-type littermates (99).

Mutations located outside the BRICHOS domain may result in proSP-C that is routed to the plasma membrane of alveolar type II cells rather than to lamellar bodies, and then trafficked to early endosomes, and inefficiently or not processed to mature SP-C (100,101). Either the lack of mature SP-C or chronically perturbed alveolar type II cell metabolism may contribute to disease pathophysiology, although precise mechanisms remain to be determined. Depending on the nature and location of the mutation and its particular mechanism for causing disease, different treatment strategies may be necessary. Agents designed to help stabilize misfolded proteins or facilitate transit from the ER to the Golgi may be ineffective for mutations that result in abnormal targeting of proSP-C to the plasma membrane (100).

The incidence and prevalence of lung disease due to *SFTPC* mutations are unknown. The p.Ile73Thr mutation was not found on > 8000 alleles in samples obtained from a neonatal screening program (76). Only one individual with an *SFTPC* mutation was identified in a group of adults with sporadic pulmonary fibrosis or ILD, although 25% of familial pulmonary fibrosis kindreds were found to have an *SFTPC* mutation as the basis for disease in another study (102,103). Multiple *SFTPC* coding variants are listed in public databases, including some known or likely to cause disease, but the associated pulmonary phenotypes are not available. The majority of published subjects with *SFTPC* mutations developed lung disease after the neonatal period. Overall *SFTPC* mutations appear to be a very rare cause of lung disease and an even rarer cause of neonatal RDS.

NKX2.1 Haploinsufficiency

The gene *NKX2.1* encodes a transcription factor essential for thyroid gland development called thyroid transcription factor 1 (TTF-1). It is also expressed in other tissues, including the central nervous system and lung, where it is necessary for expression of many genes, including those encoding SP-A, SP-B, SP-C, and ABCA3 (104). Chromosomal deletions involving the *NKX2.1* locus have been observed in newborns with severe RDS and hypothyroidism, and loss-of-function mutations on one *NKX2.1* allele have also been

associated with the same phenotype (105,106). *NKX2.1* mutations were also recognized as the cause of an autosomal dominantly inherited movement disorder, benign familial chorea. It is now clear that individuals with mutations in or deletions of this gene may have a combination of findings that include pulmonary disease (neonatal RDS, chronic lung disease, and recurrent pulmonary infections), hypothyroidism, and various neurological manifestations (developmental delay, hypotonia, ataxia, dysarthria), a constellation that has been termed “brain–thyroid–lung syndrome” (107). Affected individuals with *NKX2.1* mutations may have manifestations in only one organ system, including the lungs. The pulmonary phenotypes are still incompletely described, but range from severe neonatal lung disease with pathology findings of disrupted lung development, to onset of ILD later in childhood (108).

Based on studies of the functional effects of mutations in vitro and the observation that deletions of one copy of *NKX2.1* are associated with disease, the primary mechanism whereby *NKX2.1* mutations cause disease is haploinsufficiency (105). It is possible that some mutations may result in a gain of function, and specific mutations may have variable effects on different downstream target genes that are important in mediating the phenotype, which might depend on which target genes are most impacted by a given mutation (107). Loss-of-function mutations leading to

decreased expression of *ABCA3* or *SP-B* would explain an RDS phenotype, decreased *SP-C* expression could lead to an ILD phenotype, and decreased expression of pulmonary collectins (*SP-A*, *SP-D*) contribute to recurrent infection, given their role in host defense (108).

The incidence and prevalence of lung disease due to *NKX2.1* mutations are unknown. Population-based studies of the frequency of such mutations have not yet been carried out. Public databases list variants that are potentially disease causing, but phenotypic correlates are not readily available.

Lung Pathology of Genetic Surfactant Disorders

Lung histopathology findings associated with genetic disorders of surfactant metabolism may be distinctive, but are not specific for the gene involved (Figure 8-6) (109). A common finding in neonates or young infants is an accumulation of granular, eosinophilic material filling distal airspaces, a finding similar to that observed in older subjects with alveolar proteinosis syndromes where surfactant material accumulates in the airspaces due to impaired catabolism. The underlying alveolar architecture is usually preserved in subjects with alveolar proteinosis syndromes, whereas in surfactant production disorders there is often prominent alveolar type II cell hyperplasia,

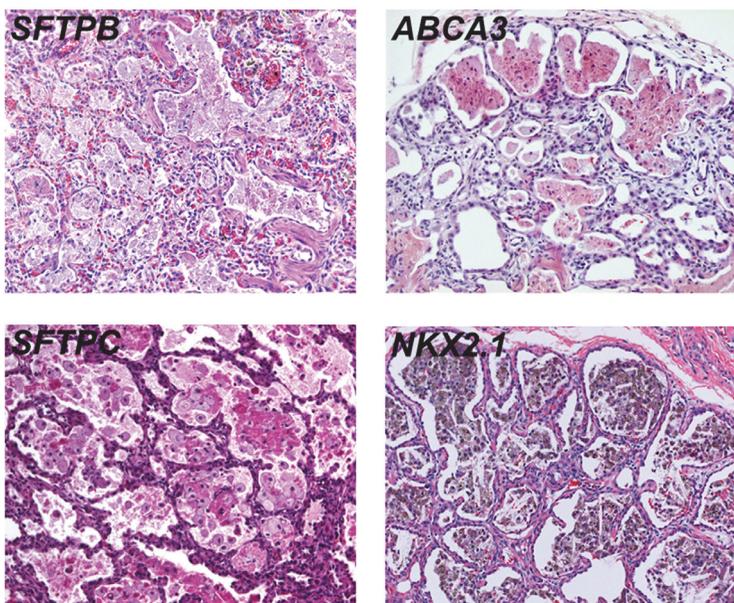


Figure 8-6. Representative lung histopathology sections (hematoxylin and eosin stain) from infants with genetic surfactant disorders. The specific gene involved is indicated on each panel. Similar findings are observed with each genetic disorder, including extracellular accumulations of proteinaceous material and macrophages, alveolar type II cell hyperplasia, and interstitial thickening.

mesenchymal thickening, and variable degrees of fibrosis. In older children the findings of proteinosis are less prominent, and variable accumulations of foamy macrophages within airspaces may be observed, with the lung histopathology interpreted as desquamative interstitial pneumonitis, chronic pneumonitis of infancy, or nonspecific interstitial pneumonia.

Electron microscopy studies may provide information on the specific genetic defect. The lamellar bodies in SP-B-deficient infants are disorganized with multiple vesicles within the organelle as opposed to orderly stacked membranes. In ABCA3-deficient infants normal lamellar bodies may not be seen, with small, dense bodies with eccentrically placed electron-dense cores found in the type II cells.

Other Surfactant-Related Genes and Lung Disease

Mutations in other genes involving surfactant function and metabolism have been reported, but have not been associated with lung disease in the perinatal period. Mutations in one of the genes encoding SP-A, *SFTPA2*, cause familial pulmonary fibrosis and lung cancer, likely from a gain-of-toxic function mechanism (110,111). Mutations in the genes (*CSFR2A*, *CSFR2B*) encoding the receptor for GM-CSF lead to impaired alveolar macrophage function and alveolar proteinosis in children and young adults, but have not been reported as causes of perinatal lung disease (112).

Gaps and the Future

Although much has been learned regarding the composition and function of surfactant, virtually nothing is known about the transcriptional networks that integrate molecular pathways involved

in prenatal type II cell maturation, surfactant protein and lipid synthesis, and alveolar defense. How these molecular networks “sense” alveolar pool size in the postnatal lung and modulate transcriptional pathways to balance surfactant synthesis with surfactant secretion, recycling, and degradation is completely unknown. Likewise, it is unclear to what extent regulatory networks involved in type II cell maturation are also involved in alveolar repair and reconstitution of surfactant homeostasis following lung injury. These critical knowledge gaps are reflected in the paucity of new therapies for lung immaturity and chronic lung diseases.

Clinically, surfactants isolated from adult animal lungs are widely available and effective. Surfactants containing only synthetic lipids and the surfactant proteins have been tested extensively in animal models and are very effective (17). The lipophilic surfactant proteins B and C are altered to increase stability or used as shorter peptides. These synthetic surfactants will result in more standardized products for clinical use, but clinical responses will probably be similar. A major effort is directed at the development of better ways to deliver surfactant to the preterm lung that can avoid intubation and mechanical ventilation.

Infants who present with a severe and progressive RDS-like syndrome may have a genetic basis for surfactant deficiency. Once infection and other causes of severe respiratory failure are excluded, then tests for abnormalities of *SFTPB*, *SFTPC*, *ABCA3*, or *NKx2-1* may be diagnostic. No doubt rarer causes of respiratory failure in the newborn will be discovered. Presently these four diagnoses will not explain the respiratory failure in many infants who have yet to have identified genetic or developmental abnormalities that influence surfactant.

References

- 1 Clements JA. Surfactant tension of lung extracts. *Proc Soc Exp Biol Med.* 1957;95:170.
- 2 Avery ME, Mead J. Surface properties in relation to atelectasis and hyaline membrane disease. *AMA J Dis Child.* 1959;97:517–523.
- 3 Clements JA, Platzker AC, Tierney DF, et al. Assessment of the risk of the respiratory distress syndrome by a rapid test for surfactant in amniotic fluid. *New Engl Med.* 1972;18:1077–1081.
- 4 Gluck L, Kulovich M, Borer RC, Brenner PH, Anderson GG, Spellacy WN. Diagnosis of the respiratory distress syndrome by amniocentesis. *Am J Ob Gyn.* 1971;109:440–445.
- 5 Hallman M, Kulovich M, Kirkpatrick E, Sugarman RG, Gluck L. Phosphatidylinositol and phosphatidylglycerol in amniotic fluid: indices of lung maturity. *Am J Obstet Gynecol.* 1976; 125:613–617.

- 6 Liggins GC, Howie RN. A controlled trial of antepartium glucocorticoid treatment for prevention of RDS in premature infants. *Pediatrics*. 1972;50:515–525.
- 7 Gregory GA, Kitterman JA, Phibbs RH, Tooley WA, Hamilton WK. Treatment of the idiopathic respiratory distress system with continuous positive airway pressure. *N Engl J Med*. 1971;284:1333–1340.
- 8 Enhörning G, Robertson B. Lung expansion in the premature rabbit fetus after tracheal deposition of surfactant. *Pediatr*. 1972; 50:58–66.
- 9 Fujiwara T, Chida S, Watabe Y, Maeta H, Morita Ta, Abe T. Artificial surfactant therapy in hyaline-membrane disease. *Lancet*. 1980;1:55–59.
- 10 Whitsett JA, Wert SE, Weaver TE. Alveolar surfactant homeostasis and the pathogenesis of pulmonary disease. *Annu Rev Med*. 2010;61:105–119.
- 11 McCormack FX, Whitsett JA. The pulmonary collectins, SP-A and SP-D, orchestrate innate immunity in the lung. *J Clin Invest*. 2002;109:707–712.
- 12 Sano H, Kuroki Y. The lung collectins, SP-A and SP-D, modulate pulmonary innate immunity. *Mol Immunol*. 2005;42:279–287.
- 13 Korfhagen TR, Bruno MD, Ross GF, et al. Altered surfactant function and structure in SP-A gene targeted mice. *Proc Natl Acad Sci U S A*. 1996;93:9594–9599.
- 14 Kingma PS, Whitsett JA. In defense of the lung: surfactant protein A and surfactant protein D. *Curr Opin Pharmacol*. 2006;6:277–283.
- 15 Kuypers E, Collins JJ, Kramer BW, et al. Intra-amniotic LPS and antenatal betamethasone: inflammation and maturation in preterm lamb lungs. *Am J Physiol Lung Cell Mol Physiol*. 2012;302:L380–389.
- 16 Ikegami M, Whitsett JA, Jobe AH, Ross G, Fisher J, Korfhagen T. Surfactant metabolism in SP-D gene ablated mice. *Am J Physiol Lung Cell Mol Physiol*. 2000;279:L468–L476.
- 17 Sato A, Whitsett JA, Scheule RK, Ikegami M. Surfactant protein-D inhibits lung inflammation caused by ventilation in premature newborn lambs. *Am J Respir Crit Care Med*. 2010;181:1098–1105.
- 18 Weaver TE, Conkright JJ. Function of surfactant proteins B and C. *Annu Rev Physiol*. 2001;63:555–578.
- 19 Clark JC, Wert SE, Bachurski CJ, et al. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc Natl Acad Sci U S A*. 1995;92:7794–7798.
- 20 Ikegami M, Whitsett JA, Martis PC, Weaver TE. Reversibility of lung inflammation caused by SP-B deficiency. *Am J Physiol Lung Cell Mol Physiol*. 2005;289: L962–970.
- 21 Glasser SW, Burhans MS, Korfhagen TR, et al. Generation of an SP-C deficient mouse by targeted gene inactivation. *Am J Respir Crit Care Med*. 2000;161:A43.
- 22 Ridsdale R, Na CL, Xu Y, Greis KD, Weaver T. Comparative proteomic analysis of lung lamellar bodies and lysosome-related organelles. *PLoS One*. 2011;6:e16482.
- 23 Wright JR, Benson BJ, Williams MC, Goerke J, Clements JA. Protein composition of rabbit alveolar surfactant subfractions. *Biochim Biophys Acta*. 1984;791:320–332.
- 24 Guillems M, De Kleer I, Henri S, et al. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *Exp Med*. 2013;210:1977–1992.
- 25 Kramer BW, Joshi SN, Moss TJ, et al. Endotoxin-induced maturation of monocytes in preterm fetal sheep lung. *Am J Physiol Lung Cell Mol Physiol*. 2007;293: L345–353.
- 26 Hillman N, Polglase GR, Pillow JJ, Saito M, Kallapur SG, Jobe AH. Inflammation and lung maturation from stretch injury in fetal preterm sheep. *AM J Physiol Lung Cell Mol Physiol*. 2011;300:L232–L241.
- 27 Young SL, Fram EK, Spain CL, Larson EW. Development of type-II pneumocytes in rat lung. *Am J Physiol*. 1991;260: L113–L122.
- 28 Rebello CM, Jobe AH, Eisele JW, Ikegami M. Alveolar and tissue surfactant pool sizes in humans. *Am J Respir Crit Care Med*. 1996;154:625–628.
- 29 Jacobs H, Jobe A, Ikegami M, Jones S. Surfactant phosphatidylcholine source, fluxes, and turnover times in 3-day-old, 10-day-old, and adult rabbits. *J Biol Chem*. 1982;257:1805–1810.
- 30 Jacobs H, Jobe A, Ikegami M, Conaway D. The significance of reutilization of surfactant phosphatidylcholine. *J Biol Chem*. 1983;258:4156–4165.
- 31 Jacobs H, Jobe A, Ikegami M, Miller D, Jones S. Reutilization of phosphatidylcholine analogues by the pulmonary surfactant system. The lack of specificity. *Biochim Biophys Acta*. 1984;793:300–309.
- 32 Yoshida M, Ikegami M, Reed JA, Chroneos ZC, Whitsett JA. GM-CSF regulates protein and lipid catabolism by alveolar

- macrophages. *Am J Physiol.* 2001;280:L379–L386.
- 33 Jobe AH, Ikegami M, Jacobs HC, Jones SJ. Surfactant pool sizes and severity of respiratory distress syndrome in prematurely delivered lambs. *Am Rev Respir Dis.* 1983;127:751–755.
- 34 Mulrooney N, Champion Z, Moss TJ, Nitsos I, Ikegami M, Jobe AH. Surfactant and physiological responses of preterm lambs to continuous positive airway pressure. *Am J Respir Crit Care Med.* 2005;171:1–6.
- 35 Jobe AH. Why surfactant works for respiratory distress syndrome. *NeoReviews.* 7 2006: e95–105.
- 36 Jackson JC, Palmer S, Truog WE, Standaert TA, Murphy JH, Hodson WA. Surfactant quantity and composition during recovery from hyaline membrane disease. *Pediatr Res.* 1986;20:1243–1247.
- 37 Hallman M, Merritt TA, Akino T, Bry K. Surfactant protein-A, phosphatidylcholine, and surfactant inhibitors in epithelial lining fluid – correlation with surface activity, severity of respiratory distress syndrome, and outcome in small premature infants. *Am Rev Respir Dis.* 1991;144:1376–1384.
- 38 Bunt JE, Zimmerman LJ, Wattimena D, Beek RH, Sauer PJ, Carmielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotope. *Am J Respir Crit Care Med.* 1998;157:810–814.
- 39 Torresin M, Zimmermann LJ, Cogo PE, et al. Exogenous surfactant kinetics in infant respiratory distress syndrome: a novel method with stable isotopes. *Am J Respir Crit Care Med.* 2000;161:1584–1589.
- 40 Carnielli VP, Zimmermann LJ, Hamvas A, Cogo PE. Pulmonary surfactant kinetics of the newborn infant: novel insights from studies with stable isotopes. *J Perinatol.* 2009;29(suppl 2):S29–37.
- 41 Tomazela DM, Patterson BW, Hanson E, et al. Measurement of human surfactant protein-B turnover in vivo from tracheal aspirates using targeted proteomics. *Anal Chem.* 2010;82:2561–2567.
- 42 Rider ED, Jobe AH, Ikegami M, Sun B. Different ventilation strategies alter surfactant responses in preterm rabbits. *J Appl Physiol.* 1992;73:2089–2096.
- 43 Bilek AM, Dee KC, Gaver DP. Mechanisms of surface-tension-induced epithelial cell damage in a model of pulmonary airway reopening. *J Appl Physiol.* 2003;94:770–783.
- 44 Rider ED, Ikegami M, Whitsett JA, Hull W, Absolom D, Jobe AH. Treatment responses to surfactants containing natural surfactant proteins in preterm rabbits. *Am Rev Respir Dis.* 1993;147:669–676.
- 45 Davis AJ, Jobe AH, Häfner D, Ikegami M. Lung function in premature lambs and rabbits treated with a recombinant SP-C surfactant. *Am J Respir Crit Care Med.* 1998;157:553–559.
- 46 Ueda T, Ikegami M. Change in properties of exogenous surfactant in injured rabbit lung. *Am J Respir Crit Care Med.* 1996;153:1844–1849.
- 47 Ueda T, Ikegami M, Jobe AH. Developmental changes of sheep surfactant: in vivo function and in vitro subtype conversion. *J Appl Physiol.* 1994;76:2701–2706.
- 48 Jobe A, Ikegami M. Surfactant for the treatment of respiratory distress syndrome. *Am Rev Respir Dis.* 1987;136:1256–1275.
- 49 Pettenazzo A, Oguchi K, Seidner S, Ikegami M, Berry D, Jobe A. Clearance of natural surfactant phosphatidylcholine from 3-day-old rabbit lungs: effects of dose and species. *Pediatr Res.* 1986;20:1139–1142.
- 50 Ikegami M, Berry D, Elkady T, Pettenazzo A, Seidner S, Jobe A. Corticosteroids and surfactant change lung function and protein leaks in the lungs of ventilated premature rabbits. *J Clin Invest.* 1987;79:1371–1378.
- 51 Pinkerton KE, Lewis JF, Rider ED, et al. Lung parenchyma and type II cell morphometrics: effect of surfactant treatment on preterm ventilated lamb lungs. *J Appl Physiol.* 1994;77:1953–1960.
- 52 Fanaroff AA, Stoll BJ, Wright LL, et al. Trends in neonatal morbidity and mortality for very low birthweight infants. *Am J Obstet Gynecol.* 2007;196:147 e141–148.
- 53 Stoll BJ, Hansen NI, Bell EF, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics.* 2010;126:443–456.
- 54 Bancalari EH, Jobe AH. The respiratory course of extremely preterm infants: a dilemma for diagnosis and terminology. *J Pediatr.* 2012;161:585–588.
- 55 Roberts D, Dalziel S. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst Rev.* 2006;3:CD004454.
- 56 Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet.* 2008;371:75–84.
- 57 Jobe AH, Newnham JP, Willet KE, et al. Endotoxin

- induced lung maturation in preterm lambs is not mediated by cortisol. *Am J Respir Crit Care Med.* 2000;162:1656–1661.
- 58 Cole TJ, Blendy JA, Monaghan AP, et al. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Gene Dev.* 1995;9:1608–1621.
- 59 Muglia L, Jacobson L, Dikkes P, Majzoub JA. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. *Nature.* 1995;373:427–432.
- 60 Mendelson CR, Boggaram V. Hormonal and developmental regulation of pulmonary surfactant synthesis in fetal lung. *Baillieres Clin Endocrinol Metab.* 1990;4:351–378.
- 61 Jobe AH. Animal models of antenatal corticosteroids: clinical implications. *Clin Obstet Gynecol.* 2003;46:174–189.
- 62 Ikegami M, Polk D, Jobe A. Minimum interval from fetal betamethasone treatment to postnatal lung responses in preterm lambs. *Am J Obstet Gynecol.* 1996;174:1408–1413.
- 63 Seidner S, Pettenazzo A, Ikegami M, Jobe A. Corticosteroid potentiation of surfactant dose response in preterm rabbits. *J Appl Physiol.* 1988;64:2366–2371.
- 64 Ikegami M, Polk D, Tabor B, Lewis J, Yamada T, Jobe A. Corticosteroid and thyrotropin-releasing hormone effects on preterm sheep lung function. *J Appl Physiol.* 1991;70:2268–2278.
- 65 Crowther CA, Doyle LW, Haslam RR, Hiller JE, Harding JE, Robinson JS. Outcomes at 2 years of age after repeat doses of antenatal corticosteroids. *New Engl Med.* 2007;357:1179–1189.
- 66 ACTOBAT. Australian collaborative trial of antenatal thyrotropin-releasing hormone (ACTOBAT) for prevention of neonatal respiratory disease. *Lancet.* 1995;345:877–882.
- 67 Machado LU, Fiori HH, Baldisserotto M, Ramos Garcia PC, Vieira AC, Fiori RM. Surfactant deficiency in transient tachypnea of the newborn. *J Pediatr.* 2011;159:750–754.
- 68 Noguee LM. Alterations in SP-B and SP-C expression in neonatal lung disease. *Annu Rev Physiol.* 2004;66:601–623.
- 69 Noguee LM, Garnier G, Dietz HC, et al. A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. *J Clin Invest.* 1994;93:1860–1863.
- 70 Noguee LM, Wert SE, Profitt SA, Hull WM, Whitsett JA. Allelic heterogeneity in hereditary SP-B deficiency. *Am J Respir Crit Care Med.* 2000;161:973–981.
- 71 Dunbar AE, 3rd, Wert SE, Ikegami M, et al. Prolonged survival in hereditary surfactant protein B (SP-B) deficiency associated with a novel splicing mutation. *Pediatr Res.* 2000;48:275–282.
- 72 Ballard PL, Noguee LM, Beers MF, et al. Partial deficiency of surfactant protein B in an infant with chronic lung disease. *Pediatrics.* 1995;96:1046–1052.
- 73 Nesslein LL, Melton KR, Ikegami M, et al. Partial SP-B deficiency perturbs lung function and causes airspace abnormalities. *Am J Physiol Lung Cell Mol Physiol.* 2005;288:L1154–L1161.
- 74 Beers MF, Hamvas A, Moxley MA, et al. Pulmonary surfactant metabolism in infants lacking surfactant protein B. *Am J Respir Cell Mol Biol.* 2000;22:380–391.
- 75 Tredano M, Cooper DN, Stuhmann M, et al. Origin of the prevalent SFTPB indel g.1549C > GAA (121ins2) mutation causing surfactant protein B (SP-B) deficiency. *Am J Med Gen A.* 2006;140:62–69.
- 76 Garmany TH, Wambach JA, Heins HB, et al. Population and disease-based prevalence of the common mutations associated with surfactant deficiency. *Pediatr Res.* 2008;63:645–649.
- 77 Garmany TH, Moxley MA, White FV, et al. Surfactant composition and function in patients with ABCA3 mutations. *Pediatr Res.* 2006;59:801–805.
- 78 Li J, Ikegami M, Na CL, et al. N-terminally extended surfactant protein (SP) C isolated from SP-B-deficient children has reduced surface activity and inhibited lipopolysaccharide binding. *Biochemistry.* 2004;43:3891–3898.
- 79 Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res.* 2001;11:1156–1166.
- 80 Shulenin S, Noguee LM, Annilo T, Wert SE, Whitsett JA, Dean M. ABCA3 gene mutations in newborns with fatal surfactant deficiency. *New Engl J Med.* 2004;350:1296–1303.
- 81 Fitzgerald ML, Xavier R, Haley KJ, et al. ABCA3 inactivation in mice causes respiratory failure, loss of pulmonary surfactant, and depletion of lung phosphatidylglycerol. *J Lipid Res.* 2007;48:621–632.
- 82 Cheong N, Zhang H, Madesh M, et al. ABCA3 is critical for lamellar body biogenesis in

- vivo. *J Biol Chem.* 2007;282:23811–23817.
- 83 Ban N, Matsumura Y, Sakai H, et al. ABCA3 as a lipid transporter in pulmonary surfactant biogenesis. *J Biol Chem.* 2007;282:9628–9634.
- 84 Bullard JE, Wert SE, Whitsett JA, Dean M, Noguee LM. ABCA3 mutations associated with pediatric interstitial lung disease. *Am J Respir Crit Care Med.* 2005;172:1026–1031.
- 85 Wambach JA, Casey AM, Fishman MP, et al. Genotype-phenotype correlations for infants and children with ABCA3 deficiency. *Am J Respir Crit Care Med.* 2014;189:1538–1543.
- 86 Hallik M, Annilo T, Ilmoja ML. Different course of lung disease in two siblings with novel ABCA3 mutations. *Eur J Pediatr.* 2014;173(12):1553–1556.
- 87 Campo I, Zorzetto M, Mariani F, et al. A large kindred of pulmonary fibrosis associated with a novel ABCA3 gene variant. *Respir Res.* 2014;15:43.
- 88 Matsumura Y, Ban N, Ueda K, Inagaki N. Characterization and classification of ATP-binding cassette transporter ABCA3 mutants in fatal surfactant deficiency. *J Biol Chem.* 2006;281:34503–34514.
- 89 Matsumura Y, Ban N, Inagaki N. Aberrant catalytic cycle and impaired lipid transport into intracellular vesicles in ABCA3 mutants associated with nonfatal pediatric interstitial lung disease. *Am J Physiol Lung Cell Mol Physiol.* 2008;295:L698–707.
- 90 Cheong N, Madesh M, Gonzales LW, et al. Functional and trafficking defects in ATP binding cassette A3 mutants associated with respiratory distress syndrome. *J Biol Chem.* 2006;281:9791–9800.
- 91 Wambach JA, Wegner DJ, Depass K, et al. Single ABCA3 mutations increase risk for neonatal respiratory distress syndrome. *Pediatrics.* 2012;130:e1575–1582.
- 92 Baekvad-Hansen M, Nordestgaard BG, Dahl M. Heterozygosity for E292V in ABCA3, lung function and COPD in 64,000 individuals. *Respir Res.* 2012;13:67.
- 93 Thomas AQ, Lane K, Phillips J, 3rd, et al. Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. *Am J Respir Crit Care Med.* 2002;165:1322–1328.
- 94 Gower WA, Noguee LM. Surfactant dysfunction. *Paediatr Respir Rev.* 2011;12:223–229.
- 95 Cameron HS, Somaschini M, Carrera P, et al. A common mutation in the surfactant protein C gene associated with lung disease. *J Pediatr.* 2005;146:370–375.
- 96 Mulugeta S, Maguire JA, Newitt JL, Russo SJ, Kotorashvili A, Beers MF. Misfolded BRICHOS SP-C mutant proteins induce apoptosis via caspase-4- and cytochrome c-related mechanisms. *Am J Physiol Lung Cell Mol Physiol.* 2007;293:L720–729.
- 97 Maguire JA, Mulugeta S, Beers MF. Endoplasmic reticulum stress induced by surfactant protein C BRICHOS mutants promotes proinflammatory signaling by epithelial cells. *Am J Respir Cell Mol Biol.* 2011;44:404–414.
- 98 Bridges JP, Xu Y, Na CL, Wong HR, Weaver TE. Adaptation and increased susceptibility to infection associated with constitutive expression of misfolded SP-C. *J Cell Biol.* 2006;172:395–407.
- 99 Lawson WE, Cheng DS, Degryse AL, et al. Endoplasmic reticulum stress enhances fibrotic remodeling in the lungs. *Proc Natl Acad Sci U S A.* 2011;108:10562–10567.
- 100 Stewart GA, Ridsdale R, Martin EP, et al. 4-phenylbutyric acid treatment rescues trafficking and processing of a mutant surfactant protein-C. *Am J Respir Cell Mol Biol.* 2012;47:324–331.
- 101 Beers MF, Hawkins A, Maguire JA, et al. A nonaggregating surfactant protein C mutant is misdirected to early endosomes and disrupts phospholipid recycling. *Traffic.* 2011;12:1196–1210.
- 102 van Moersel CH, van Oosterhout MF, Barlo NP, et al. Surfactant protein C mutations are the basis of a significant portion of adult familial pulmonary fibrosis in a Dutch cohort. *Am J Respir Crit Care Med.* 2010;182:1419–1425.
- 103 Lawson WE, Grant SW, Ambrosini V, et al. Genetic mutations in surfactant protein C are a rare cause of sporadic cases of IPF. *Thorax.* 2004;59:977–980.
- 104 Boggaram V. Thyroid transcription factor-1 (TTF-1/Nkx2.1/TTF1) gene regulation in the lung. *Clin Sci.* 2009;116:27–35.
- 105 Krude H, Schutz B, Biebermann H, et al. Choreoathetosis, hypothyroidism, and pulmonary alterations due to human NKX2-1 haploinsufficiency. *J Clin Invest.* 2002;109:475–480.
- 106 Devriendt K, Vanhole C, Matthijs G, de Zegher F. Deletion of thyroid transcription factor-1 gene in an infant with neonatal thyroid dysfunction and respiratory

- failure. *New Engl J Med.* 1998;338:1317–1318.
- 107 Guillot L, Carre A, Szinnai G, et al. NKX2-1 mutations leading to surfactant protein promoter dysregulation cause interstitial lung disease in “Brain-Lung-Thyroid Syndrome.” *Hum Mutat.* 2010;31:E1146–1162.
- 108 Hamvas A, Deterding RR, Wert SE, et al. Heterogeneous pulmonary phenotypes associated with mutations in the thyroid transcription factor gene NKX2-1. *Chest.* 2013;144:794–804.
- 109 Wert SE, Whitsett JA, Noguee LM. Genetic disorders of surfactant dysfunction. *Pediat Dev Pathol.* 2009;12:253–274.
- 110 Maitra M, Cano CA, Garcia CK. Mutant surfactant A2 proteins associated with familial pulmonary fibrosis and lung cancer induce TGF-beta1 secretion. *Proc Natl Acad Sci U S A.* 2012;109:21064–21069.
- 111 Wang Y, Kuan PJ, Xing C, et al. Genetic defects in surfactant protein A2 are associated with pulmonary fibrosis and lung cancer. *Am J Hum Gen.* 2009; 84:52–59.
- 112 Suzuki T, Sakagami T, Young LR, et al. Hereditary pulmonary alveolar proteinosis: pathogenesis, presentation, diagnosis, and therapy. *Am J Respir Crit Care Med.* 2010;182:1292–1304.

Initiation of Breathing at Birth

Arjan B. te Pas and Stuart Hooper

Abstract

Recent animal and human studies have challenged some of the prevailing concepts regarding the major physiological changes that characterize the transition to life after birth. In this chapter we will explain in detail how the breathing effort initiates a pulmonary and hemodynamic cascade of events that is of vital importance for survival after birth. The role of cord clamping and its effects on the hemodynamic transition will be discussed. This chapter also covers the pathophysiology of preterm infants failing transition and the current strategies caregivers can use to support them. Finally, we describe how positive pressure ventilation (PPV) can cause not only lung injury, but can also adversely affect cardiovascular function and initiate a systemic inflammatory cascade that can injure the immature brain.

Keywords:

Birth, newborn, breathing, lung liquid, lung aeration, pulmonary vascular resistance, cord clamping, respiratory distress syndrome, lung injury, brain injury

Introduction

Breathing initiates the physiological changes that characterize the transition to life after birth. The respiratory efforts made by the infant must be sufficient to clear the liquid so that air can enter the distal airways and pulmonary gas exchange can begin. The infant then uses breathing patterns, largely characterized by braking of expiratory gas flow, to maintain functional residual capacity (FRC). The FRC increases during each inspiration and is maintained by braking during expiration. Because lung aeration is the trigger for dilation of the pulmonary vascular bed and a marked increase in pulmonary blood flow (PBF), breathing is also important for a successful hemodynamic transition. The increase in pulmonary venous return to the left atrium is critical for replacing the loss of umbilical venous return from the placenta when the umbilical cord is clamped. Our understanding of the physiology of transition is mostly based on animal studies and extrapolation from human fetal data from the 1970s. However, recent animal and human studies have challenged some of the prevailing concepts of transition as well as the causes and consequences for when this transition fails.

Although most preterm infants make respiratory efforts at birth, failure to aerate the lungs, recruit an FRC, and establish adequate gas exchange is most common in preterm infants. The weak respiratory muscles and surfactant

deficiency makes them unable to generate sufficient inspiratory pressures to overcome the high surface tension and frictional forces to achieve effective lung aeration. The compliant rib cage reduces the efficiency of the diaphragmatic contractions and is not able to resist lung recoil, leading to small tidal volumes and low FRCs. In addition, the small surface area and thick air/blood gas barrier further impair gas exchange. As epithelial sodium channel expression is absent in the immature lung, the lung is unable to reabsorb sodium, thereby increasing the likelihood of liquid reentering the airways.

Consequently, preterm infants, particularly those born prior to 29 weeks of gestation, often require respiratory support at birth. The initiation of respiratory support in the delivery room, typically with intermittent positive pressure ventilation (PPV), via a face mask or after intubation, is often critical to ensure a successful respiratory transition. Caregivers prefer noninvasive ventilation to support the transition to avoid intubation and mechanical ventilation at birth, as mechanical ventilation is associated with an increased risk of lung and brain injury. Although different noninvasive strategies have been tested clinically, the mechanisms and effects of mask ventilation on lung aeration and respiratory function at birth are not well understood. The development of the best respiratory strategies at birth is not only important to reduce the risk of lung injury, but to also minimize the adverse effects of ventilation

on the cardiovascular system and brain injury. Lung injury with the initiation of an inflammatory response within the lung can have direct and indirect effects on the cardiovascular system, systemic circulation, and cerebral circulation. In this chapter we will discuss the physiology of pulmonary transition at birth, the clinical supporting strategies when transition fails, and the injury that can result.

Respiratory Drive

Fetal breathing movements (FBMs) can be observed as early as 12 weeks of gestation (1) and are vital for normal lung growth and development. FBMs have similarities with breathing activity after birth, are sleep-state dependent, and are controlled by descending output from the respiratory center. This output is influenced by numerous physiological stimuli, including hypoxia and hypercapnia, and activates the diaphragm and other inspiratory muscles such as adductor and abductor muscles of the larynx (1,2), FBMs are restricted to periods of fetal activity and are discontinuous, occurring <50% of the time (1). FBMs primarily occur during a state resembling rapid eye movement sleep, whereas during episodes of “quiet sleep,” fetuses are largely apneic, with an adducted glottis (3). Most FBMs generate transpulmonary pressures of <20 cmH₂O, and as the chest wall is very compliant, regions of the chest wall collapse during inspiration as the diaphragm contracts (4). As a result, liquid tidal volumes are small and difficult to measure (2). Fetuses near term can make large inspiratory efforts (>30 cmH₂O) (5), demonstrating that they are capable of generating the large transpulmonary pressures needed to rapidly aerate the lungs after birth (6). Leading up to and particularly during active labor, the incidence of FBMs is reduced. Although the mechanisms are not well understood, the release of prostaglandins from the placenta and adenosine from the liver and placenta may suppress FBMs (1,7).

The factors that trigger the onset of large inspiratory efforts at birth are not clear but are thought to include activation of chemoreceptors, increased PaCO₂ levels, loss of inhibitory factors on respiratory center activity and physical stimuli (light, temperature, and handling). During parturition the infant may become hypercapnic,

which is a powerful stimulant for respiratory drive both before and after birth and could contribute to the large respiratory efforts. Although central and peripheral chemoreceptors are active in the fetus (8), their sensitivity may be tonically suppressed by factors released from the placenta into the fetal circulation (9). When the umbilical cord is cut at birth and the placenta is removed from the fetal circulation, there may be an increased ventilatory sensitivity to CO₂ due to the removal of an inhibitory factor of placental origin. Such factors could include prostaglandin E₂ (PGE₂), adenosine and progesterone metabolites (9, 10), which are thought to act at the level of the brain stem (11).

At birth, the infant is also exposed to lower environmental temperatures resulting in increased heat loss. The fetus will also be exposed to greatly increased external sensory stimuli, and the behavioral state will change to arousal, which could also be attributed to the removal of placental factors (12). Data on physical stimuli are scarce, although cooling lambs at birth elicits normal quiet breathing, but no large initial inspiratory efforts (13). In contrast, painful stimuli elicit gasps in anesthetized lambs with an intact umbilical cord, but not sustained respiratory movements (14).

During parturition, the infant may become mildly hypoxic. Although hypoxia is a stimulus for respiratory drive in adults, it remains questionable if hypoxia contributes to the increased respiratory drive at birth (5,15). Hypoxia is a potent inhibitor of FBM prenatally due to direct inhibitory neural input to the respiratory center from a region located in the upper lateral pons (16). Although it is unclear when this inhibitory effect is removed, the hypoxic sensitivity is low in newborns shortly after birth (5). Then during the first few weeks after birth, hypoxia increasingly stimulates respiratory drive due a temporal change in O₂ sensitivity (5). Thus, immediately after birth, hypoxia may inhibit respiratory drive and cause adduction of the glottis, particularly in preterm infants. Indeed, in preterm lambs, maturation of the increase in O₂ sensitivity is delayed (5). On the other hand, hyperoxia could also inhibit the chemoreceptors, as a delay in onset of breathing was observed in asphyxiated infants and animals resuscitated with 100% oxygen (17). Although the pathways are largely unidentified, lung volume receptors

(pulmonary stretch receptors) may have essential role in breathing at birth (18,19). Volume receptive vagal feedback at end-expiration, which is normally maintained by an adequate FRC, is essential for continuous breathing in the newborn.

Airway Liquid Clearance and Lung Aeration at Birth

Airway Liquid Clearance Before Labor

In utero, lung development is largely dependent on the volume of liquid retained within the fetal airways. This liquid is secreted by the pulmonary epithelium and maintains the lungs in a distended state at a volume that is greater than the volume of air in the lung (FRC) after birth. It is questionable whether lung liquid clearance normally begins days before labor onset because the proposed mechanisms such as a reduction in lung liquid secretion rates cannot be verified experimentally (20). Indeed, a reduction in fetal lung liquid secretion rates simply results in a simultaneous reduction in liquid loss via the trachea, resulting in no net change in lung liquid volume (21,22). Similarly, other studies have failed to show a decrease in lung liquid volumes before labor onset unless the pregnancy was associated with reduced amniotic fluid volumes (20). Based on these findings (23,24), the suggestion that lung liquid volumes decreased in the week before birth may have been based on an experimental artifact (amniotic fluid loss following fetal surgery) (4). More recent studies have shown that healthy fetuses near term with normal amniotic fluid volumes do not have a decrease in lung liquid volumes (20).

As the fetal respiratory system is very compliant, only small changes in transpulmonary pressures are needed to cause large changes in lung liquid volumes late in gestation. Thus, simple changes in fetal posture, which markedly increase the transpulmonary pressure gradient, will increase lung liquid loss (23). For instance, lung liquid loss could occur from decreases in intrauterine volume, caused by amniotic liquid loss, the presence of a twin, or nonlabor uterine contractions. These pregnancy related events can increase flexion of the fetal trunk, increase abdominal pressure, elevate the diaphragm, and increase lung liquid efflux via the trachea (4).

Airway Liquid Clearance During Labor

The “vaginal squeeze” leading to large “gushes” of liquid following delivery of the infant’s head is still considered an important mechanism for airway liquid clearance by perinatal caregivers (25). However, as delivery of the chest offers little resistance as it passes through the birth canal compared to the head and shoulders makes it unlikely that a “vaginal squeeze” per se significantly influences liquid clearance (26). Instead, large amounts of liquid can be lost in response to changes in fetal posture caused by membrane rupture and amniotic fluid loss in association with uterine contractions and shortening of the myometrium. These events increase transpulmonary pressure gradients and lung liquid loss in both sheep (23) and humans (27). Indeed, marked reductions in airway liquid volumes were observed shortly after labor onset in sheep (indicated by uterine EMGs), many hours before the second stage of labor commences (20).

Until recently, the primary mechanism driving airway liquid clearance was thought to involve activation of amiloride-inhibitable epithelial Na^+ channels (ENaCs) (2,28), which reverses the osmotic gradient across the pulmonary epithelium that normally drives fetal lung liquid secretion. Increased release of circulating adrenaline and arginine vasopressin (AVP) during the stress of active labor induces a cAMP-mediated activation of ENaCs (2). This leads to Na^+ and Na^+ -linked chloride ion flux across the pulmonary epithelium, from lumen into the interstitium, reversing the osmotic gradient and liquid movement across the epithelium (28). Indeed, adrenaline and AVP can inhibit fetal lung liquid secretion and initiate liquid reabsorption, but only late in gestation. Furthermore, the ability of adrenaline and AVP to stimulate lung liquid reabsorption increases in an exponential-like manner close to term and is dependent on the actions of both cortisol and triiodothyronine (T3) (29–32). However, this mechanism is thought to be inactive during preterm birth, which is consistent with the finding that RNA transcripts for epithelial sodium channels (ENaC) are virtually undetectable in the lung of preterm infants (33). Although this may partially explain why preterm infants commonly have liquid retained in the airways, the majority of preterm infants are able to aerate their lungs and begin ventilation within

minutes of birth, presumably in the absence of this mechanism (34).

Airway Liquid Clearance at Birth

Although adrenaline-induced ENaC activation likely plays a role, it is unlikely to be the primary mechanism for airway liquid clearance at birth (35,36). Indeed, ENaC activation-induced liquid clearance is orders of magnitude too slow to clear the airways of liquid within seconds to minutes after birth (37,38). X-ray images (see following) demonstrate that lung aeration occurs at a rate of $\sim 3\text{mL/kg/sec}$ during inspiration (36,39), which is considerably greater than the maximum reabsorption rates that can be achieved with pharmacological doses of adrenaline ($\sim 10\text{mL/kg/h}$) (28,40,41). As a result, adrenaline would need to be elevated for hours to clear the airways of all liquid (28,40,41). Sustained high levels of adrenaline would also result in a sustained tachycardia, which does not occur in healthy infants after birth (42). In addition, although ENaC knockout mice (43,44) have respiratory failure, they can survive for hours after birth (33,43). As lung wet weights were increased, it was assumed that adrenaline-induced activation of Na reabsorption was disrupted by deletion of the α -ENaC gene (43). However, these newborn mice also did not feed well and had poor costal retractions, indicating that energy supply and inspiratory activity

were likely reduced (33). Indeed, deletion of β - and γ -ENaC subunits did not cause respiratory failure in newborn pups, despite reducing ENaC activity sixfold (33). Similarly, infants that have gene mutations that markedly reduce ENaC activity (pseudoaldosteronism) do not have respiratory failure at birth (45). Furthermore, although ENaC inhibition with amiloride delays liquid clearance in experimental animals (46,47), it does not prevent lung liquid clearance from occurring (48).

Recently phase-contrast X-ray imaging was used to investigate in detail airway liquid clearance after birth (35,36,49,50). Phase-contrast X-ray imaging uses both absorption and the refractive index differences between air and water to produce contrast, allowing air/water boundaries to be visualized (51). As the lung is 80% air filled at FRC, this technique is ideal for imaging the lung, allowing the small airways (including alveoli) to be resolved with a high degree of spatial resolution. By phase contrast x-ray imaging, amiloride had no effect on airway liquid clearance or FRC recruitment during mechanical ventilation (50). Instead, amiloride increased the rate of liquid reentry into the airways when the lung was at FRC, thereby decreasing FRC between inflations (Figure 9-1). Na^+ reabsorption after birth may help to keep the airways cleared of liquid and to maintain FRC after the liquid has been cleared (50).

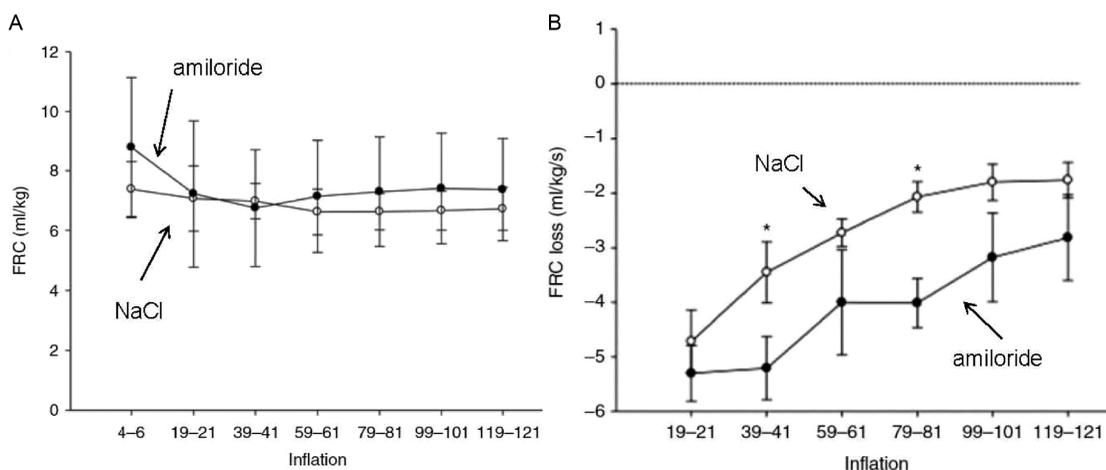


Figure 9-1. (A) Changes in FRC of rabbit pups (30 days) treated with either an epithelial sodium channel blocker (amiloride; filled circles) or saline (open circles) and mechanically ventilated from birth relative to mechanical breath number on the x-axis. No difference in FRC was observed between the amiloride and saline-treated groups. (B) Rate of change in FRC during the expiratory phase, in rabbit pups (30 days) ventilated from birth and treated with either an epithelial sodium channel blocker (amiloride; filled circles) or saline (open circles); a negative value indicates a net reduction in FRC. The decrease in FRC was significantly greater in the amiloride-treated group. Data redrawn from Siew, Wallace, Allison, et al. *Pediatr Res.* 2013 Apr;73 (4 Pt 1):443–449.

Airway Liquid Clearance After Birth

Phase-contrast X-ray image sequences and simultaneous plethysmography of spontaneously breathing term newborn rabbits at birth demonstrate that lung liquid clearance occurs during inspiration (36,39). The increase in transpulmonary hydrostatic pressure gradients generated during inspiration are likely responsible for the majority of airway liquid clearance at birth. The X-ray movie sequences clearly show that the air/liquid interface only moves distally toward the terminal airways during inspiration and, although some proximal movement can occur during expiration, little or no distal movement occurs between breaths (36,39) (Figure 9-2). Thus, liquid moves from the airways into the surrounding tissue during inspiration, and little or no liquid reenters during expiration. As a result, FRC accumulates with each breath and the FRC volume increase equals the volume of liquid leaving the airways (36,39) (Figure 9-3). These findings confirm the causal link between spontaneous breathing and FRC accumulation after birth reported using a variety of different animal models (44,52).

Inspiration causes expansion-induced pressure reductions in both the intrapleural space and perialveolar interstitial tissue (36), which

generates a pressure gradient between the airways and surrounding tissue (across the airway wall) and between the lower and upper airways. These pressure gradients drive liquid movement distally through the airways and across the epithelium and into the tissue. Within the perialveolar tissue, the liquid forms into perivascular fluid cuffs, from where it is gradually cleared via the pulmonary vasculature and lymphatics (53). As this can take hours, the retention of liquid within the tissue causes pulmonary interstitial tissue pressures to transiently (~4 h) increase (54) (Figure 9-4) and the chest wall to expand immediately after birth (39) (Figure 9-5). Chest wall expansion is required to accommodate the increase in gas volume as well as the volume of liquid within the interstitial tissue that resided within the airways before lung aeration (39). This understanding provides a rational explanation for why an infant needs to have a compliant chest wall at birth. That is, a compliant chest wall will minimize the increase in interstitial tissue pressure associated with increases in intrathoracic volume. If the chest wall was not compliant, the increased pressure required to increase intrathoracic volumes would necessarily increase interstitial tissue pressures to much higher levels and increase the likelihood of liquid reentry into the airways at FRC.

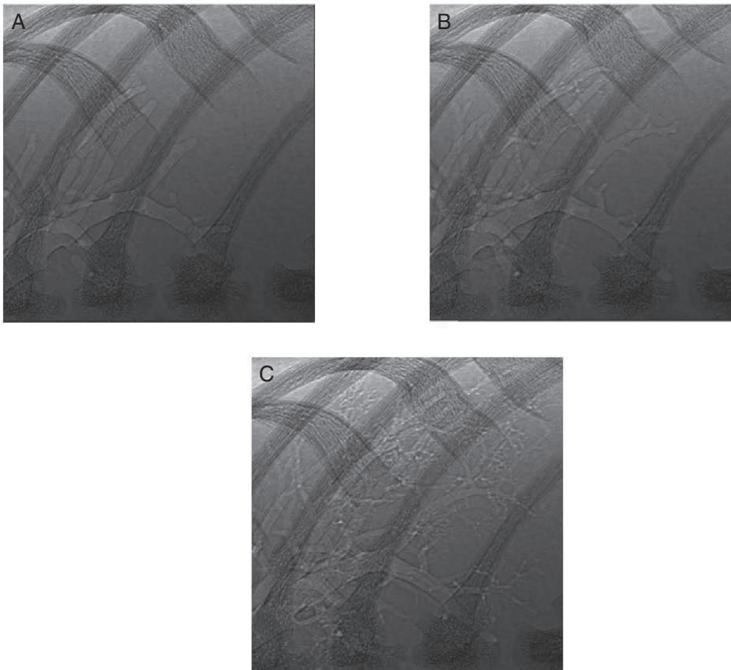


Figure 9-2. Phase-contrast X-ray images acquired during lung aeration in a spontaneously breathing term rabbit pup. The three images show a close-up view of the non-dependent lower right bronchus for consecutive breaths. (A) Before inspiration, the larger airways are aerated, and the air-liquid interface is visible in the large proximal airways. (B) Following the subsequent inspiration, some smaller airways have aerated, and the air-liquid interface has moved more distally. In between inspirations there is very little or no proximal or distal movement of the air-liquid interface (C).

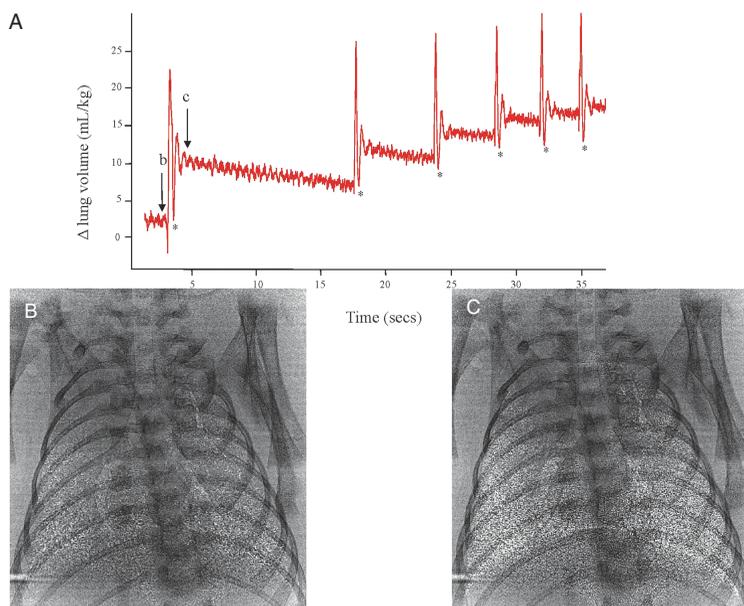


Figure 9-3. Plethysmograph recording of the first breaths of a spontaneously breathing term rabbit pup after birth. Each rapid large increase in lung volume (in mL/kg) is an inspiratory effort and results in a stepwise accumulation of FRC after each breath. The phase contrast X-ray images were acquired immediately before (image B) and immediately after (image C) an inspiration effort, demonstrating the degree of lung aeration achieved with one breath. Data redrawn from Siew, Wallace, Kitchen, et al. *J Appl Physiol*. 2009 Jun;106(6):1888–1895. Following the next inspiration, some terminal airways become visible.

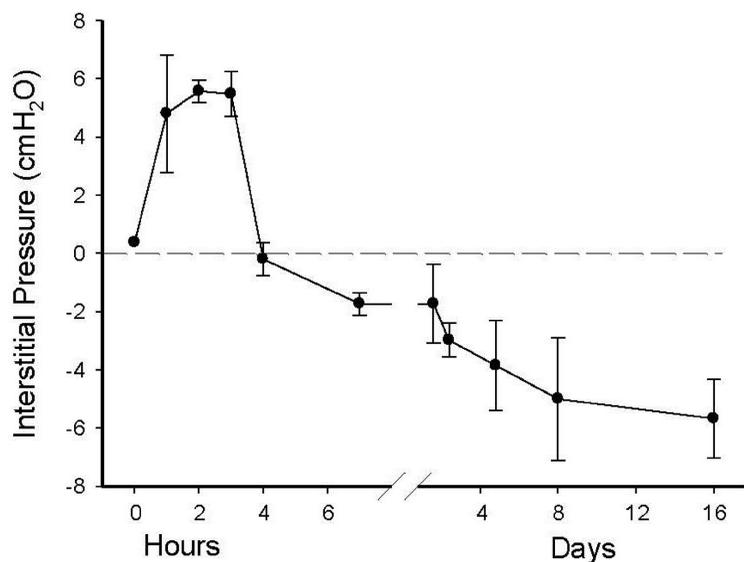


Figure 9-4. Pulmonary interstitial tissue pressure measured in a term rabbit lung immediately after birth. As the lung aerates, liquid leaves the airways and enters the interstitial tissue compartment at a much greater rate than it is cleared from the tissue by the lymphatics and blood vessels. As a result, pressures initially increase before they decrease and remain subatmospheric. Data redrawn from Miserocchi, Poskurica Del. *J Appl Physiol* (1985). 1994 Nov;77(5):2260–2268.

Small transient (~4h) increases in interstitial tissue pressure normally occur at birth (54), which facilitates liquid reentry into the airways. This is consistent with a gradual decline in FRC that was noted between breaths (36,50), but the rate of liquid reentry is considerably slower than the rate at which it leaves the airways during inspiration, largely due to the differences in pressure gradients (36, 50). Furthermore, the more airway liquid present at birth, the greater the volume of liquid that must be accommodated in the interstitial tissue compartment. As this compartment has a fixed volume, this will lead to higher interstitial tissue pressures and a greater potential for the

reentry of liquid into the airways. This explains why infants born by caesarean section are more likely to have “wet lung” or transient tachypnea of the newborn (44). To prevent reentry of lung liquid these infants perform expiratory braking maneuvers (grunting) and have tachypnea. Continuous positive airway pressure (CPAP) is an effective treatment for “wet lung.”

The Consequences of Lung Recoil

Although lung volumes are commonly thought to be similar after birth and during fetal life, the generation of surface tension when air enters

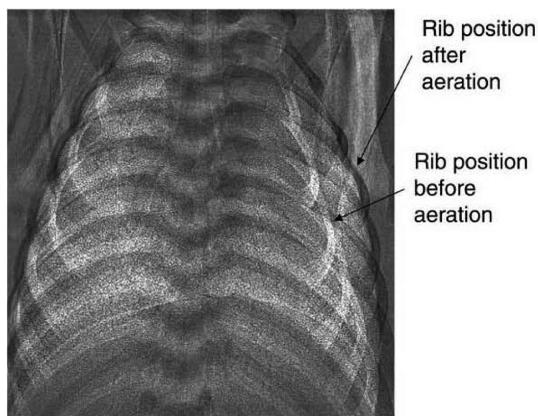


Figure 9-5. Phase-contrast X-ray imaging showing rib positions before and after lung aeration. The ribs are positioned more horizontally and displaced laterally after lung aeration, indicating a substantially increased thoracic volume caused by the increase in air volume (without the loss of liquid, which has accumulated in interstitium)

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the lung increases lung recoil and causes resting lung volumes to decrease after birth, despite the presence of surfactant. As air is compressible, it is less able to oppose the increase in lung recoil caused by surface tension and, combined with the absence of the distending influence of lung liquid, leads to a reduction in lung expansion (2, 4). This reduction helps to explain a number of physiological changes that occur after birth. For instance, the increase in lung recoil and the partial collapse of the lung away from the chest wall explains why intrapleural pressures become subatmospheric after birth (55). Before birth, intrapleural pressures are similar to ambient (amniotic fluid) pressure (55), but within hours of birth, they decrease to 2–4 cmH₂O below atmospheric pressure (54,55). This indicates that the mechanical load experienced by the chest wall has increased with the increase in lung recoil, which likely is important for stiffening the chest wall after birth (56). Similarly, as the liquid is cleared from the interstitial tissue space after birth, interstitial tissue pressures decrease and become subatmospheric and are similar to intrapleural pressures (54). As a result, the interstitial tissue/capillary wall transmural pressures must increase to facilitate capillary recruitment and expansion, which will help sustain the reduction in pulmonary vascular resistance (PVR) after birth (see below).

In addition, the decrease in lung expansion has a profound effect on alveolar epithelial cell (AEC)

populations (57). Although increased fetal lung expansion increases the proportions of type-I AECs (57), a reduction in lung expansion leads to increased transdifferentiation of type I AECs into type-II AECs (surfactant-producing cells) (58). The proportion of type-I AECs decrease from 60–65% in the fetus to ~30% in the newborn, whereas the proportion of type-II cells increase from ~30% to 50–55% after birth (59).

Hemodynamic Consequences of Lung Aeration

The fetal circulation has two shunts, the foramen ovale (FO) and ductus arteriosus (DA), which allow both ventricles to work independently and to supply output to the systemic circulation. The FO allows venous return to bypass the right side of the heart and directly enter the left atrium, whereas the DA shunts blood from the main pulmonary artery into the descending aorta. As a result, in fetal sheep, the right ventricle (RV) provides 66% of combined ventricular output, with 33% coming from the left ventricle (LV) (60). Before birth PVR is high and PBF is low (61), and only a small proportion (~10%) of RV output flows through the lungs, with the majority bypassing the lungs and entering the systemic circulation through the DA (right-to-left shunting) (60). Fetal PBF is not persistently low, as is commonly reported, but can vary 10-fold, depending on fetal activity, particularly late in gestation (62). For instance, FBMs significantly increase PBF due to a decrease in PVR, which is thought to result from an increase in the capillary/interstitial tissue transmural pressure (62). This increase in transmural pressure causes capillary distension and recruitment, which increases PBF in close association with inspiratory effort. Each individual FBM causes large changes in the PBF waveform, although the predominant effect is a marked reduction in the amount of retrograde flow during diastole, which is indicative of reduced PVR (62) (Figure 9-6).

The consequence of a high PVR and a low PBF in the fetus is that pulmonary venous return is unable to provide sufficient preload to sustain LV output. Instead, preload for the LV is primarily derived from umbilical venous return in the fetus, which flows via the ductus venos, inferior vena cava, and FO directly into the left atrium (60).

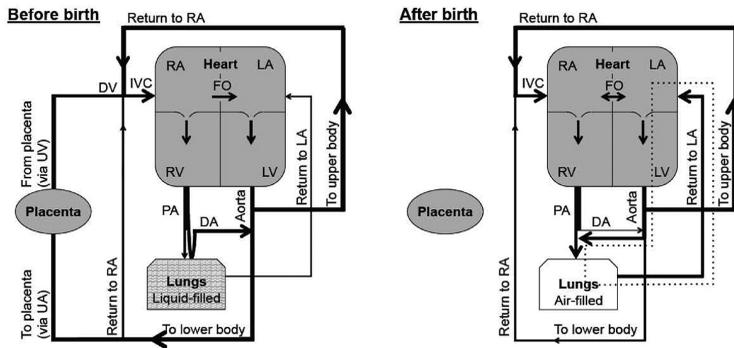


Figure 9-6. A diagrammatic representation of the circulations before and after birth. Lung aeration after the disconnection of the infant from the placental circulation increases PBF, reverses ductal flow, and establishes a LV-lung-LV short circuit (dotted lines) that maintains LV output. UA; umbilical artery. UV; umbilical vein. DV; ductus venosus. IVC; inferior vena cava. RA; right atrium. LA; left atrium. RV; right ventricle. FO; foramen ovale. LV; left ventricle. PA; pulmonary artery. DA; ductus arteriosus.

As the umbilical circulation is a low resistance vascular bed, it receives ~30–50% of the combined ventricular output of the fetus (60). As a consequence, umbilical venous return is large (30–50% of total) and supplies the majority of LV preload in the fetus. Thus, clamping the umbilical cord at birth provides a major disturbance to the fetal circulation, particularly to afterloads and the supply of venous return and preload for both ventricles (63). Specifically, removal of the low-resistance umbilical circulation with cord clamping markedly increases downstream peripheral resistance (63). This results in ~30% increase in arterial blood pressure over the first four heartbeats after cord clamping, which in turn causes a transient, pressure-driven increase in cerebral blood flow (63). However, both RV and LV output then markedly decrease (by ~50%), mostly due to the loss of umbilical venous return caused by umbilical cord clamping (63, 64). This large reduction in preload decreases cardiac output and causes a transient decrease in blood pressure (63, 64). Cardiac output remains low, due to the low preload, until ventilation increases PBF. At this time, the rapid and large increase in PBF restores preload to the LV by increasing pulmonary venous return, thereby markedly increasing cardiac output. The increase in PBF also likely restores some preload to the RV, presumably via left-to-right flow through the FO, as RV output also rapidly increases (63).

The increase in PBF at birth is essential for pulmonary gas exchange and to replace umbilical venous return as the primary source of preload for the LV. The increase in systemic vascular resistance with umbilical cord clamping and decrease in PVR with lung aeration has the net effect to decrease PVR below systemic vascular resistance with a reversal in blood flow (from

right-to-left to left-to-right) through the DA (64). Within ~10–20 mins of the onset of ventilation in lambs, the extent of the left-to-right shunting through the DA is so large that the LV contributes up to ~50% of PBF, resulting in a substantial LV-lung-LV short circuit within the systemic circulation (64). The consequence of this is an increase in cardiac output, which in turn causes a rebound increase in arterial pressure and carotid blood flow (63). Thus, following umbilical cord occlusion, the loss of umbilical venous return and preload causes substantial reductions in cardiac output (CO), which cannot be restored until the lung aerates and PBF increases. This represents a major disturbance to the fetal circulatory system, resulting in large swings in CO that cause large changes in blood pressures and flows. To avoid these large swings in CO, umbilical cord clamping should be delayed until after ventilation has commenced and PBF has increased. Delayed cord clamping allows the source of preload for the left ventricle to switch from the umbilical circulation to pulmonary venous return without significant interruption (63). As a result, CO does not decrease following cord clamping, and heart rates remain much higher (63). Interestingly, the 30% increase (over four heartbeats) in arterial blood pressure caused by cord clamping is also greatly reduced when cord clamping follows lung aeration, indicating that the decrease in PVR can mostly compensate for the increase in systemic vascular resistance caused by cord clamping (63). This suggestion is consistent with the finding that, following lung aeration, despite a decrease in PVR, shunting through the DA remains right-to-left while the umbilical cord is open (unclamped) (63). This indicates that downstream resistance in the systemic circulation remains lower than the

pulmonary circulation. However, immediately (within seconds) after the cord is clamped, flow through the DA switches and becomes left-to-right, indicating that after removal of the umbilical circulation, downstream resistance is lower in the pulmonary circulation than in the systemic circulation (63).

Changes in the Ductus Arteriosus Flow at Birth

After birth, the large decrease in PVR and increase in systemic vascular resistance, reverses the pressure gradient across the DA and blood predominantly shunts from left-to-right (from the systemic to the pulmonary circulation) (64). As a result, retrograde flow in the left and right pulmonary arteries quickly decreases (within minutes of birth), resulting in only forward flow through the pulmonary arteries even during diastole (64). However, the DA flow profile is complex with small amounts of right-to-left shunting persisting during peak systole, whereas for the remainder of the cardiac cycle blood flows mostly left-to-right (64). This transition occurs in ventilated lambs and in spontaneously breathing term infants born by caesarean section (65). While the DA remains open, blood will pass between the pulmonary and systemic circulations, depending on the pressure gradient across this vessel, thereby preventing any substantial decrease in pulmonary arterial pressure. Functional closure of the DA begins within hours of birth in lambs and is clearly evident by the substantial decrease in both absolute DA flow, but most particularly by the pulsatile DA flow caused by ventricular contraction and relaxation (64). Although mediators responsible for DA closure have been identified, it is interesting to speculate whether the anatomic relationship between the pulmonary artery, DA and the descending aorta could be involved. Indeed, considerable turbulence must occur at this site when blood flow through the DA switches from right-to-left to left-to-right, which could elicit the local release of endothelial-derived vasoactive factors that contribute to constriction of the DA.

Strategies to Support Preterm Infants Failing Transition

For the majority of infants, the transition to newborn life is uneventful, and no intervention by the

caregiver is needed. However, approximately 3–5% of infants require some form of intervention, usually respiratory support at birth (66). On most occasions resuscitation is needed because respiratory drive is absent (asphyxia) or is insufficient due to immaturity (preterm infants). Adequate ventilation is then the key to successful resuscitation, and chest compressions or medication are rarely needed (67).

Preterm infants (< 32 weeks' gestational age) have more difficulties aerating their lungs than term infants (38), and approximately 60% of preterm infants need respiratory support at birth (68). Although most preterm infants breathe at birth, hypoxia during labor and at birth can inhibit their respiratory drive. Similarly, inspiratory efforts that are too weak to generate pressures to overcome the high surface tension and frictional forces associated with moving liquid through their airways will not achieve effective lung aeration. In addition, their respiratory muscles are underdeveloped, which contributes to their inability to generate sufficient pressure when there is insufficient surfactant (38). Further, the compliant chest wall leads to inward deformation during diaphragmatic contraction, thereby reducing the inspired tidal volume. The chest wall is also unable to resist lung recoil, which reduces resting lung gas volumes at end expiration (69, 70). Also adrenaline driven ENaC activation, which assists in preventing lung liquid returning to the airways, is largely absent in preterm infants, who are less able than term infants to maintain airways free of liquid (71,72).

Facilitating Lung Liquid Clearance and Lung Aeration in Preterm Infants

As the resistance to liquid flow through an airway is about 100 times greater than air, for a given pressure gradient the flow of liquid will be orders of magnitude slower than air. To overcome this higher resistance, either higher pressures or longer inflation times (i.e., greater pressure-time integral) are needed. To avoid the use of high pressures, a theoretically better alternative is to prolong the time over which the inflation pressure is applied (sustained inflation) (73). Our natural tendency is to think that inspiration should be brief and followed by expiration, as the function of expiration is to clear the lungs of CO₂.

However, when the distal airways are liquid filled, no gas exchange can occur, and so expiration serves no purpose.

In a series of preterm animal studies, sustained inflation pressures for up to 20 s duration aerated the lungs from the first inflation. Sustained inflations were also associated with more uniform aeration across the lung, a larger FRC and more consistent volume during consecutive tidal ventilation (74–76). Similarly, sustained inflations of 5 s were effective at aerating the lung of intubated asphyxiated term infants (77). Currently only limited clinical data on the effects of sustained inflations for ventilating preterm infants at birth are available. Lindner reported in a retrospective cohort study the use of a sustained inflation of 15–20 s in a noninvasive approach in very preterm infants (78). The study was repeated in a small randomized trial comparing a sustained inflation of 15 s with standard PPV. Although recruitment was slow and the study was stopped early, there was a trend for a lower rate of subsequent CPAP-failure in the sustained inflation group (79). Harling et al. randomly assigned preterm infants ($n = 52$) to a 2 versus a 5 s initial sustained inflation and found no difference in pro-inflammatory pulmonary cytokines as the primary outcome (80). Te Pas et al. used a sustained inflation of 10 s followed by CPAP in a delivery room strategy with a nasal tube and neopuff as compared to standard mask and bag ventilation. The rate of infants needing intubation and mechanical ventilation and the rate of bronchopulmonary dysplasia (BPD) decreased, but the specific contribution of sustained inflation was not isolated in this latter study (81). More recently a strategy using initial sustained inflations with positive end expiratory pressure (PEEP)/CPAP immediately after birth reduced the rate of intubation/mechanical ventilation and BPD in preterm infants as compared to historical controls using intermittent positive pressure ventilation (IPPV)/CPAP (82).

No specific recommendations have been made for the use of sustained inflations in international resuscitation guidelines. There are concerns of overinflation, which can cause lung injury in animals when using high inflation pressures (83, 84). However, sustained inflation did not cause overdistention in the imaging studies that accurately monitored lung distension (74, 75). Similarly the concern that a sustained inflation

will impede the increase in PBF at birth was not confirmed in a preterm lamb study. The increase of PBF at birth was similar in lambs given a sustained inflation (40 cmH₂O for 1 min or to a volume of 20 mL/kg) or ventilated with conventional PPV (76). However, oxygenation and respiratory function were markedly improved in the sustained inflation group. Similarly, no relevant adverse effects using sustained inflations, such as increased rates of pulmonary air leaks, intraventricular hemorrhage (IVH) or negative hemodynamic effects were reported in the randomized trials (79, 81, 82). On the contrary, Fuchs et al. demonstrated with near-infrared spectroscopy (NIRS) measurements that sustained inflation in preterm infants at birth increased cerebral oxygen saturation at least as fast as in full-term infants not requiring any respiratory support (85). In preterm lambs a sustained inflation is just as injurious as conventional PPV, although the peak inflating pressures used in that study were 50 cmH₂O, which may have resulted in some overdistension (86). Another recent preterm lamb study indicated that a prolonged (over minutes) lung recruitment strategy that used stepwise increases (to 20 cmH₂O) and decreases in PEEP was a more effective strategy than a sustained inflation for improving respiratory function at birth (87). However, considering that this strategy will be applied for minutes and that high PEEP levels can have severe effects on PBF (see following), this approach is likely to have a major detrimental impact on cardiovascular function. Further studies are needed to assess the appropriate pressure and inflation duration that will effectively aerate the lung without causing injury.

Maintaining FRC after Birth

To maintain FRC immediately after birth, preterm infants have to oppose two major counterforces: the increase in lung recoil caused by surface tension and supra-atmospheric pressures within the interstitial tissue that promote liquid reentry into the airways. Preterm infants frequently use expiratory braking maneuvers to prevent distal airway collapse and/or to prevent liquid from entering the airways (37,50). The infant actively expires against a closed/adducted glottis, thereby sustaining a supra-atmospheric pressure in the airways to prevent loss of FRC. In addition, by reducing surface

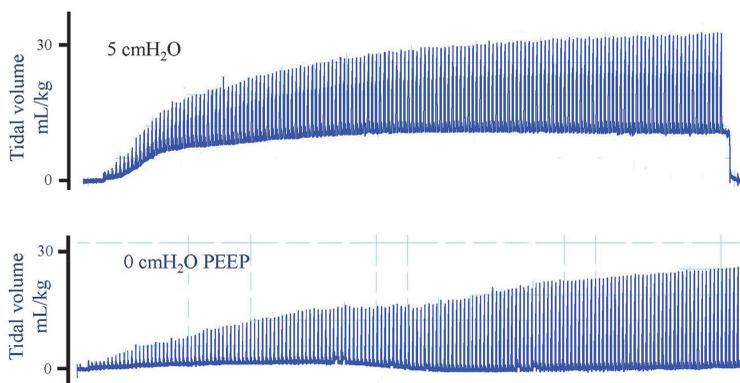


Figure 9-7. Plethysmography recordings of preterm rabbit pups ventilated from birth with (top panel) or without (bottom panel) PEEP. When ventilated with 5 cmH₂O, PEEP lung volume at end expiration increases after which it remains stable. In contrast, when a pup was ventilated without PEEP (0 cmH₂O), no FRC accumulated. The estimated dead space volume is indicated by the dotted line, indicating that volume in the lung only increased above the dead space volume briefly during inspiration with 0 PEEP. As result, gas exchange is restricted to the brief period during inflation and does not occur throughout the respiratory cycle. Data redrawn from Sievw, te Pas, Wallace, et al. *J Appl Physiol.* 2009 May;106 (5):1487–1493.

tension within the surface film lining the internal alveolar surface, surfactant makes the pressure within this film less negative and thereby reduces the transepithelial pressure gradient for alveolar flooding. When the respiratory effort and surfactant are insufficient, an external end expiratory pressure can be applied via mask or tube as a CPAP or PEEP during ventilation (Figure 9-7) (73). There are very little data on the optimal PEEP/CPAP level at birth, but undoubtedly the pressure will vary between infants and with the time after birth. Indeed, the optimum PEEP/CPAP level will be a counterbalance between respiratory and cardiovascular effects. For instance, in preterm lambs ventilated from birth, PEEP levels above 8 cmH₂O improve oxygenation but adversely affect PBF by increasing PVR. As a result the PBF waveform is markedly altered, reestablishing retrograde flow during diastole, which is characteristic of the fetal state (88). Recent phase-contrast X-ray imaging studies have shown that a more uniform distribution of ventilation can be achieved by initiating ventilation with a high PEEP level of 10 cmH₂O (89). After lung aeration, small and stepped reductions in PEEP result in more uniform changes in ventilation than do starting with lower PEEP levels and then increasing the PEEP (89). To avoid mechanical ventilation-associated injury, many caregivers prefer the gentle approach of using noninvasive ventilation. Several large randomized trials demonstrated that early CPAP after birth is a good alternative for endotracheal intubation, mechanical ventilation, and surfactant treatment. However, in the trials this “early” CPAP is applied after most of the infants were stabilized with PPV (90).

The Use of Oxygen

Hyperoxemia (high oxygen levels in blood) can lead to hyperoxia (high oxygen concentrations in tissue), causing oxidative stress and tissue injury (91,92). Excessive oxygen exposure should be avoided in infants during stabilization at birth. Meta-analyses indicate that resuscitation of term infants at birth with air significantly reduced mortality compared with infants resuscitated with 100% oxygen (91–96). Recently updated international resuscitation guidelines now recommend that respiratory support in term infants should start with air (92,97,98). Less clinical data are available for preterm infants, although hyperoxia at birth may increase the risk of BPD (99). As a result, current recommendations suggest using oxygen judiciously during stabilization of preterm infants at birth (92,97,98). On the other hand, hypoxia is known to inhibit breathing in the fetus (16) due to a direct inhibitory input into the respiratory center from higher brain centers (16). Although a temporal change in O₂ sensitivity occurs in days/weeks after birth (5) and most preterm infants breathe at birth (34,100), it is not known when the switch from respiratory suppression to stimulation occurs in response to hypoxia. It is possible that hypoxia immediately after birth will cause a weakened or absent respiratory drive, particularly in preterm infants, who are essentially exteriorized fetuses. Indeed, maturation of the hypoxic sensitivity for breathing is delayed in preterm lambs (5).

The optimal inspired oxygen content required to avoid hypoxia as well as hyperoxia remains unclear. In small randomized trials, hyperoxia

occurred more often in preterm infants when resuscitation started with high oxygen concentrations (99, 101–103). However, most infants started with 21% or 30% oxygen required increases in inspired oxygen levels to meet the target oxygen saturation (SpO_2) in the first minutes of life (99,101–103). However, these studies were performed before the normograms of SpO_2 percentiles (104) were introduced and higher target ranges were used (99, 101–103).

It is commonly assumed that the sensitivity of the pulmonary vasculature to oxygen differs between term and preterm infants and persistent hypoxia is caused by a failure of the pulmonary vasculature to sufficiently dilate at birth in the absence of supplemental oxygen (103). However, experimental studies have shown that the decrease in PVR at birth is mostly related to ventilation onset and that oxygen has a much smaller impact, which appeared to be additive with ventilation (76,105,106). Also, Sobotka et al. found that increasing FiO_2 to 1.0 in lambs who were hypoxic and difficult to ventilate markedly improved blood oxygenation, but had no effect on ventilation variables (lung compliance), $PaCO_2$, or PBF (76). This supports the hypothesis that increased oxygenation after increasing the FiO_2 to 1.0 is achieved by increasing the partial pressure gradient for oxygen diffusion across the air/blood barrier to compensate for a limited surface area and associated ventilation perfusion mismatch (76).

Mask Ventilation

The success of ventilation strategies to support respiratory function at birth in the newborn is largely dependent on the technique and strategy applied. In most neonatal units, the initial approach is to use noninvasive ventilation that is applied using a face mask. In studies using respiratory function measurements, face mask ventilation was difficult, and the delivered tidal volumes were often inadequate (34,107). Mask leak and obstruction frequently occur and together with the use of inadequate pressures, the tidal volumes are often below dead space volume (34,107). Furthermore, although experimental studies have demonstrated beneficial effects of applying an initial sustained inflation at birth, it has been difficult to duplicate these benefits with mask ventilation in preterm infants at birth due to similar problems (108).

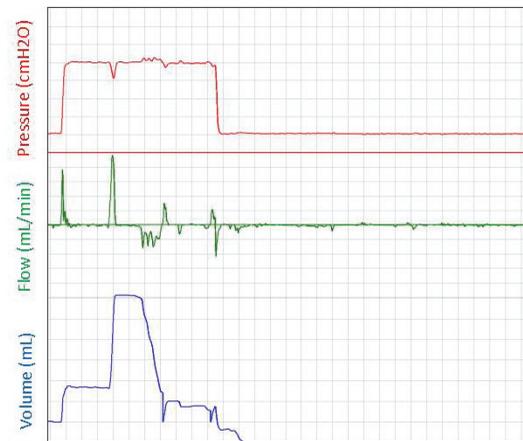


Figure 9-8. A respiratory function recording of an initial sustained inflation given to an apneic preterm infant at birth. The pressure is displayed in the top panel (cmH₂O), gas flow into and out of the face mask is displayed in the middle panel (mL/min), and the gas volume (mL) moving into the lung is displayed in the bottom panel. Large breaths are observed when the sustained pressure is applied, but the infant remains apneic after the sustained inflation. Data redrawn from van Vonderen, Hooper, Hummler, Lopriore, te Pas. *J Pediatr.* 2014 Nov;165(5):903–908.

Although the source of the obstruction is unclear, it is possible that during the pressure inflation the infant's glottis is closed. As a result, when the infants take a breath during the sustained inflation, the glottis opens, and large volumes enter the lung. In some apneic infants, breaths appeared to be triggered during the sustained inflation but then the infant remained apneic. It is possible that applying a pressure to the pharynx during the sustained inflation may have induced a respiratory reflex that stimulated breathing, as demonstrated in cats (109,110), and that this stimulus was removed following the sustained inflation (Figure 9-8). It is also possible that the pressures used for the sustained inflations were too low to overcome the resistance of the liquid-filled lung. Before we can translate the beneficial effects of a sustained inflation into clinical practice, we need to develop more effective strategies that will allow the inflation pressures developed during a sustained inflation to reach the airways.

Considering the difficulties caregivers have with the use of face masks, alternative interfaces have been suggested, including a nasal tube (111,112). The recently completed MOUNTAIN trial compared the effect of face masks versus nasal tubes during noninvasive ventilation at birth (113). As the rates of intubation were similar

between the groups and complications of using each interface were infrequent, the trial concluded that either interface could be used and that a nasal tube may be a good alternative to a face mask (113). However, measurements of respiratory function during nasal tube ventilation showed that the tidal volumes were often too low. The low tidal volumes resulted from high rates of leak and obstruction as compared with face mask ventilation. Leak-free ventilation was more difficult with a nasal tube, even when the contralateral nostril and mouth were closed. It is also possible that the tip of the tube was against the posterior wall of the nasopharynx, causing obstruction.

Recent studies have demonstrated that during resuscitation at birth the mask ventilation given was not effective until the infant took a breath (34, 107). However, breathing is difficult to observe and is therefore often missed clinically, especially in preterm infants covered in a wrap to prevent hypothermia (114). Respiratory function monitoring (RFM) can be used to detect spontaneous breathing (34,114,115), and although the spontaneous breaths are not always at a sufficient rate or tidal volume for detection, spontaneous breaths usually yield larger tidal volumes than mechanical inflations. In addition, when a spontaneous breath coincides with the mechanical inflation, the resulting tidal volume is much larger. Although the presence of breathing in preterm infants likely influences the caregiver's decision for whether additional ventilation is required, caution is needed when giving these inflations. In particular, when the breaths and inflations occur in and out of synchrony in a random manner, problems can arise. When inflations and spontaneous breaths coincide, inadvertently high transpulmonary pressures can occur, resulting in high tidal volumes and an increased risk for lung injury and air leaks (34,107,116). On the other hand, when they do not coincide, the inflation pressure may be too low to produce a sufficient tidal volume. The caregiver then may increase the inflation pressure, which may be injurious when the spontaneous breath occurs with the mechanical breath (117).

Ventilation Induced Injury at Birth

Although most clinical trials in delivery room management have failed to show a decrease in morbidity and mortality, there is much

experimental data demonstrating that the respiratory support given at birth can injure the preterm infant, with potentially lifelong consequences. In addition, large clinical studies in very preterm infants reported higher mortality rates and an increased risk of lung and brain injury with increasing levels of delivery room resuscitation (118–120). At birth, the lungs of very preterm infants are uniquely susceptible to injury because they are structurally immature, surfactant deficient, liquid filled, and not supported by a stiff chest wall (38). The gas volume and compliance of the lung in newborn infants greatly changes over the first few breaths as airway liquid is replaced with air. This highlights the large differences in regional lung mechanics that occur when the lung only partially aerates (121). Spontaneously breathing infants develop high transthoracic pressures over the first few breaths, and relatively high positive pressures are required to initiate mechanical ventilation (6). However, compliance rapidly increases as more of the lung aerates, resulting in lower pressure requirements to achieve a functional tidal volume (V_T) with subsequent breaths. In addition, although the lung, heart, and brain are often considered independently, they are intimately linked, particularly during transition at birth. Treatments aimed to provide respiratory support for the lungs can have severe adverse consequences for the preterm heart and brain (122). For example, PPV not only can cause lung injury, but also can adversely affect the cardiovascular system, systemic circulation, and cerebral circulation. Similarly, inflammation resulting from lung injury can induce a systemic inflammatory response that includes the brain (122).

Lung Injury During Clearance of Lung Liquid and Lung Aeration

At birth, the liquid-filled immature lungs of preterm infants are very vulnerable to injury. When tidal ventilation is applied to a partially liquid-filled lung, the inflation pressure and the short inflation time (usually < 0.5 s) may be sufficient to only inflate aerated lung regions. In liquid-filled regions, the pressure-time integral of normal ventilation is far too low to overcome the resistance to moving liquid distally through the airways. Instead, gas only flows into aerated regions, overdistending them and causing

regional volutrauma. For example, 5 mL/kg equates to 15 mL/kg if only one-third of the lung is aerated and the remaining two-thirds are liquid filled. Combined with a compliant chest wall, regional overdistension injury at a given airway pressure may be large.

The site of injury in the lung is not restricted to the distal gas exchange regions, as smaller conducting airways that contain little collagen are also susceptible to injury (123,124). In preterm sheep, airway stretch occurs during initiation of ventilation, and initial injury is localized primarily to the bronchioles and respiratory bronchioles (124). Also, the shear stress caused by the movement of the air/liquid interface across the epithelial cells can distort and injure the epithelium of the small airways. As the initial ventilation may be given before much of the endogenous surfactant is secreted, there may be no protective effect of surfactant (125).

Preterm infants have a smaller inspiratory reserve volume than term infants, and therefore the volume difference between FRC and total lung capacity (TLC) is smaller (126). Preterm infants have an FRC of 11 mL/kg and a TLC of 19 mL/kg (13), while term infants have an FRC of ~20 mL/kg and a TLC of 43–52 mL/kg (126,127). Thus, in preterm infants, a tidal volume above 8 mL/kg may distend the lung above TLC and cause injury (123,128). This is because spontaneously breathing preterm infants have a mean tidal volume of 4.4 (range 2.6–7.2) mL/kg (37). As five large rapid inflations at birth can cause lung injury (83) and abolish the benefits conferred with surfactant treatment (125), it is often assumed that overinflation per se is the root cause of ventilation-induced lung injury. Indeed, injurious ventilation, using large tidal volumes induces acute phase injury response genes (129). However, the tidal volumes used in those studies were very large, either 15 mL/kg (129) or 35–40 mL/kg (83), and therefore, it is not surprising that they caused injury. As high inflation rates, causing large shear stress, are also injurious (130), it is not known whether it is large tidal volumes per se or the high rate at which these volumes are achieved that is primarily responsible for the injury.

Currently caregivers use pressure-limited devices for resuscitation in the delivery room, but the tidal volume is not measured. Instead of measuring volume, caregivers rely on chest

excursions, which are not a good indicator of tidal volume (128). Many infants can receive inappropriate V_T within minutes of birth (117). Indeed, Dawson et al. measured the V_T delivered during resuscitation of preterm infants at birth with a T-piece or self-inflating device. The tidal volume ranged from 0 to >30 mL/kg (131), with the majority (85%) of preterm infants receiving excessively high V_T (> 8 mL/kg) (131).

It will be difficult to define the safe tidal volume range during mask ventilation of preterm infants at birth for two main reasons. First, the current recommendation of the safe range of tidal volumes (4–8 mL/kg) is based on measurements of spontaneous breathing and intubated and ventilated infants (132). However, during mask ventilation, the complete respiratory system is pressurized and ventilated, which includes the lungs, the trachea, and the nasopharynx. Some gas also may enter the esophagus. During an inflation given via a mask or nasal tube, the nasopharynx is pressurized, leading to a volume displacement, which does not occur during a spontaneous breath (132). As such this volume displacement has to be taken into account when measuring volumes during mask ventilation (132). Mask ventilation may be more effective if the caregiver aims for larger tidal volumes than would be targeted in intubated infants. Thus, a different range of safe tidal volumes should be defined for ventilation given via a mask or an ET tube to avoid inadequate but also excessive and injurious tidal volumes (132).

Lung Injury Following Lung Aeration

Repeated collapse and reopening of distal airways will expose the lung to high shear forces leading to injury (atelectrauma) (133), which initiates an inflammatory cascade that includes cytokine release and the recruitment of activated leukocytes to the lungs (133). The damage is characterized by epithelial disruption, hyaline membrane formation, airway cell loss, increased alveolar capillary permeability, surfactant dysfunction, decreased lung compliance, and poor gas exchange (134). This sequence emphasizes the importance of applying an adequate PEEP/CPAP level at birth to prevent airway collapse. In surfactant-treated preterm lambs, PPV without PEEP was associated with reduced compliance and oxygenation compared with ventilation with

PEEP of 4 to 7 cmH₂O (135). In addition, preterm lambs ventilated with a PEEP of 8 cmH₂O had a significantly lower oxygen requirement by 10 minutes of age compared with lambs ventilated with no PEEP (136), and the improvement in oxygenation occurred more rapidly than with surfactant administration (137).

Hemodynamic Consequences

Ventilation can alter pulmonary venous return and, as a result, cause rapid changes in left ventricular output and systemic arterial pressure. As instantaneous cerebral blood flow is pressure passive in the immature brain at birth, large, rapid fluctuations in cardiac output cause large swings in cerebral blood flow (63). Experimental and clinical studies showed the correlation between mean airway pressure and altered PBF leading to destabilization of systemic arterial flows (138–141). In addition, high airway pressures can also compress the heart directly and reduce cardiac performance and ventricular output (141, 142). Changing the PEEP level can significantly change the systemic blood flow in preterm infants (143). Echo Doppler studies in the first 24 h after birth in preterm infants (< 29 weeks) demonstrated a strong association of low SVC flow with cerebral injury, including IVH and long-term neurodevelopmental disability (144,145).

In preterm lambs, large fluctuations in cerebral blood flow occurred when high V_T ventilation was given for the first 15 minutes after birth (122), which was accompanied by impaired cerebral autoregulation (cerebral vasoparalysis) and increased vascular extravasation (leakage), a precursor to cerebral hemorrhage (122). Optimizing the initial respiratory support at birth (immediate surfactant, initial sustained inflation followed by tidal ventilation of 7 mL/kg) significantly reduced the risk of brain injury by improving CBF stability and cerebral oxygenation and reducing vascular permeability (76,122). It is not ventilation per se that is injurious, but the poorly controlled use of PPV in the delivery room that increases the risk of brain injury.

Systemic Consequences of Lung Injury

Initial ventilation in preterm lambs with higher than normal tidal volumes causes pulmonary inflammation (146) and subsequently a systemic

inflammatory cascade (123,147,148). Indeed, increased levels of pro-inflammatory cytokines IL-8, IL-1, and TNF and decreased anti-inflammatory cytokine IL-10 were measured in preterm and term newborns 2 h after initiation of mechanical ventilation (149). The circulating cytokines elicited and exacerbated an inflammatory response within the brain (150,151), which resulted in increased infiltrating inflammatory cells, activation of resident microglia, increased oxidative stress, and subsequent diffuse white matter gliosis within the periventricular white matter, subcortical white matter, and corpus callosum (122,152). The pattern of white matter injury associated with increased systemic pro-inflammatory mediators is consistent with that in infants with PVL associated with high plasma or cerebrospinal fluid cytokine levels (153,154). Increased systemic pro-inflammatory cytokines not only cause direct cerebral injury but may also alter postnatal hemodynamics as cord-blood IL-6 levels are inversely related to systolic, mean, and diastolic blood pressures (155). Also the gray matter can be injured with high tidal ventilation, as demonstrated in animal experiments (148,156,157), and the injury was reduced with less-invasive ventilation (156,157).

Oxygen Injury

Randomized controlled trials have shown that resuscitating asphyxiated newborn infants with air reduces mortality compared with resuscitation with 100% oxygen (95). In a recent meta-analysis, high levels (60%) of oxygen increased the incidence of BPD when compared to a low FiO₂ level (30%) (158). A high fraction of inspired oxygen (FiO₂) is toxic to lung tissue in both animals and humans, which is evident in both term and preterm subjects when treated with a high FiO₂ for prolonged periods (134). Very preterm infants are particularly susceptible to free-radical damage because antioxidant mechanisms are not fully developed until the third trimester (159). Prolonged exposure to hyperoxia was associated with leukocyte activation and sequestration in the neonatal rat lung (160). A high FiO₂ may contribute to arrested alveolar development in very preterm infants who develop BPD. Even a short period of hyperoxia in preterm infants at birth increased oxidative stress and inflammation, and preterm infants initially exposed to a high FiO₂ had an

increased risk for BPD (99). These findings suggest that in the first minutes after birth, avoiding the use of high oxygen concentrations may reduce acute lung injury, particularly in very preterm infants. Oxygen saturation should be monitored, and oxygen should be titrated to achieve the desired saturation.

Knowledge Gaps and Future Research

Understanding the physiological process in pulmonary and hemodynamic transition at birth is vital for designing the most effective and least-injurious way to support infants at birth when transition fails. The use of new innovative methods in experimental studies and a renewed interest in performing physiological observations in the delivery room has led to more insight into our understanding of transition after birth. It also has become clear that inappropriate ventilation and cord clamping can have a significant impact on the lungs, heart, and brain with long-term consequences, especially in preterm infants.

Care in the delivery room has improved, but this is also a work in progress. There are still gaps in our knowledge concerning the transition, and the least injurious but most effective support in preterm infants still needs to be defined. The

following research questions need to be answered in the future.

- 1) What are the mechanisms of switching from FBMs to breathing with air after birth? Is there a hierarchy in factors that increase the respiratory drive? What is the role of oxygen in this? How can we stimulate breathing when respiratory drive is insufficient?
- 2) What is the role of interstitial sensory receptors in perialveolar lung tissue in the pulmonary and hemodynamic transition at birth?
- 3) What is the role of the larynx during noninvasive ventilation?
- 4) What is the mechanism of the placental to infant blood transfusion before cord clamping? We need to know how factors, such as gravity, influence the placental-to-infant transfusion of blood and the cardiovascular transition at birth. Furthermore, we need to know how uterine contractions influence the benefits of DCC to ascertain the most appropriate timing of uterotonic administration.
- 5) Which range of tidal volumes can be considered "safe" when noninvasive ventilation is applied to preterm infants at birth and which ventilation strategy is optimal?

Reference List

- 1 Harding R. Fetal breathing movements. In: Crystal R, West J, Weibel E, Barnes P, eds. *The Lung: Scientific Foundations*. New York: Lippincott-Raven; 1997:2093.
- 2 Hooper SB, Harding R. Fetal lung liquid: a major determinant of the growth and functional development of the fetal lung. *Clin Exp Pharmacol Physiol*. 1995 Apr;22(4):235–247.
- 3 Dawes GS, Fox HE, Leduc BM, Liggins GC, Richards RT. Respiratory movements and rapid eye movement sleep in the foetal lamb. *J Physiol*. 1972 Jan;220(1):119–143.
- 4 Harding R, Liggins GC. Changes in thoracic dimensions induced by breathing movements in fetal sheep. *Reprod Fertil Dev*. 1996;8(1):117–124.
- 5 Davey MG, Moss TJ, McCrabb GJ, Harding R. Prematurity alters hypoxic and hypercapnic ventilatory responses in developing lambs. *Respir Physiol*. 1996 Aug;105(1–2):57–67.
- 6 Vyas H, Field D, Milner AD, Hopkin IE. Determinants of the first inspiratory volume and functional residual capacity at birth. *Pediatr Pulmonol*. 1986 Jul;2(4):189–193.
- 7 Koos BJ, Maeda T, Jan C. Adenosine A(1) and A(2A) receptors modulate sleep state and breathing in fetal sheep. *J Appl Physiol* (1985). 2001 Jul;91(1):343–350.
- 8 Jansen AH, Chernick V. Fetal breathing and development of control of breathing. *J Appl Physiol* (1985). 1991 Apr;70(4):1431–1446.
- 9 Thorburn GD. The placenta and the control of fetal breathing movements. *Reprod Fertil Dev*. 1995;7(3):577–594.
- 10 Crossley KJ, Nicol MB, Hirst JJ, Walker DW, Thorburn GD. Suppression of arousal by progesterone in fetal sheep. *Reprod Fertil Dev*. 1997;9(8):767–773.
- 11 Tai TC, MacLusky NJ, Adamson SL. Ontogenesis of prostaglandin E2 binding sites

- in the brainstem of the sheep. *Brain Res.* 1994 Jul 25;652(1):28–39.
- 12 Adamson SL. Regulation of breathing at birth. *J Dev Physiol.* 1991 Jan;15(1):45–52.
- 13 Dawes GS. Oxygen supply and consumption on late fetal life and the onset of breathing at birth. In: Fenn WO, Rahn H, eds. *Handbook of Physiology: Sec 2. Respiration.* Washington DC: American Physiological Society; 1965:1313–1328.
- 14 Harned HS Jr, Wolkoff AS, Pickrell J, MacKinney LG. Hemodynamic observations during birth of the lamb. Studies of the unanesthetized full-term animal. *Am J Dis Child.* 1961 Aug;102:180–189.
- 15 Rigatto H, Brady JP, de la Torre Verduzco R. Chemoreceptor reflexes in preterm infants: I. The effect of gestational and postnatal age on the ventilatory response to inhalation of 100% and 15% oxygen. *Pediatrics.* 1975 May;55(5):604–613.
- 16 Gluckman PD, Johnston BM. Lesions in the upper lateral pons abolish the hypoxic depression of breathing in unanaesthetized fetal lambs in utero. *J Physiol.* 1987 Jan;382:373–383.
- 17 Bookatz GB, Mayer CA, Wilson CG, et al. Effect of supplemental oxygen on reinitiation of breathing after neonatal resuscitation in rat pups. *Pediatr Res.* 2007 Jun;61(6):698–702.
- 18 Wong KA, Bano A, Rigaux A, et al. Pulmonary vagal innervation is required to establish adequate alveolar ventilation in the newborn lamb. *J Appl Physiol (1985).* 1998 Sep;85(3):849–859.
- 19 Harding R. State-related and developmental changes in laryngeal function. *Sleep.* 1980;3(3–4):307–322.
- 20 Lines A, Hooper SB, Harding R. Lung liquid production rates and volumes do not decrease before labor in healthy fetal sheep. *J Appl Physiol (1985).* 1997 Mar;82(3):927–932.
- 21 Dickson KA, Harding R. Restoration of lung liquid volume following its acute alteration in fetal sheep. *J Physiol.* 1987 Apr;385:531–543.
- 22 Hooper SB, Dickson KA, Harding R. Lung liquid secretion, flow and volume in response to moderate asphyxia in fetal sheep. *J Dev Physiol.* 1988 Oct;10(5):473–485.
- 23 Harding R, Hooper SB, Dickson KA. A mechanism leading to reduced lung expansion and lung hypoplasia in fetal sheep during oligohydramnios. *Am J Obstet Gynecol.* 1990 Dec;163(6 Pt 1):1904–1913.
- 24 Kitterman JA, Ballard PL, Clements JA, Mescher EJ, Tooley WH. Tracheal fluid in fetal lambs: spontaneous decrease prior to birth. *J Appl Physiol Respir Environ Exerc Physiol.* 1979 Nov;47(5):985–989.
- 25 Riley CA, Boozer K, King TL. Antenatal corticosteroids at the beginning of the 21st century. *J Midwifery Women's Health.* 2011 Nov;56(6):591–597.
- 26 Bland RD. Loss of liquid from the lung lumen in labor: more than a simple “squeeze.” *Am J Physiol Lung Cell Mol Physiol.* 2001 Apr;280(4):L602–L605.
- 27 Albuquerque CA, Smith KR, Saywers TE, Johnson C, Cock ML, Harding R. Relation between oligohydramnios and spinal flexion in the human fetus. *Early Hum Dev.* 2002 Jul;68(2):119–126.
- 28 Olver RE, Ramsden CA, Strang LB, Walters DV. The role of amiloride-blockable sodium transport in adrenaline-induced lung liquid reabsorption in the fetal lamb. *J Physiol.* 1986 Jul;376:321–340.
- 29 Barker PM, Brown MJ, Ramsden CA, Strang LB, Walters DV. The effect of thyroidectomy in the fetal sheep on lung liquid reabsorption induced by adrenaline or cyclic AMP. *J Physiol.* 1988 Dec;407:373–383.
- 30 Wallace MJ, Hooper SB, Harding R. Effects of elevated fetal cortisol concentrations on the volume, secretion, and reabsorption of lung liquid. *Am J Physiol.* 1995 Oct;269(4 Pt 2):R881–R887.
- 31 Wallace MJ, Hooper SB, Harding R. Role of the adrenal glands in the maturation of lung liquid secretory mechanisms in fetal sheep. *Am J Physiol.* 1996 Jan;270(1 Pt 2):R33–R40.
- 32 Barker PM, Markiewicz M, Parker KA, Walters DV, Strang LB. Synergistic action of triiodothyronine and hydrocortisone on epinephrine-induced reabsorption of fetal lung liquid. *Pediatr Res.* 1990 Jun;27(6):588–591.
- 33 Hummler E, Planes C. Importance of ENaC-mediated sodium transport in alveolar fluid clearance using genetically-engineered mice. *Cell Physiol Biochem.* 2010;25(1):63–70.
- 34 Schilleman K, van der Pot CJ, Hooper SB, Lopriore E, Walther FJ, te Pas AB. Evaluating manual inflations and breathing during mask ventilation in preterm infants at birth. *J Pediatr.* 2013 Mar;162(3):457–463.
- 35 Hooper SB, Kitchen MJ, Siew ML, et al. Imaging lung aeration and lung liquid clearance at birth using phase contrast X-ray imaging. *Clin Exp Pharmacol Physiol.* 2009 Jan;36(1):117–125.

- 36 Siew ML, Wallace MJ, Kitchen MJ, et al. Inspiration regulates the rate and temporal pattern of lung liquid clearance and lung aeration at birth. *J Appl Physiol*. 2009 Jun;106(6):1888–1895.
- 37 te Pas AB, Davis PG, Kamlin CO, Dawson J, O'Donnell CP, Morley CJ. Spontaneous breathing patterns of very preterm infants treated with continuous positive airway pressure at birth. *Pediatr Res*. 2008 Apr;64(3):281–285.
- 38 te Pas AB, Davis PG, Hooper SB, Morley CJ. From liquid to air: breathing after birth. *J Pediatr*. 2008 May;152(5):607–611.
- 39 Hooper SB, Kitchen MJ, Wallace MJ, Yagi N, Uesugi K, Morgan MJ, et al. Imaging lung aeration and lung liquid clearance at birth. *FASEB J*. 2007 Oct;21(12):3329–3337.
- 40 Hooper SB, Harding R. Effect of beta-adrenergic blockade on lung liquid secretion during fetal asphyxia. *Am J Physiol*. 1989 Oct;257 (4 Pt 2):R705–R710.
- 41 Walters DV, Olver RE. The role of catecholamines in lung liquid absorption at birth. *Pediatr Res*. 1978 Mar;12(3):239–242.
- 42 Dawson JA, Kamlin CO, Wong C, et al. Changes in heart rate in the first minutes after birth. *Arch Dis Child Fetal Neonatal Ed*. 2010 May;95(3):F177–F181.
- 43 Hummler E, Barker P, Gatzky J, et al. Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nat Genet*. 1996 Mar;12(3):325–328.
- 44 Jain L, Eaton DC. Physiology of fetal lung fluid clearance and the effect of labor. *Semin Perinatol*. 2006 Feb;30(1):34–43.
- 45 Bonny O, Rossier BC. Disturbances of Na/K balance: pseudohypoaldosteronism revisited. *J Am Soc Nephrol*. 2002 Sep;13(9):2399–2414.
- 46 Olver RE, Robinson EJ. Sodium and chloride transport by the tracheal epithelium of fetal, new-born and adult sheep. *J Physiol*. 1986 Jun;375:377–390.
- 47 Wallace MJ, Hooper SB, Harding R. Regulation of lung liquid secretion by arginine vasopressin in fetal sheep. *Am J Physiol*. 1990 Jan;258 (1 Pt 2):R104–R111.
- 48 O'Brodovich H, Hannam V, Seear M, Mullen JB. Amiloride impairs lung water clearance in newborn guinea pigs. *J Appl Physiol (1985)*. 1990 Apr;68(4):1758–1762.
- 49 Kitchen MJ, Lewis RA, Morgan MJ, et al. Dynamic measures of regional lung air volume using phase contrast x-ray imaging. *Phys Med Biol*. 2008 Nov 7;53(21):6065–6077.
- 50 Siew ML, Wallace MJ, Allison BJ, et al. The role of lung inflation and sodium transport in airway liquid clearance during lung aeration in newborn rabbits. *Pediatr Res*. 2013 Apr;73 (4 Pt 1):443–449.
- 51 Kitchen MJ, Lewis RA, Yagi N, et al. Phase contrast X-ray imaging of mice and rabbit lungs: a comparative study. *Br J Radiol*. 2005 Nov;78(935):1018–1027.
- 52 Mortola JP, Fisher JT, Smith B, Fox G, Weeks S. Dynamics of breathing in infants. *J Appl Physiol Respir Environ Exerc Physiol*. 1982 May;52(5):1209–1215.
- 53 Bland RD, McMillan DD, Bressack MA, Dong L. Clearance of liquid from lungs of newborn rabbits. *J Appl Physiol Respir Environ Exerc Physiol*. 1980 Aug;49(2):171–177.
- 54 Miserocchi G, Poskurica BH, Del FM. Pulmonary interstitial pressure in anesthetized paralyzed newborn rabbits. *J Appl Physiol (1985)*. 1994 Nov;77(5):2260–2268.
- 55 Avery ME, Cook CD. Volume-pressure relationships of lungs and thorax in fetal, newborn, and adult goats. *J Appl Physiol*. 1961 Nov;16:1034–1038.
- 56 Davey MG, Johns DP, Harding R. Postnatal development of respiratory function in lambs studied serially between birth and 8 weeks. *Respir Physiol*. 1998 Jul;113(1):83–93.
- 57 Flecknoe SJ, Crossley KJ, Zuccala GM, et al. Increased lung expansion alters lung growth but not alveolar epithelial cell differentiation in newborn lambs. *Am J Physiol Lung Cell Mol Physiol*. 2007 Feb;292(2):L454–L461.
- 58 Flecknoe SJ, Wallace MJ, Harding R, Hooper SB. Determination of alveolar epithelial cell phenotypes in fetal sheep: evidence for the involvement of basal lung expansion. *J Physiol*. 2002 Jul 1;542(Pt 1):245–253.
- 59 Flecknoe SJ, Wallace MJ, Cock ML, Harding R, Hooper SB. Changes in alveolar epithelial cell proportions during fetal and postnatal development in sheep. *Am J Physiol Lung Cell Mol Physiol*. 2003 Sep;285(3):L664–L670.
- 60 Rudolph AM. Fetal and neonatal pulmonary circulation. *Annu Rev Physiol*. 1979;41:383–395.
- 61 Hooper SB. Role of luminal volume changes in the increase in pulmonary blood flow at birth in sheep. *Exp Physiol*. 1998 Nov;83(6):833–842.
- 62 Polglase GR, Wallace MJ, Grant DA, Hooper SB. Influence of fetal breathing movements on pulmonary hemodynamics in fetal sheep.

- Pediatr Res.* 2004 Dec;56(6):932–938.
- 63 Bhatt S, Alison BJ, Wallace EM, et al. Delaying cord clamping until ventilation onset improves cardiovascular function at birth in preterm lambs. *J Physiol.* 2013 Apr 15;591(Pt 8):2113–2126.
- 64 Crossley KJ, Allison BJ, Polglase GR, Morley CJ, Davis PG, Hooper SB. Dynamic changes in the direction of blood flow through the ductus arteriosus at birth. *J Physiol.* 2009 Oct 1;587 (Pt 19):4695–4704.
- 65 van Vonderen JJ, Roest AA, Siew ML, et al. Noninvasive measurements of hemodynamic transition directly after birth. *Pediatr Res.* 2014 Mar;75(3):448–452.
- 66 Vento M, Saugstad OD. Resuscitation of the term and preterm infant. *Semin Fetal Neonatal Med.* 2010 Aug;15(4):216–222.
- 67 Perlman JM, Risser R. Cardiopulmonary resuscitation in the delivery room. Associated clinical events. *Arch Pediatr Adolesc Med.* 1995 Jan;149(1):20–25.
- 68 te Pas AB, Walther FJ. Ventilation of very preterm infants in the delivery room. *Current Pediatric Reviews.* 2006;2(3):187–197.
- 69 Gerhardt T, Bancalari E. Chestwall compliance in full-term and premature infants. *Acta Paediatr Scand.* 1980 May;69(3):359–364.
- 70 Heldt GP, McIlroy MB. Dynamics of chest wall in preterm infants. *J Appl Physiol.* 1987 Jan;62(1):170–174.
- 71 Barker PM, Olver RE. Invited review: Clearance of lung liquid during the perinatal period. *J Appl Physiol (1985).* 2002 Oct;93(4):1542–1548.
- 72 Barker PM, Gowen CW, Lawson EE, Knowles MR. Decreased sodium ion absorption across nasal epithelium of very premature infants with respiratory distress syndrome. *J Pediatr.* 1997 Mar;130(3):373–377.
- 73 Hooper SB, Siew ML, Kitchen MJ, te Pas AB. Establishing functional residual capacity in the non-breathing infant. *Semin Fetal Neonatal Med.* 2013 Dec;18(6):336–343.
- 74 te Pas AB, Siew M, Wallace MJ, et al. Effect of sustained inflation length on establishing functional residual capacity at birth in ventilated premature rabbits. *Pediatr Res.* 2009 Sep;66(3):295–300.
- 75 te Pas AB, Siew M, Wallace MJ, et al. Establishing functional residual capacity at birth: the effect of sustained inflation and positive end-expiratory pressure in a preterm rabbit model. *Pediatr Res.* 2009 May;65(5):537–541.
- 76 Sobotka KS, Hooper SB, Allison BJ, et al. An initial sustained inflation improves the respiratory and cardiovascular transition at birth in preterm lambs. *Pediatr Res.* 2011 Jul;70(1):56–60.
- 77 Vyas H, Milner AD, Hopkin IE, Boon AW. Physiologic responses to prolonged and slow-rise inflation in the resuscitation of the asphyxiated newborn infant. *J Pediatr.* 1981 Oct;99(4):635–639.
- 78 Lindner W, Vossbeck S, Hummler H, Pohlandt F. Delivery room management of extremely low birth weight infants: spontaneous breathing or intubation? *Pediatrics.* 1999 May;103(5 Pt 1):961–967.
- 79 Lindner W, Hogel J, Pohlandt F. Sustained pressure-controlled inflation or intermittent mandatory ventilation in preterm infants in the delivery room? A randomized, controlled trial on initial respiratory support via nasopharyngeal tube. *Acta Paediatr.* 2005 Mar;94(3):303–309.
- 80 Harling AE, Beresford MW, Vince GS, Bates M, Yoxall CW. Does sustained lung inflation at resuscitation reduce lung injury in the preterm infant? *Arch Dis Child Fetal Neonatal Ed.* 2005 Sep;90(5):F406–F410.
- 81 te Pas AB, Walther FJ. A randomized, controlled trial of delivery-room respiratory management in very preterm infants. *Pediatrics.* 2007 Aug;120(2):322–329.
- 82 Lista G, Fontana P, Castoldi F, Cavigioli F, Dani C. Does sustained lung inflation at birth improve outcome of preterm infants at risk for respiratory distress syndrome? *Neonatology.* 2010 Jul 9;99(1):45–50.
- 83 Bjorklund LJ, Ingimarsson J, Curstedt T, John J, Robertson B, Werner O, et al. Manual ventilation with a few large breaths at birth compromises the therapeutic effect of subsequent surfactant replacement in immature lambs. *Pediatr Res.* 1997 Sep;42(3):348–355.
- 84 Ingimarsson J, Bjorklund LJ, Curstedt T, Larsson A, Robertson B, Werner O. A lung recruitment maneuver immediately before rescue surfactant therapy does not affect the lung mechanical response in immature lambs with respiratory distress syndrome. *Acta Anaesthesiol Scand.* 2003 Sep;47(8):968–972.
- 85 Fuchs H, Lindner W, Buschko A, Trischberger T, Schmid M, Hummler HD. Cerebral oxygenation in very low birth weight infants supported with sustained lung inflations after birth. *Pediatr Res.* 2011 Aug; 70(2):176–180.
- 86 Hillman NH, Kemp MW, Noble PB, Kallapur SG, Jobe AH. Sustained inflation at

- birth did not protect preterm fetal sheep from lung injury. *Am J Physiol Lung Cell Mol Physiol.* 2013 Sep 15;305(6):L446–L453.
- 87 Tingay DG, Bhatia R, Schmolzer GM, Wallace MJ, Zahra VA, Davis PG. Effect of sustained inflation vs. stepwise PEEP strategy at birth on gas exchange and lung mechanics in preterm lambs. *Pediatr Res.* 2014 Feb;75(2):288–294.
- 88 Polglase GR, Morley CJ, Crossley KJ, et al. Positive end-expiratory pressure differentially alters pulmonary hemodynamics and oxygenation in ventilated, very premature lambs. *J Appl Physiol.* 2005 Oct;99(4):1453–1461.
- 89 Kitchen MJ, Siew ML, Wallace MJ, et al. Changes in positive end-expiratory pressure alter the distribution of ventilation within the lung immediately after birth in newborn rabbits. *PLoS One.* 2014;9(4):e93391.
- 90 Schmolzer GM, Kumar M, Pichler G, Aziz K, O'Reilly M, Cheung PY. Non-invasive versus invasive respiratory support in preterm infants at birth: systematic review and meta-analysis. *BMJ.* 2013;347:f5980.
- 91 Higgins RD, Bancalari E, Willinger M, Raju TN. Executive summary of the workshop on oxygen in neonatal therapies: controversies and opportunities for research. *Pediatrics.* 2007 Apr;119(4):790–6.
- 92 Vento M, Saugstad OD. Oxygen supplementation in the delivery room: updated information. *J Pediatr.* 2011 Feb;158(2 suppl):e5–e7.
- 93 Hellstrom-Westas L, Forsblad K, Sjors G, et al. Earlier Apgar score increase in severely depressed term infants cared for in Swedish level III units with 40% oxygen versus 100% oxygen resuscitation strategies: a population-based register study. *Pediatrics.* 2006 Dec;118(6):e1798–e1804.
- 94 Rabi Y, Rabi D, Yee W. Room air resuscitation of the depressed newborn: a systematic review and meta-analysis. *Resuscitation.* 2007 Mar;72(3):353–63.
- 95 Saugstad OD, Ramji S, Soll RF, Vento M. Resuscitation of newborn infants with 21% or 100% oxygen: an updated systematic review and meta-analysis. *Neonatology.* 2008;94(3):176–82.
- 96 Tan A, Schulze A, O'Donnell CP, Davis PG. Air versus oxygen for resuscitation of infants at birth. *Cochrane Database Syst Rev.* 2005;(2):CD002273.
- 97 Perlman JM, Wyllie J, Kattwinkel J, et al. Part 11: neonatal resuscitation: 2010 International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science with Treatment Recommendations. *Circulation.* 2010 Oct 19;122(16 suppl 2):S516–S538.
- 98 Wyllie J, Perlman JM, Kattwinkel J, et al. Part 11: Neonatal resuscitation: 2010 International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science with Treatment Recommendations. *Resuscitation.* 2010 Oct;81(suppl 1):e260–e287.
- 99 Vento M, Moro M, Escrig R, et al. Preterm resuscitation with low oxygen causes less oxidative stress, inflammation, and chronic lung disease. *Pediatrics.* 2009 Sep;124(3):e439–e449.
- 100 O'Donnell CP, Kamlin CO, Davis PG, Morley CJ. Crying and breathing by extremely preterm infants immediately after birth. *J Pediatr.* 2010 May;156(5):846–847.
- 101 Escrig R, Arruza L, Izquierdo I, et al. Achievement of targeted saturation values in extremely low gestational age neonates resuscitated with low or high oxygen concentrations: a prospective, randomized trial. *Pediatrics.* 2008 May;121(5):875–881.
- 102 Rabi Y, Singhal N, Nettel-Aguirre A. Room-air versus oxygen administration for resuscitation of preterm infants: the ROAR study. *Pediatrics.* 2011 Aug;128(2):e374–e381.
- 103 Wang CL, Anderson C, Leone TA, Rich W, Govindaswami B, Finer NN. Resuscitation of preterm neonates by using room air or 100% oxygen. *Pediatrics.* 2008 Jun;121(6):1083–1089.
- 104 Dawson JA, Kamlin CO, Vento M, et al. Defining the reference range for oxygen saturation for infants after birth. *Pediatrics.* 2010 Jun;125(6):e1340–e1347.
- 105 Lakshminrusimha S, Steinhorn RH, Wedgwood S, et al. Pulmonary hemodynamics and vascular reactivity in asphyxiated term lambs resuscitated with 21 and 100% oxygen. *J Appl Physiol.* 2011 Nov;111(5):1441–1447.
- 106 Teitel DF, Iwamoto HS, Rudolph AM. Changes in the pulmonary circulation during birth-related events. *Pediatr Res.* 1990 Apr;27(4 Pt 1):372–378.
- 107 Schmolzer GM, Dawson JA, Kamlin CO, O'Donnell CP, Morley CJ, Davis PG. Airway obstruction and gas leak during mask ventilation of preterm infants in the delivery room. *Arch Dis Child Fetal Neonatal Ed.* 2011 Jul;96(4):F254–F257.

- 108 van Vonderen JJ, Hooper SB, Hummler HD, Lopriore E, te Pas AB. Effects of a sustained inflation in preterm infants at birth. *J Pediatr*. 2014 Nov;165(5):903–908.
- 109 Tomori Z, Benacka R, Donic V. Mechanisms and clinicophysiological implications of the sniff- and gasp-like aspiration reflex. *Respir Physiol*. 1998 Oct;114(1):83–98.
- 110 Tomori Z, Donic V, Benacka R, Jakus J, Gresova S. Resuscitation and auto resuscitation by airway reflexes in animals. *Cough*. 2013;9(1):21.
- 111 Capasso L, Capasso A, Raimondi F, Vendemmia M, Araimo G, Paludetto R. A randomized trial comparing oxygen delivery on intermittent positive pressure with nasal cannulae versus facial mask in neonatal primary resuscitation. *Acta Paediatr*. 2005 Feb;94(2):197–200.
- 112 Segedin E, Torrie J, Anderson B. Nasal airway versus oral route for infant resuscitation. *Lancet*. 1995 Aug 5;346(8971):382.
- 113 Kamlin CO, Schilleman K, Dawson JA, et al. Mask versus nasal tube for stabilization of preterm infants at birth: a randomized controlled trial. *Pediatrics*. 2013 Aug;132(2):e381–e388.
- 114 Schilleman K, Siew ML, Lopriore E, Morley CJ, Walther FJ, te Pas AB. Auditing resuscitation of preterm infants at birth by recording video and physiological parameters. *Resuscitation*. 2012 Feb 6.
- 115 van Vonderen JJ, Roest AA, Siew ML, Walther FJ, Hooper SB, te Pas AB. Measuring physiological changes during the transition to life after birth. *Neonatology*. 2014 Feb 6;105(3):230–242.
- 116 Greenough A, Dimitriou G, Prendergast M, Milner AD. Synchronized mechanical ventilation for respiratory support in newborn infants. *Cochrane Database Syst Rev*. 2008;(1):CD000456.
- 117 Schmolzer GM, te Pas AB, Davis PG, Morley CJ. Reducing lung injury during neonatal resuscitation of preterm infants. *J Pediatr*. 2008 Dec;153(6):741–745.
- 118 Finer NN, Horbar JD, Carpenter JH. Cardiopulmonary resuscitation in the very low birth weight infant: the Vermont Oxford Network experience. *Pediatrics*. 1999 Sep;104(3 Pt 1):428–434.
- 119 Shah PS. Extensive cardiopulmonary resuscitation for VLBW and ELBW infants: a systematic review and meta-analyses. *J Perinatol*. 2009 Oct;29(10):655–661.
- 120 DeMauro SB, Roberts RS, Davis P, Alvaro R, Bairam A, Schmidt B. Impact of delivery room resuscitation on outcomes up to 18 months in very low birth weight infants. *J Pediatr*. 2011 Oct;159(4):546–550.
- 121 Vyas H, Milner AD, Hopkins IE. Intrathoracic pressure and volume changes during the spontaneous onset of respiration in babies born by cesarean section and by vaginal delivery. *J Pediatr*. 1981 Nov;99(5):787–791.
- 122 Polglase GR, Miller SL, Barton SK, et al. Initiation of resuscitation with high tidal volumes causes cerebral hemodynamic disturbance, brain inflammation and injury in preterm lambs. *PLoS One*. 2012;7(6):e39535.
- 123 Hillman NH, Moss TJ, Kallapur SG, et al. Brief, large tidal volume ventilation initiates lung injury and a systemic response in fetal sheep. *Am J Respir Crit Care Med*. 2007 Sep 15;176(6):575–581.
- 124 Hillman NH, Polglase GR, Pillow JJ, Saito M, Kallapur SG, Jobe AH. Inflammation and lung maturation from stretch injury in preterm fetal sheep. *Am J Physiol Lung Cell Mol Physiol*. 2011 Feb;300(2):L232–L241.
- 125 Ingimarsson J, Bjorklund LJ, Curstedt T, et al. Incomplete protection by prophylactic surfactant against the adverse effects of large lung inflations at birth in immature lambs. *Intensive Care Med*. 2004 Jul;30(7):1446–1453.
- 126 Bjorklund LJ, Vilstrup CT, Larsson A, Svenningsen NW, Werner O. Changes in lung volume and static expiratory pressure-volume diagram after surfactant rescue treatment of neonates with established respiratory distress syndrome. *Am J Respir Crit Care Med*. 1996 Oct;154(4 Pt 1):918–923.
- 127 Vilstrup CT, Bjorklund LJ, Werner O, Larsson A. Lung volumes and pressure-volume relations of the respiratory system in small ventilated neonates with severe respiratory distress syndrome. *Pediatr Res*. 1996 Jan;39(1):127–133.
- 128 Stenson BJ, Boyle DW, Szyld EG. Initial ventilation strategies during newborn resuscitation. *Clin Perinatol*. 2006 Mar;33(1):65–82.
- 129 Wallace MJ, Probyn ME, Zahra VA, et al. Early biomarkers and potential mediators of ventilation-induced lung injury in very preterm lambs. *Respir Res*. 2009;10:19.
- 130 Bach KP, Kuschel CA, Hooper SB, et al. High bias gas flows increase lung injury in the ventilated preterm lamb. *PLoS One*. 2012;7(10):e47044.
- 131 Dawson JA, Schmolzer GM, Kamlin CO, et al. Oxygenation

- with T-piece versus self-inflating bag for ventilation of extremely preterm infants at birth: a randomized controlled trial. *J Pediatr*. 2011 Jun;158(6):912–918.
- 132 van Vonderen JJ, Hooper SB, Krabbe VB, Siew ML, te Pas AB. Monitoring tidal volumes in preterm infants at birth: mask versus endotracheal ventilation. *Arch Dis Child Fetal Neonatal Ed*. 2015 Jan;100(1):F43–F46.
- 133 Jobe AH, Ikegami M. Mechanisms initiating lung injury in the preterm. *Early Hum Dev*. 1998 Nov;53(1):81–94.
- 134 Clark RH, Gerstmann DR, Jobe AH, Moffitt ST, Slutsky AS, Yoder BA. Lung injury in neonates: causes, strategies for prevention, and long-term consequences. *J Pediatr*. 2001 Oct;139(4):478–486.
- 135 Michna J, Jobe AH, Ikegami M. Positive end-expiratory pressure preserves surfactant function in preterm lambs. *Am J Respir Crit Care Med*. 1999 Aug;160(2):634–639.
- 136 Probyn ME, Hooper SB, Dargaville PA, et al. Positive end expiratory pressure during resuscitation of premature lambs rapidly improves blood gases without adversely affecting arterial pressure. *Pediatr Res*. 2004 Aug;56(2):198–204.
- 137 Crossley KJ, Morley CJ, Allison BJ, et al. Blood gases and pulmonary blood flow during resuscitation of very preterm lambs treated with antenatal betamethasone and/or Curosurf: effect of positive end-expiratory pressure. *Pediatr Res*. 2007 Jul;62(1):37–42.
- 138 Polglase GR, Hooper SB, Gill AW, et al. Cardiovascular and pulmonary consequences of airway recruitment in preterm lambs. *J Appl Physiol (1985)*. 2009 Apr;106(4):1347–1355.
- 139 Kluckow M, Evans N. Relationship between blood pressure and cardiac output in preterm infants requiring mechanical ventilation. *J Pediatr*. 1996 Oct;129(4):506–512.
- 140 Polglase GR, Moss TJ, Nitsos I, Allison BJ, Pillow JJ, Hooper SB. Differential effect of recruitment maneuvers on pulmonary blood flow and oxygenation during HFOV in preterm lambs. *J Appl Physiol (1985)*. 2008 Aug;105(2):603–610.
- 141 Mirro R, Busija D, Green R, Leffler C. Relationship between mean airway pressure, cardiac output, and organ blood flow with normal and decreased respiratory compliance. *J Pediatr*. 1987 Jul;111(1):101–106.
- 142 Biondi JW, Schulman DS, Soufer R, et al. The effect of incremental positive end-expiratory pressure on right ventricular hemodynamics and ejection fraction. *Anesth Analg*. 1988 Feb;67(2):144–151.
- 143 de Waal KA, Evans N, Osborn DA, Kluckow M. Cardiorespiratory effects of changes in end expiratory pressure in ventilated newborns. *Arch Dis Child Fetal Neonatal Ed*. 2007 Nov;92(6):F444–F448.
- 144 Kluckow M, Evans N. Low superior vena cava flow and intraventricular haemorrhage in preterm infants. *Arch Dis Child Fetal Neonatal Ed*. 2000 May;82(3):F188–F194.
- 145 Miletin J, Dempsey EM. Low superior vena cava flow on day 1 and adverse outcome in the very low birthweight infant. *Arch Dis Child Fetal Neonatal Ed*. 2008 Sep;93(5):F368–F371.
- 146 Polglase GR, Hillman NH, Pillow JJ, et al. Positive end-expiratory pressure and tidal volume during initial ventilation of preterm lambs. *Pediatr Res*. 2008 Nov;64(5):517–522.
- 147 Chiumello D, Pristine G, Slutsky AS. Mechanical ventilation affects local and systemic cytokines in an animal model of acute respiratory distress syndrome. *Am J Respir Crit Care Med*. 1999 Jul;160(1):109–116.
- 148 Quilez ME, Fuster G, Villar J, et al. Injurious mechanical ventilation affects neuronal activation in ventilated rats. *Crit Care*. 2011;15(3):R124.
- 149 Bohrer B, Silveira RC, Neto EC, Procianny RS. Mechanical ventilation of newborns infant changes in plasma pro- and anti-inflammatory cytokines. *J Pediatr*. 2010 Jan;156(1):16–19.
- 150 Noori S, McCoy M, Anderson MP, Ramji F, Seri I. Changes in cardiac function and cerebral blood flow in relation to peri/intraventricular hemorrhage in extremely preterm infants. *J Pediatr*. 2014 Feb;164(2):264–270.
- 151 Threlkeld SW, Lynch JL, Lynch KM, Sadowska GB, Banks WA, Stonestreet BS. Ovine proinflammatory cytokines cross the murine blood-brain barrier by a common saturable transport mechanism. *Neuroimmunomodulation*. 2010;17(6):405–410.
- 152 Polglase GR, Nitsos I, Baburamani AA, et al. Inflammation in utero exacerbates ventilation-induced brain injury in preterm lambs. *J Appl Physiol (1985)*. 2012 Feb;112(3):481–489.
- 153 Viscardi RM, Muhumuza CK, Rodriguez A, et al. Inflammatory markers in intrauterine and fetal blood and cerebrospinal fluid

- compartments are associated with adverse pulmonary and neurologic outcomes in preterm infants. *Pediatr Res.* 2004 Jun;55(6):1009–1017.
- 154 Volpe JJ. Postnatal sepsis, necrotizing enterocolitis, and the critical role of systemic inflammation in white matter injury in premature infants. *J Pediatr.* 2008 Aug;153(2):160–163.
- 155 Yanowitz TD, Jordan JA, Gilmour CH, et al. Hemodynamic disturbances in premature infants born after chorioamnionitis: association with cord blood cytokine concentrations. *Pediatr Res.* 2002 Mar;51(3):310–316.
- 156 Loeliger M, Inder T, Cain S, et al. Cerebral outcomes in a preterm baboon model of early versus delayed nasal continuous positive airway pressure. *Pediatrics.* 2006 Oct;118(4):1640–1653.
- 157 Albertine KH. Brain injury in chronically ventilated preterm neonates: collateral damage related to ventilation strategy. *Clin Perinatol.* 2012 Sep;39(3):727–740.
- 158 Saugstad OD, Aune D, Aguar M, Kapadia V, Finer N, Vento M. Systematic review and meta-analysis of optimal initial fraction of oxygen levels in the delivery room at ≤ 32 weeks. *Acta Paediatr.* 2014 Jul;103(7):744–751.
- 159 Frank L, Sosenko IR. Development of lung antioxidant enzyme system in late gestation: possible implications for the prematurely born infant. *J Pediatr.* 1987 Jan;110(1):9–14.
- 160 Torbati D, Tan GH, Smith S, et al. Multiple-organ effect of normobaric hyperoxia in neonatal rats. *J Crit Care.* 2006 Mar;21(1):85–93.
- 161 Siew ML, te Pas AB, Wallace MJ, et al. Positive end-expiratory pressure enhances development of a functional residual capacity in preterm rabbits ventilated from birth. *J Appl Physiol.* 2009 May;106(5):1487–1493.

Perinatal Modifiers of Lung Structure and Function

Suhas G. Kallapur and Sailesh Kotecha

Abstract

The fetal lung progresses through a series of orchestrated developmental events that ultimately leads to a structurally and a functionally mature lung at term birth. However, a number of insults during fetal gestation can lead to aberrant lung growth and/or lung injury. The focus in this chapter is on events that occur after the fetus is of viable gestation rather than the early embryonic insults that ultimately lead to bronchopulmonary dysplasia, lung hypoplasia, or other clinically relevant adverse lung outcomes. Reviews of experimental animal studies are presented in the context of how these studies inform us of likely pathways of lung injury documented in clinical studies. The major perinatal insults discussed are chorioamnionitis, drugs and toxins, and intrauterine growth restriction or nutritional insults. The chapter also integrates how the developmental immune system modulates the lung injury and inflammation that leads to poor functional lung outcomes.

Keywords:

Chorioamnionitis, intraamniotic inflammation, lung development, respiratory distress syndrome, bronchopulmonary dysplasia

Introduction

Lung growth and development of the fetus are critically dependent on the intrauterine environment (Figure 10-1). Factors include the space available to the fetus to grow and breathe. Any factors affecting either the intra- or extrathoracic space, fetal lung fluid, or fetal breathing movements will result in altered lung development. Antenatal infection including chorioamnionitis is associated with preterm birth but also primes the preterm infant to further lung injury when exposed to mechanical ventilation and/or oxygen therapy. Maternal or placental diseases will affect the delivery of nutrients and oxygen thus will affect fetal lung growth directly or indirectly via poor somatic growth as occurs with intrauterine growth restriction (IUGR). Toxins such as nicotine in cigarette smoke directly affect lung development and function but also indirectly by affecting somatic growth. Discussed next are the major antenatal determinants that influence lung growth and function.

Lung Growth

The lung bud arises as an outgrowth of the foregut endoderm. Thereafter lung development occurs as

a result of epithelial–mesenchyme interactions and branching morphogenesis (1). The stages and the timetable for human lung development are embryonic stage (3–7 weeks), pseudoglandular (5–17 weeks), canalicular (16–26 weeks), saccular (24–38 weeks), and alveolar (36 weeks to six months postnatal) (2). The lung is ready to function as an efficient gas exchange organ at birth as a result of the complex fetal lung developmental processes. The timing of different intrauterine insults will affect lung development in different ways, resulting in physiological consequences to the fetus and the newborn. This chapter will review the impact of common intrauterine exposures on fetal lung development. We will present current concepts from animal experiments and clinical studies.

Chorioamnionitis

Chorioamnionitis in humans is an ascending infection of genital organisms to the choriodecidual space or the chorioamnion space through the cervix. Organisms are thought to spread diffusely through the choriodecidual or the chorioamnion plane and then invade the amniotic cavity. However, a recent study using molecular microbiologic techniques in human placentae demonstrated that the initial event is a localized choriodecidual

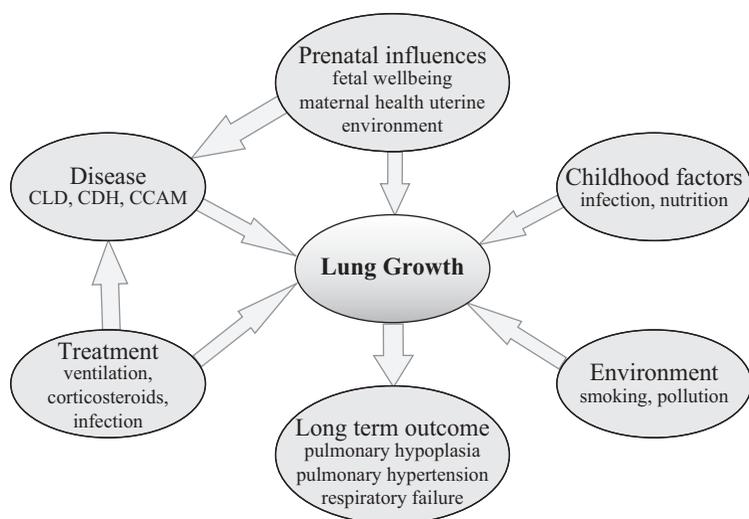


Figure 10-1. Causes of poor lung growth. Lung growth is complex and modulated by multiple processes. Poor lung growth may result from maternal, fetal, placental, environmental, and/or genetic factors. Reprinted with permission from Kotecha *Paediatr Respir Rev.* 1: 308–313, 2000.

infection, which then invades the amniotic cavity, thereby infecting amniotic fluid and the fetus prior to diffuse choriodecidual inflammation (3). This sequence is consistent with experiments in the rhesus macaque demonstrating that localized choriodecidual infection with live Group B streptococci did not trigger preterm labor until the amniotic fluid was colonized. However, a transient choriodecidual infection can induce cytokine production in the amniotic fluid, which results in fetal lung inflammation without overt infection of amniotic fluid or preterm labor. Therefore animal models of chorioamnionitis resulting from injection of inflammatory agents or organisms into the amniotic fluid reproduce the pathology of chorioamnionitis. We will review experiments in which experimental animals were given intra-amniotic or intrauterine agonists/organisms. We will not review experiments with maternal intraperitoneal or intravascular injection of agonists because these models reflect maternal septicemia or bacteremia, which are rare events in human chorioamnionitis.

Animal Models of Chorioamnionitis

Models of chorioamnionitis have been described with intrauterine injection of agonists or live bacteria in the mouse and the rabbit. Chorioamnionitis can also be induced by intra-amniotic injection in the sheep using agonists including IL-1 β , IL-1 α , lipopolysaccharide (LPS – ligand for TLR4), live *Ureaplasma parvum*, and live *Candida albicans*. In the Rhesus macaque,

intra-amniotic injection of Group B streptococci, *Ureaplasma parvum*, IL-1 β , or TNF cause chorioamnionitis and preterm labor. In the sheep, intra-amniotic injection of PamCysK4 (ligand for TLR2) induced weak fetal lung inflammation, but poly I:C (TLR3 ligand) did not cause inflammation. Interestingly intra-amniotic injection of TNF α (4), IL-6 or IL-8 (5) did not induce lung inflammation in fetal sheep, and intra-amniotic injection of IL-6 or IL-8 did not induce preterm labor in the rhesus macaque (6). These experiments demonstrate relative specificity or potency of responses to different inflammatory agents.

Using broad-range microbe-specific PCR assays, the common organisms in the amniotic fluid of women with preterm labor and intact membranes or with preterm rupture of membranes were *Ureaplasma*, *Mycoplasma*, and *Fusobacterium* (7). The strongest evidence that *Ureaplasma* can cause preterm labor is from experiments in the rhesus macaque. Intra-amniotic injection of *Ureaplasma parvum* or the related organism *Mycoplasma hominis* induced chorioamnionitis, fetal inflammation, and preterm labor (8). In the sheep, *Ureaplasma parvum* is not cleared from the amniotic compartment after IA injection but does not cause preterm labor (9). In the amniotic fluid of sheep, *Ureaplasmas* have antigenic variations with changes in the multibanded antigen and extensive horizontal gene transfer resulting in hybrid forms of *Ureaplasma* serovars, implying unstable genotypes during the course of infection. These

antigenic changes perhaps evolved as a mechanism to evade host immune system. Curiously, chorioamnionitis caused by *Ureaplasma* is of variable intensity in fetal sheep – ranging from minimal to severe, but without differences in the titer of the organism in amniotic fluid (9,10). Similarly in the human, *Ureaplasma* can be recovered without severe chorioamnionitis or progressive preterm labor. *Candida* spp. colonization/infection of the lower genital tract is fairly common in women. In addition, *Candida* spp. can also be recovered in amniotic fluid from women particularly with an indwelling intrauterine device and/or cervical cerclage. In contrast to *Ureaplasma* spp, intra-amniotic injection of a clinical isolate of live *Candida albicans* caused severe fetal inflammation and death in untreated sheep (11).

Chorioamnionitis and Maturation of the Fetal Lung

One of the striking and counterintuitive effects of intra-amniotic LPS or IL-1 in the fetal sheep is the increase in pulmonary surfactant lipids (Figure 10-2A) and surfactant-associated proteins A, B, and C (Figure 10-2B) (12,13). The increase in airway surfactant and a lung structural maturation results in improved lung mechanics in the preterm resulting in “clinical lung maturation” (Figure 10-2C). This effect of chorioamnionitis-induced lung maturation is more potent than that resulting from maternal administration of

betamethasone alone in the sheep (Figure 10-3). Further, the combination of LPS and betamethasone caused the largest increase in lung volume (see following section on combined effects of chorioamnionitis and antenatal steroids). Intra-amniotic injection of LPS with tracheal ligation prevented the lung maturation (14,15), but fetal intratracheal infusion of LPS or IL-1 resulted in increased lung maturation, demonstrating the sufficiency of direct airway contact with LPS or IL-1 for lung maturation (16,17).

The precise signaling that results in fetal lung maturation is not known. However, intramuscular injection of an anti-CD18 antibody largely decreased the inflammatory cell influx into the lungs of fetal sheep and significantly decreased the lung maturation (18). Thus, inflammatory cell influx was critically required to mediate the clinical lung maturation induced by inflammatory agonist exposure in the sheep. Similarly, human IL-1 receptor antagonist decreased the IA LPS induced lung maturation (19). These experiments in the fetal sheep demonstrate that IL-1 signaling is central to the lung maturation induced by LPS. The numbers of inflammatory cells recruited to the lung correlated with the surfactant lipid pool size, suggesting that it will be difficult to separate the inflammatory effects of these agonists from the beneficial lung maturation effects. Similar changes of lung maturation with increases in surfactant proteins and type II cell numbers were found after exposure of fetal mouse or rabbit lungs to LPS or IL-1 β .

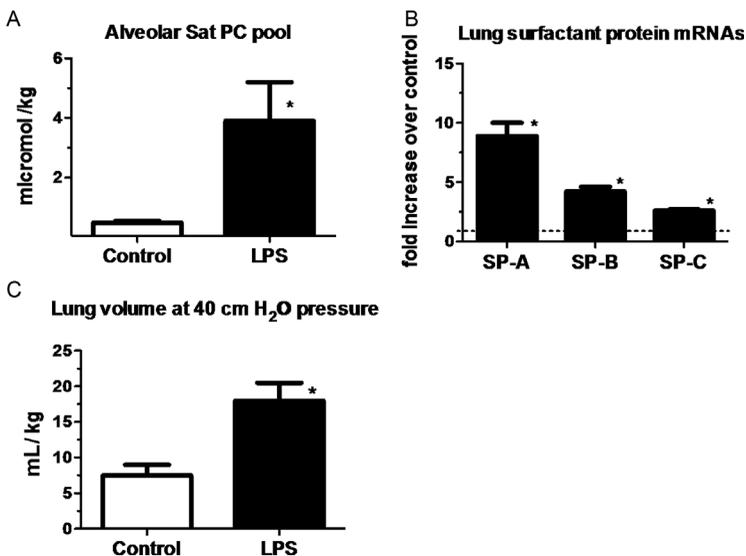


Figure 10-2. Intra-amniotic LPS caused a “clinical lung maturation” in preterm fetal sheep.

Measurements were done on lung and bronchoalveolar lavage fluid of fetal sheep after exposure to intra-amniotic LPS for 7 d (panels a & c) or 2 d (panel B). Exposure to LPS increased (A) surfactant lipid (saturated phosphatidylcholine) pool size normalized to body weight. (B) Induction of mRNAs for surfactant protein A, B, and C compared to control (value normalized to 1). (C) Lung volumes measured at 40 cmH₂O pressure expressed relative to body weight. (* $p < 0.05$ vs. control) (Data redrawn from Kallapur, Willet, Jobe, Ikegami, Bachurski. *AM J Physiol Lung Cell Mol Physiol.* 2001;280:L527–L536 and Bachurski, Ross, Ikegami, Kramer, Jobe. *Am J Physiol Lung Cell Mol Physiol.* 2001;280:L279–285).

Lung volume at 40 cm H₂O pressure

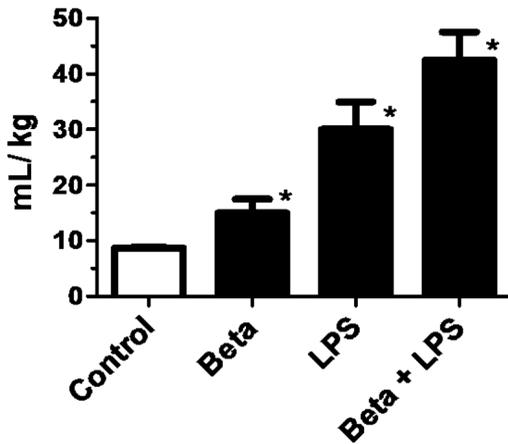


Figure 10-3. Combined exposure of antenatal betamethasone and intra-amniotic LPS to pregnant ewe induced a more potent “clinical lung maturation.”

Pregnant ewes were given intramuscular betamethasone (Beta) 7 d before delivery or intra-amniotic LPS 14 d before delivery or the combined exposures of Beta + LPS. Fetuses delivered at 80% gestation had lung volumes measured at 40 cmH₂O pressure at autopsy. Note the higher lung volumes induced by LPS compared to Beta. However, the most potent response was in animals receiving the combined exposures of LPS and Beta. (* $p < 0.05$ vs. control) (Data redrawn from Kuypers, Collins, Kramer, et al. *Am J Physiol Lung Cell Mol Physiol.* 2012;302: L380–389).

Chorioamnionitis – Inflammation/ Injury of the Fetal Lung

Despite the beneficial lung maturation, intra-amniotic injection of pro-inflammatory agonists interferes with normal lung development. In the sheep, intra-amniotic LPS decreased alveolar numbers, caused thinning of the alveolar septae, and increased the size of the alveoli (Figure 10-4A) (20). An important feature of alveolarization is the expression of elastin that are the sites of secondary septation. Intra-amniotic LPS induced an aberrant and decreased expression of elastin foci (Figure 10-4B) (21). Because elastin deposition at the leading edge of alveolar septae is critical for secondary septation, these results are consistent with decreased alveolar septation in fetuses exposed to experimental chorioamnionitis. This inhibition of formation of terminal respiratory units also occurred in fetal mouse lung explants treated with LPS. In this explant model, LPS inhibited FGF-10 expression, which required macrophage NF- κ B signaling (22). Intra-amniotic LPS in the preterm fetal sheep also inhibited several genes critical for vascular development, including *VEGF-A*,

VEGFR2, and *NOSIII* (23). Intra-amniotic LPS caused smooth muscle hypertrophy in the resistance arterioles and adventitial fibroblast proliferation of the fetal sheep lung (Figure 10-4C,D) (23). These changes of vascular remodeling resulted in increased pulmonary vascular resistance (24).

In the sheep, intra-amniotic injection of *U. parvum* increased lung surfactant pools and induced a clinical lung maturation, although less consistently than LPS or IL-1 (25). Intra-amniotic *U. parvum* also induced mild lung inflammation with mixed neutrophil and monocytic infiltration and low cytokine expression in the fetal lung. However, *Ureaplasma* caused a small decrease in elastic foci in secondary alveolar septae, impaired alveolar development, and increased smooth muscle around bronchioles and pulmonary artery/arterioles, similar to the changes caused by LPS. In contrast to what happens in the sheep, intra-amniotic *Ureaplasma parvum* (and the related organism *Mycoplasma hominis*) caused severe inflammation in the fetal rhesus macaque lung, characterized by increased neutrophils and macrophages and alveolar type II cell proliferation (8). These results suggest species differences in susceptibility to *Ureaplasma*.

In experiments simulating the effects of intra-partum exposure to inflammation, fetal lung inflammation induced changes similar to bronchopulmonary dysplasia (BPD) in transgenic mice. Human IL-1 β expressed in airway epithelial cells during the latter part of mouse gestation caused neutrophil and monocyte influx into the lung, respiratory insufficiency with increased postnatal mortality (26). IL-1 β disrupted alveolar septation with aberrant alpha-smooth muscle actin and elastin deposition in the septa of distal airspaces. IL-1 β also disrupted pulmonary vascular development.

Although antenatal infection causes aberrant pulmonary development, these changes are modest. For instance, the lung inflammation induced by chorioamnionitis in most experimental models is microscopic in nature and likely will not be visualized by X-ray imaging. Further, the delayed alveolar development apparent when fetuses are delivered seven days after intra-amniotic exposure to a pro-inflammatory agonist is no longer evident when fetuses are delivered twenty to thirty days later (27). These results imply that the fetus has a remarkable ability to

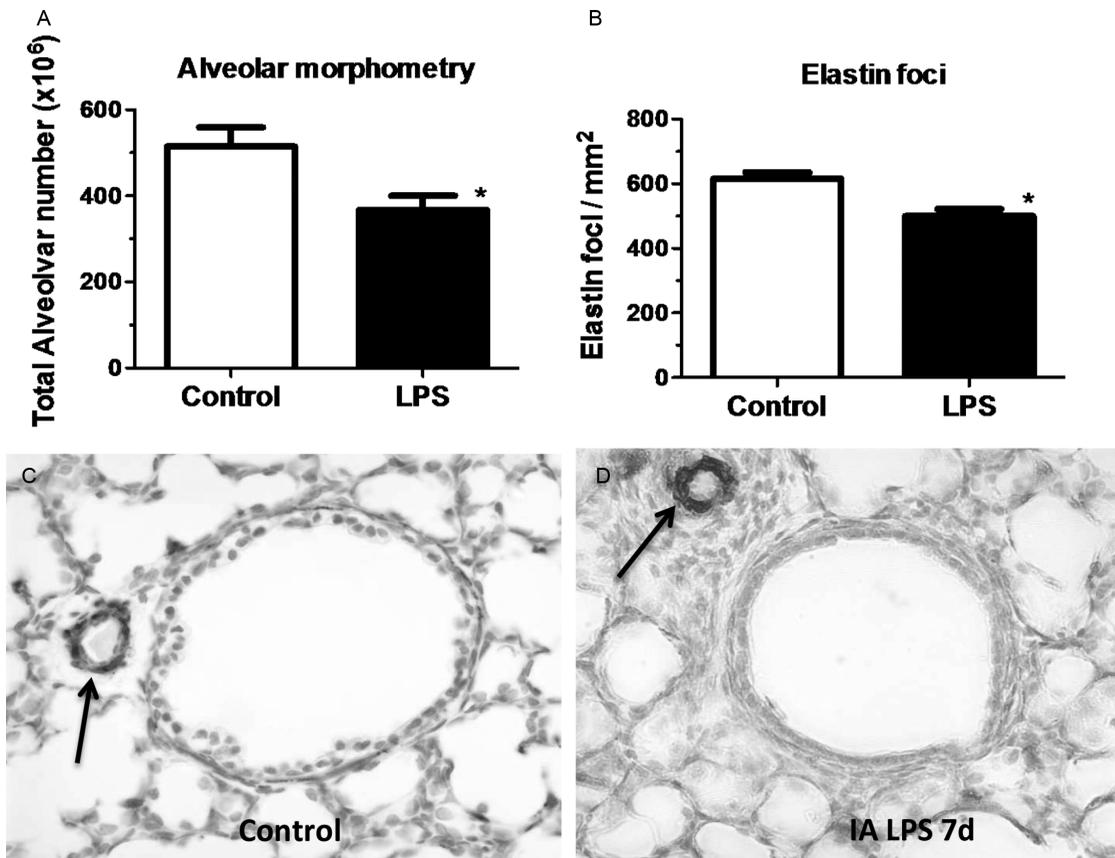


Figure 10-4. LPS-induced chorioamnionitis inhibited lung alveolar and vascular development in fetal sheep. Pregnant sheep were given intra-amniotic LPS 7 d prior to preterm delivery at 80% gestation. Lung morphometry was performed using inflation-fixed fetal lung. Compared to control, LPS exposed lungs had (A) decreased alveolar numbers (B) decreased elastin foci. (C–D) Compared to control fetuses, LPS-exposed fetuses had increased pulmonary arteriolar α -smooth muscle actin staining (arrow showing brown staining) indicating media hypertrophy. Note the adventitial fibrocyte proliferation surrounding the arteriole in LPS exposed lung. (* $p < 0.05$ vs. control) (Data redrawn from (20,21,23).

repair the lung in utero. Understanding the mechanisms of lung repair following intra-uterine injury is clearly important.

Innate Immune Responses in the Fetal Lung Exposed to Chorioamnionitis

The fetal lung responds to chorioamnionitis with inflammation, induced “lung maturation,” and aberrant alveolar and vascular development. The central question is how the naïve immune system of the fetal lung responds to an intrauterine inflammatory challenge. The sentinel immune cell of the lung is the alveolar macrophage. In adult humans and animals, macrophages are located in the airspaces directly in contact with the alveolar hypophase. Fetuses do not normally have alveolar macrophages. In mice, macrophages can be

detected in the lung interstitium from early gestation, while in other species, including nonhuman primates and sheep, very few mature macrophages are found in the fetal lung. In all species, mature alveolar macrophages begin populating the lung in large numbers postnatally with the onset of air breathing. Immature lung monocytes from preterm sheep have a minimal response (IL6 secretion) to an in vitro challenge to LPS and do not respond to TNF α (28). However, intra-amniotic LPS matures lung monocytes by stimulating GM-CSF and PU.1 expression in the fetal lung (Figure 10-5A,B). These monocytes migrate into the fetal alveolar spaces and respond vigorously to both LPS and TNF α in vitro (Figure 10-5C) (28). Thus exposure to a pro-inflammatory agonist in the amniotic fluid is a potent stimulus for maturation and responsiveness of monocytes in the lung.

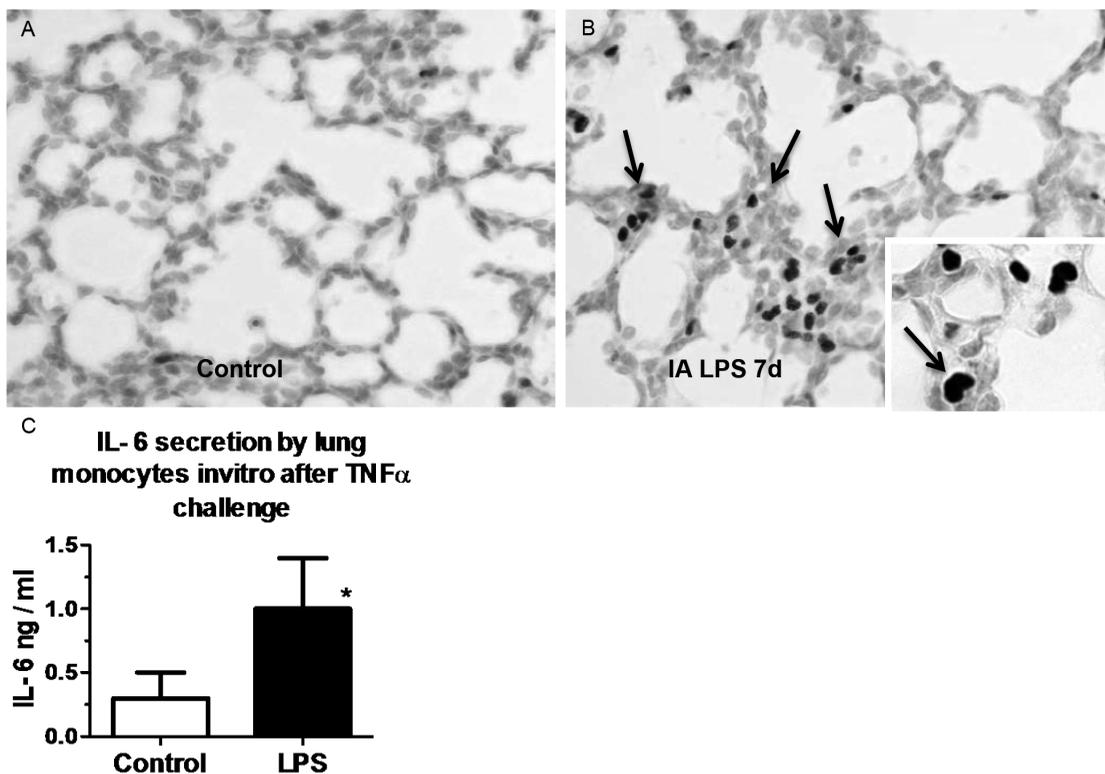


Figure 10-5. Functional maturation of lung monocytes in fetuses exposed to LPS induced chorioamnionitis. Pregnant sheep were given intra-amniotic LPS 7 d prior to preterm delivery at 80% gestation. (A,B) Immunohistology staining for PU.1 (a transcription factor necessary for maturation of monocyte) in fetal lung. Note increased monocyte PU.1 staining (arrow) after LPS exposure. (C) Lung monocytes were purified from fetuses and cultured in vitro in the presence of TNF α . Control preterm fetal lung monocyte responded with minimal IL-6 secretion, while fetuses exposed to intra-amniotic LPS responded with increased IL-6 secretion signifying a functional maturation of lung monocytes. (* $p < 0.05$ vs. control) (Data redrawn from Kramer, Joshi, Moss, et al. *Am J Physiol Lung Cell Mol Physiol.* 2007;293: L345–353).

Intra-amniotic LPS also can cause an innate immune tolerance in the fetus. In adult animals and humans, endotoxin tolerance is the suppression of LPS signaling caused by a complex reprogramming of inflammatory responses. As part of endotoxin tolerance, pro-inflammatory cytokine expression is downregulated, while there is no change or an increase in the expression of anti-inflammatory genes, antimicrobial genes, and genes mediating phagocytosis. In the preterm fetal sheep, exposure to intra-amniotic LPS 2 d before delivery induces a robust expression of cytokines in the fetal lung. However, if the fetus is exposed to two intra-amniotic LPS injections of the same dose 7 d and 2 d prior to delivery, the fetal lung is refractory to the second LPS injection (Figure 10-6A) (29). Interestingly, both lung and blood monocytes are refractory to an in vitro challenge with LPS.

The phenomenon of innate immune tolerance is not restricted to exposure to LPS. Exposure to

intra-amniotic *Ureaplasma parvum* almost completely abolished responsiveness of the fetal lung to LPS, implying a profound immune paralysis in the fetal lung induced by *Ureaplasma* exposure (Figure 10-6B) (30). The lung and blood monocytes from fetal sheep exposed to two injections of intra-amniotic LPS were also refractory to stimulation by a host of other TLR agonists, including PamCysK4 (TLR2), flagellin (TLR5), and CpG-DNA (TLR9) (31), demonstrating a cross tolerance to multiple TLRs. Other interactive phenomenon between antenatal LPS and postnatal inflammatory insults have also been reported. In rats, intra-amniotic LPS alone induced aberrant lung development and pulmonary hypertension. When these fetal rats exposed to LPS were exposed to postnatal moderate hyperoxia, the lung abnormalities were no longer evident. However, exposure to postnatal severe hyperoxia further enhanced the pulmonary abnormalities induced by antenatal LPS (32). Thus the

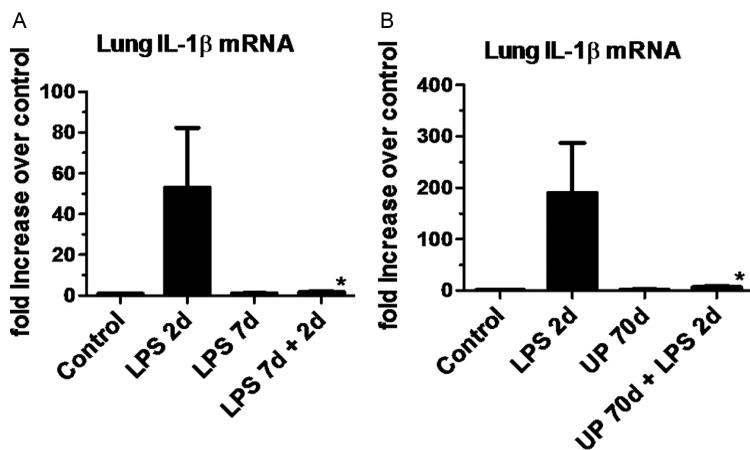


Figure 10-6. Endotoxin tolerance on repeated exposures to intra-amniotic LPS or *Ureaplasma parvum*. (a) Pregnant ewes were given intra-amniotic (IA) LPS either 2 d or 7 d or combined 7 d + 2 d prior to preterm delivery at 80% gestation. Quantitative rt-PCR for IL-1 β mRNA was performed on whole-lung homogenates. Exposure to a single LPS injection increased IL-1 β mRNA expression at 2 d with a decrease at 7 d. However, a prior exposure to IA LPS at 7 d blunted subsequent response to LPS injection at 2 d prior to delivery demonstrating endotoxin tolerance. (b) In a similar experimental schema pregnant ewes were given IA LPS injection 2 d or live *Ureaplasma parvum* (UP) 70 d or the combined exposure to *Ureaplasma parvum* 70 d + LPS 2 d prior to preterm delivery. *Ureaplasma* alone did not cause a significant induction of IL-1 β mRNA expression but almost completely blunted LPS responsiveness, demonstrating a potent innate immune modulation (* $p < 0.05$ vs. LPS 2 d) (Data redrawn from Kallapur, Jobe, Ball, et al. *J Immunol.* 2007;179:8491–8499; Kallapur, Kramer, Knox, et al. *J Immunol.* 2011;187:2688–2695).

interactive phenomena between different inflammatory insults can be complex and either exacerbate or reduce the lung injury response. Because innate immune tolerance is time dependent, it is not clear how these experimental phenomena will translate into clinical scenarios, where the timing of exposure to different inflammatory insults is not known. Although the precise mechanisms of innate immune tolerance are not known, the expression of negative regulator of Toll/IL-1 signaling, IRAK-M is increased in both the lung and blood monocytes (29).

Signaling Mediators Modulating Lung Development After Antenatal Insults

Intra-amniotic injection of IL-1 induced a large expression of IL-1 β , IL-8, GM-CSF, MCP-1, and the acute phase reactant serum amyloid A3 in the lungs of fetal sheep and fetal monkeys. Interestingly, the Th1 cytokines IFN γ , IL-12, type I interferon inducible genes CXCL9 (MIG) and CXCL10 (IP-10), and the Th2 cytokines IL-4 and IL-13 were not induced. IA LPS increased expression of additional genes inducible by the type I interferon signaling, including CXCL9 (MIG) and CXCL10 (IP-10) (33). In the fetal sheep, IA LPS

increased lung expression of TLR4 and TLR2 mRNA expression but decreased TLR4 expression in the gut, demonstrating organ specific responses. Neither IA IL-1 nor LPS significantly increased expression of TNF α in the lung, and IA TNF did not induce chorioamnionitis or fetal lung inflammation (4). The counterregulatory cytokine IL-10 and IL-1 receptor antagonist are only modestly increased in the fetal lung exposed to IA IL-1 or LPS.

Some candidate genes associated with lung injury have been assessed. Caveolins (Cavs) are implicated as major modulators of lung injury and remodeling by multiple signaling pathways by virtue of their strategic positioning in the lipid rafts of plasma membranes. Intra-amniotic LPS decreased the expression of Cav-1 in the preterm fetal lung (34). The decreased expression of Cav-1 was associated with the activation of the Smad2/3, Stat 3, a-SMase/ceramide pathways, and increased expression of HO-1 (34). Consistent with activation of Smads, intra-amniotic LPS increased lung TGF- β 1 mRNA and protein expression (35). However, the lung expression of CTGF – a key pro-fibrotic protein induced by TGF- β , decreased on exposure to intra-amniotic LPS (35). Metallo-proteinases regulate the breakdown of extracellular matrix in the lung. The expression of MMP-9 was increased in sheep

given intra-amniotic LPS (36) and in transgenic mice that overexpress IL-1 β in the lung (37). Sonic Hedgehog (Shh) signaling is a major pathway directing lung development. Intra-amniotic LPS also decreased Shh mRNA levels and Gli1 protein expression in the fetal sheep (21). In a fetal mouse lung explant system, abnormal saccular lung development induced by LPS was mediated by NF κ B activation in the macrophage, resulting in IL-1 β secretion (22). The link between inflammation and abnormal lung development was FGF-10 in this mouse lung explant system, a finding that is consistent with reduced FGF-10 in the tracheal aspirates of preterm infants developing chronic lung disease (38). In a recent study, vitamin D administration prenatally and postnatally reversed intra-amniotic LPS-induced aberrant alveolar and pulmonary vascular development in rats (39). Clearly, chorioamnionitis directly and indirectly changes multiple pathways that have been linked to lung growth and development.

Vascular endothelial growth factor (VEGF) signaling also is important in fetal lung development. Women with preeclampsia have increased amniotic fluid levels of soluble VEGF receptor 1, an increased risk of delivering infants with intra-uterine growth restriction, and an increased the risk for chronic lung disease (40). Intra-amniotic injection of soluble VEGF receptor 1 in rats inhibits alveolar development and causes pulmonary hypertension postnatally (41). Hypoxia and HIF-1 are well-known regulators of VEGF expression (42). Pulmonary expression of HIF-1 α , HIF-2 α , and the downstream target of their regulation, VEGF mRNA, is impaired following RDS in neonatal lambs (43). Furthermore, high postnatal HIF-1 levels increased VEGF levels and stimulated pulmonary angiogenic factors in a preterm baboon model of chronic lung disease (44). Infants dying of chronic lung disease had lower levels of VEGF and other angiogenic factors in tracheal aspirates (45) consistent with inhibited expression of angiogenic proteins in a baboon model of chronic lung disease and fetal sheep exposed to chorioamnionitis (23,46). These studies underscore the tight linkage between pulmonary vascular development and fetal lung growth.

Human Studies of Chorioamnionitis

Chorioamnionitis in pregnant women is diagnosed clinically or after histological examination

of the placenta. Thus it is often difficult to compare rates between centers or between different populations. In one U.S. study of over 2.2 million births, the prevalence of chorioamnionitis was 9.7 per 1,000 live births with odds ratio for neonatal mortality of 1.72 (95% CI 1.2 to 2.45) (47). The association of respiratory diseases including BPD following chorioamnionitis is complex. Early reports suggested that chorioamnionitis was associated with decreased rates of RDS and increased rates of developing BPD. While a systemic review did show an association between chorioamnionitis and development of BPD, the authors concluded that there was "strong evidence of publication bias which suggests potential overestimation of the measure of association between chorioamnionitis and BPD" (48). Been and colleagues have recently reported that severity of RDS may be greater in chorioamnionitis where there is fetal involvement compared to when there is not (49). Furthermore, responses of surfactant may vary after exposure to chorioamnionitis, especially when there is fetal inflammation (50). It is thus very likely that respiratory outcomes may depend on a number of factors associated with exposure to chorioamnionitis, including timing, fetal involvement, underlying cause of the infective process, and so on. Chorioamnionitis observed in infants born preterm may be associated with adverse longer-term respiratory outcomes. Jones and colleagues classified 95 preterm born infants according to histologic examination of the placenta (51). They observed lower maximal expiratory flows at forty weeks postconceptional age but interestingly noted that this outcome was mainly due to females being affected. In the Boston prospective study of 1,096 preterm subjects, those born at less than thirty-three weeks gestation with chorioamnionitis had the greatest risk of wheezing, and physicians diagnosed asthma up to the follow-up age of 2.2 years (52). Although chorioamnionitis certainly affects lung development, further work is required to address the specific population at highest risk of future risk of respiratory compromise.

Ureaplasma and Bronchopulmonary Dysplasia (BPD)

Ureaplasma is the most commonly isolated organism from the uterine cavity of women who deliver prematurely. The organism readily transfers from

the mother to the fetus and has been shown to cause chorioamnionitis and significant neonatal morbidity. Macrolide treatment of pregnant women presenting in preterm labor did not alter newborn outcomes (53,54). Several meta-analyses found an association between pulmonary presence of *Ureaplasma* and subsequent development of BPD (55–57). The most recent report gave odds ratios for the association between the presence of pulmonary *Ureaplasma* and subsequent development of BPD – oxygen dependency for at least twenty-eight days of age of 3.04 (95% CI 2.41, 3.83) or oxygen dependency at least thirty-six weeks postconceptional age of 2.22 (95% CI 1.42, 3.47) (57).

Disappointingly, initial studies using erythromycin to determine if eradication of *Ureaplasma* in preterm infants decreased rates of BPD were unsuccessful but were markedly underpowered. More recent studies are encouraging, although they should be considered as proof of principle studies (58,59). In the first, Ballard and colleagues, reported decreased duration of mechanical ventilation for infants treated with up to six weeks of azithromycin when compared to placebo. In the other study, Ozdemir et al. treated 74 *Ureaplasma* culture-positive preterm infants with clarithromycin or placebo and noted that *Ureaplasma* was eradicated in 68.5% of the cases. Rates of BPD decreased to 2.9% in the treated group compared to 36.4% in the placebo group ($p < 0.001$). Partial eradication suggests either inadequate treatment or antibiotic resistance to the macrolide.

In addition to their anti-infective activities, macrolides are attractive as they have potent anti-inflammatory activities. Two studies have reported the potential role of azithromycin in targeting pulmonary inflammation in infants at risk of developing BPD. In the first, Ballard and colleagues treated 220 preterm infants for up to six weeks with azithromycin or placebo but did not note any differences in BPD or death between the two groups (60). In their subanalyses they noted decreased BPD rates in the *Ureaplasma* colonized infants (73% vs. 94%, $p = 0.03$). In the other study, Gharehbaghi and colleagues, treated 108 preterm infants with two weeks of azithromycin and noted decreased rates of BPD in the treated group (25% vs. 43%, $p = 0.4$) (61). These studies are encouraging, but adequately powered studies are required to assess if indeed targeting

Ureaplasma and/or pulmonary inflammation can indeed alter the causal pathway in the development of BPD.

Chorioamnionitis and Antenatal Glucocorticoids: Effects on Fetal Lung Development

After the pioneering studies of Liggins (62) and the subsequent meta-analysis by Crowley (63), antenatal maternal corticosteroid treatment became a standard clinical practice for women at risk for preterm birth. A majority of very low-birth-weight deliveries also are exposed to infection or inflammation. Thus the fetus at high risk for preterm delivery is often exposed to both chorioamnionitis and maternal glucocorticoids. The common scenario is likely a fetus exposed to chorioamnionitis initially who subsequently is exposed to maternal glucocorticoids. However it is also possible that a pregnant woman who received betamethasone will subsequently develop rupture of membrane and consequently chorioamnionitis. The combined effects of these two exposures is of great biologic interest because betamethasone is a potent anti-inflammatory agent while chorioamnionitis causes fetal inflammation. Studies in fetal sheep have evaluated the pulmonary effects of fetal exposure to chorioamnionitis and maternal betamethasone with differing orders of exposures. If intra-amniotic LPS and maternal betamethasone were given simultaneously to pregnant ewes, there was an initial suppression of fetal lung inflammation induced by LPS. Curiously, there was an unexpected amplification of pulmonary inflammation at later time points. Thus maternal betamethasone had an initial innate immune suppressive effect as expected, but the later amplification of innate immune response was not anticipated (64). Despite the modulation of inflammatory response, the physiologic effect of the combined exposure was more airway surfactant production and improved lung mechanics compared to either exposure alone (Figure 10-3). When maternal betamethasone is administered before intra-amniotic LPS in the ewes, lung inflammation was suppressed (65). Interestingly, betamethasone treatment after LPS did not counteract inflammation but enhanced lung maturation. Thus the order of exposures of intra-amniotic LPS or maternal betamethasone

had large effects on fetal lung inflammation and maturation. Extrapolation of these experimental results would support the clinical practice of giving maternal betamethasone to women with suspected chorioamnionitis because the net physiological effect of the combined exposure is for the fetal lung to be more “clinically mature.”

Fetal Growth Restriction, Maternal Nutrition, and Lung Development

Intra-uterine growth restriction (IUGR) is a relatively common complication of pregnancy. Various terms including IUGR, fetal growth restriction, small for gestational age, and low birth weight are often confused. IUGR can be constitutional or nonconstitutional and is often described as symmetrical or nonsymmetrical. The IUGR, defined by both birth weight and gestation may have a pathological basis, which may occur due to fetal, maternal, or placental conditions with additional environmental or genetic influences (66). Its origin is likely to involve deficiencies of delivery of nutrition and/or oxygen. The resultant effect on growth trajectories will depend on when these insults occur during the different stages of lung growth.

A number of different fetal exposures can result in IUGR. These include hypertensive disorders in the mother, maternal smoking and poor nutrition, multiple pregnancies, uterine malformations, and fetal intra-uterine infections. From a physiological perspective, IUGR is caused by decreased fetal perfusion due to increased placental resistance or due to fetal abnormal somatic growth. Decreased tissue perfusion causes hypoxemia, decreased delivery of nutrients, and increased cortisol concentrations, all of which can affect lung development. Fetal growth restriction is independently associated with an increased risk of BPD in preterm infants (67). Calorie or protein restriction in multiple species or placental blood restriction to fetuses are established models of fetal growth restriction. IUGR is associated with decrease lung weight and total DNA content, decreased maturation of type II cells and subsequent surfactant maturation, decreased alveoli numbers, and thickened airway walls in animal models.

A mechanism by which IUGR may interfere with lung development is via PPAR gamma, a transcription factor known to regulate epigenetic

changes, particularly of chromatin-modifying enzymes. In a rat model of IUGR, maternal supplementation of docosahexaenoic acid (DHA) increased PPAR gamma levels and restored aberrant fetal lung development (68). Another mechanism of aberrant lung development during IUGR is via retinoic acid signaling. The retinoids act on their receptors RARs and RXRs and modulate alveolar formation, branching morphogenesis and surfactant protein synthesis (69). In a rat model, fetal growth restriction induced by a low-calorie maternal diet during pregnancy induced alveolar hypoplasia that was reversible by postnatal vitamin A supplementation (70). Administration of vitamin A can reduce lung injury and BPD in sheep and humans (71,72).

Longer-term effects of IUGR are of great interest as an initial study in the UK reported increased cardiovascular disease in adults who had growth restriction during pregnancy and during the first year of life (73). Placental insufficiency may accelerate maturation of the lungs as indicated by increased lecithin/sphingomyelin ratios in amniotic fluid (74). However, it is unclear if IUGR is associated with increased or decreased rates of respiratory distress syndrome in infancy. There is increasing evidence that growth restriction is associated with decreased lung function in childhood and beyond. In adulthood, Lawlor and colleagues in a meta-analysis of eight studies of adults found that birth weight was associated with approximately 48 mL (95% CI 26 to 70) increase in FEV₁ for each kg increase in birth weight (75). Catch-up growth during childhood may improve the outlook (76), but caution is required as increased weight gain during infancy may worsen lung function (77). IUGR may be caused by fetal, maternal, or placental factors. Increasing evidence suggests that IUGR is associated with increased long-term morbidity. The challenge is to prevent IUGR where possible by health education factors, including decreasing antenatal smoking and optimal maternal nutrition.

Effects of Prenatal Exposure to Toxins on Fetal Lung Development

Environmental pollutants are well recognized to cause major lung structure and function changes. In particular, antenatal smoking has received much attention. The effects of nicotine, the major

toxin in cigarettes, on fetal lung development are complex. The animal experiments and clinical studies point to morphological effects that translate to altered lung function after the fetus is born. Although the rate of smoking during pregnancy has decreased in the past decade in the United States, about 12% of all pregnant mothers reported smoking cigarettes (78). Maternal smoking causes harmful effects on the fetus due to fetal hypoxemia induced by carboxyhemoglobin and growth restriction in addition to direct fetal effects of the components of cigarette smoke. Nicotine readily crosses the placenta and is likely to be a prime mediator of the toxic effects of smoking on the fetal lungs. Nicotine causes DNA damage and promotes oxidant injury.

Nicotine has direct effects on both the conducting airways and the parenchyma but also appears to alter the epigenome resulting in life-long pulmonary consequences. Maternal cigarette smoke exposure in rats results in fewer numbers of alveoli in exposed pups (79). In fetal monkeys, prenatal exposure to nicotine also causes lung hypoplasia and increases collagen deposition around large airways and vessels (80). Prenatal nicotine exposure of lambs and monkeys causes proximal airway obstruction (81,82). Apart from the detrimental effects of nicotine on fetal lungs, nicotine stimulated branching morphogenesis and increased expression of surfactant proteins in embryonic mouse lungs cultured *in vitro* and increased surfactant producing alveolar type II cells in monkeys (80,83).

Consistent with these experimental results, human studies have also suggested a decreased risk for respiratory distress syndrome in infants exposed to maternal smoking (84). However, infants born to mothers who smoke during pregnancy are at greater risk of sudden infant death syndrome (SIDS), decreased lung function at birth and into adulthood, increased risk of respiratory symptoms, increased diagnosis of asthma, and increased risk of bronchial hyper-responsiveness. One study showed increased inner airway wall thickening in infants who died from SIDS for mothers who smoked 20 cigarettes or more during pregnancy when compared to those who did not (85). Clinical studies have also demonstrated more restriction in pulmonary airflow in preterm infants compared with term infants (86, 87). A more recent prospective study of a twenty-one-year follow-up of 2,409 young adults

measured decreased lung spirometry (FEV_1 and $FEF_{25-75\%}$) in the young adults born to mothers who smoked during pregnancy (88). Another recent study evaluated lung function of infants whose mothers smoked cigarettes during pregnancy. Predictably, compared to healthy term infants, lung function of infants born to smokers was decreased at birth, and these infants also had increased wheezing reports at one year of age (89). This effect was particularly pronounced in mothers with homozygous mutant alleles in the $\alpha 5$ nicotinic acetylcholine receptor. Remarkably, daily vitamin C supplementation (500 mg) of smoking mothers starting in the second trimester rescued decreased lung function at birth and the incidence of wheezing at one year of age. Presumably, the salutary effect of vitamin C was due to its antioxidant effect. Thus, the effects of antenatal smoking are long lasting but do provide an opportunity to intervene either by smoking cessation or by vitamin C supplementation to decrease the long-term consequences of antenatal exposure to a noxious substance.

The fetal lung may also be affected by exposure to drugs. Antidepressant, particularly selective serotonin reuptake inhibitor (SSRI), usage has increased greatly during pregnancy. In a recent meta-analysis, the use of antidepressants during pregnancy was associated with an increased the risk for persistent pulmonary hypertension of the newborn if the drug was used in the third trimester (2.50, 1.32 to 4.73; $P = 0.005$) but not the first trimester (odds ratio 1.23, 95% confidence interval 0.58 to 2.60; $P = 0.58$) (90). Because the disease is not very common, the overall risk for neonatal pulmonary hypertension with maternal SSRI use is small when considered against the potential benefits.

Another area receiving increasing scrutiny is the exposure to environmental pollutions such as carbon particles and gases such as nitrous oxide and sulphur oxide particularly from charcoal and kerosene used for cooking and heating. Increased risk of stillbirth, decreased birth weight, and prematurity are associated with increased antenatal exposure. In one study, birth weight was decreased by 110 g for coal, 107 g for kerosene, and 78 g for biomass fuels when compared to "cleaner" fuels. Low birth weight was also increased with use of kerosene and biomass fuels (91). An increased risk of neonatal death is strongly associated with household use of coal

(OR 18.54; 95% CI: 6.31–54.45) and perhaps with kerosene (OR 2.30; 95% CI: 0.95–5.55). The effects of pollution may indirectly affect lung growth and development in the newborn, and further work is needed to assess if any of the effects directly affect lung growth and development of the fetus.

Fetal Breathing, Fetal Lung Fluid, Amniotic Fluid, and Lung Development

For optimal lung growth and development, adequate intra- and extrathoracic fluid, adequate intrathoracic space, pulmonary blood flow, and fetal breathing are required. Abnormalities of the thoracic cage or muscle disorders may also affect lung growth and development. The elegant experiments by Desai and Wigglesworth showed that transection of the phrenic nerve results in hypoplastic lungs suggesting that breathing movements are essential to lung development (92). In humans, breathing movements are observed from eleven weeks gestation and occur approximately 30% of the time in the third trimester. Breathing movements appear to be essential to maintain an adequate fetal lung fluid volume. The rhythmic movements are necessary to promote mitogenic growth factor activity. Attempts have been made to use breathing movements and patterns to predict outcomes, but their clinical use are limited by their episodic nature.

The importance of a satisfactory fetal lung fluid volume was shown by Moessinger et al., who observed that when one lobe in fetal lambs was allowed to drain freely, marked lung hypoplasia occurred (93). In contrast, ligation of the contralateral lobe resulted in lung hyperplasia. Fetal lung fluid is formed by epithelial cells that actively transport chloride ions from the interstitium to the lumen. In the near term fetal lung, the fluid production rate is 4 to 5 mL/kg/hr (94). Assuming a 3 to 4 kg fetus, the daily production of fetal lung fluid is therefore about 400 mL/day. Fetal lung fluid flows intermittently up the trachea with fetal breathing movements, and some of this fluid is swallowed while the rest mixes with the amniotic fluid. The pressure in the fetal trachea exceeds that in the amniotic fluid by about 2 mm Hg, demonstrating a resistance to outflow that maintains the fetal lung fluid volume. Although

the presence of fetal lung fluid is essential for normal lung development, its clearance is equally essential for normal neonatal respiratory adaptation. Fluid clearance after birth results from active sodium transport via the ENaC sodium channel, which can be blocked with amiloride (95). In experimental models, tracheal occlusion in late gestation can reverse much of the pulmonary hypoplasia resulting from diaphragmatic hernia, but the occlusion induces a decrease in type II cell number and causes surfactant deficiency (96).

Decreased amniotic fluid or oligohydramnios complicates about 10% of pregnancies (97). Although a number of factors are responsible for amniotic fluid volume, fetal urine is a significant contributor to amniotic fluid volume during later stages of pregnancy. Severe pulmonary hypoplasia associated with renal agenesis (Potter's syndrome), and prolonged oligohydramnios is characterized by a decrease in lung size and cell number together with narrow airways, a delay of epithelial differentiation, and surfactant deficiency (98). The importance of oligohydramnios was shown in study of 5,228 women with oligohydramnios during the third trimester (99). When compared to 20,912 unaffected pregnancies, the offspring of the oligohydramnios group had increased rates of respiratory failure and increased rates of hospitalization.

Another important cause of decreased amniotic fluid is early preterm prelabor rupture of membranes (pPROM), often occurring during the second trimester. Relatively short-term oligohydramnios caused by ruptured membranes in the sixteenth to twenty-eighth week of gestation can also result in pulmonary hypoplasia; the severity in general correlates with the length of the oligohydramnios (100). Williams and colleagues reported their two-year follow-up of fifteen survivors whose mothers had ruptured their membranes before twenty-five weeks of completed gestation (101). Surprisingly they did not note a difference in BPD between the groups, but those infants with pPROM had increased length of respiratory support and increased hospital stay. Encouragingly, these affected infants did not have neurological deficits, but they did have increased postdischarge hospital readmissions for respiratory illness.

Pulmonary hypoplasia can also result from either a restriction of lung growth or the absence of fetal breathing (102). Any reduction of the

chest cavity by a mass, effusion, or external compression can impact lung growth. Lung hypoplasia can be minimal or very severe. Infants with congenital diaphragmatic hernia have more severe hypoplasia on the ipsilateral side than on the contralateral side, although the contralateral lung may be hypoplastic (103). The lungs have decreased acinar units as well as delayed epithelial maturation with an associated surfactant deficiency.

Lung growth is dependent on the coordinated interaction between the intra- and extrathoracic volumes, adequate fetal lung volume and fluid production, and breathing movements. Disturbance of any of these factors will disrupt normal development of the fetal lung.

Knowledge gaps and the future: Although the concept of origin of cardiovascular and respiratory diseases in adults due to perturbations in early infancy is well known, the fetal origins hypothesis is still relatively new. We have reviewed a number of fetal insults including infection, inflammation, mechanical perturbations, and toxin exposure to the fetus as determinants of aberrant lung growth. Much of these observations are still at an early descriptive stage. Focused research using animal models and targeted clinical studies will be needed to unravel mechanisms of the pulmonary insult. A major area that desperately needs further study is the understanding of developmental ontogeny of the innate immune system of the fetus. How does the fetal immune system recognize and respond to its environmental challenge remains a mystery. The clinical correlate of this question is: Does the patterning of fetal immune system by exposure to infectious/inflammatory insult lead to lasting effects, and if so, what are the pathways? Pulmonary diseases such as reactive airway disease particularly resulting from prenatal exposures

likely are mediated by aberrant lung growth and aberrant immune modulation. Understanding the relative contributions of these pathways will be important to devise targeted therapies. Similar questions can be asked about how mechanical stretch mediates lung development in the fetus. At present, treatment with antimicrobials alone have not shown to substantively improve perinatal outcomes even for women with chorioamnionitis. In the future, it may be important to devise anti-inflammatory immunomodulatory therapies as adjunct to antimicrobials to optimize the perinatal outcomes including optimizing pulmonary outcomes for the fetus. Vitamin C supplementation for women who are not able to cease smoking during pregnancy might be an inexpensive but effective treatment to improve pulmonary outcomes of the fetus.

Summary

A multitude of exposures can affect fetal lung development. Understanding the physiology and basic mechanisms of fetal lung development may allow us to intervene in the future. For example, imaging studies are being used to predict the degree of lung hypoplasia in high-risk pregnancies. Unfortunately, antenatal prediction of pulmonary hypoplasia is still far from accurate. However, more sophisticated imaging modalities in the future can improve the antenatal prediction of pulmonary hypoplasia. Currently, the long-term benefits of fetal tracheal occlusion therapy to reduce pulmonary hypoplasia resulting from diaphragmatic hernia have not been proven, and this therapy remains investigative. Finally, public health measures to reduce fetal exposure to toxins that adversely affect lung development need to be intensified.

References

- Shannon JM, Deterding RR. Epithelial-mesenchymal interactions in lung development. In: McDonald JA, ed. *Lung Growth and Development*. New York, NY: Marcel Dekker; 1997:81–106.
- Pringle, KC. Human fetal lung development and related animal models. *Clin Obstet Gynecol*. 1986;29: 502–513.
- Kim MJ, Romero R, Gervasi MT, et al. Widespread microbial invasion of the chorioamniotic membranes is a consequence and not a cause of intra-amniotic infection. *Lab Invest*. 2009;89:924–936.
- Ikegami M, Moss TJ, Kallapur SG, et al. Minimal lung and systemic responses to TNF {alpha} in preterm sheep. *Am J Physiol Lung Cell Mol Physiol*. 2003;285:L121–L129.
- Kallapur SG, Moss TJ, Auten RL Jr, et al. IL-8 signaling does not mediate intra-amniotic LPS-induced inflammation and maturation in preterm fetal lamb lung. *Am J Physiol Lung Cell Mol Physiol*. 2009;297: L512–519.

- 6 Sadowsky DW, Adams KM, Gravett MG, Witkin SS, Novy MJ. Preterm labor is induced by intraamniotic infusions of interleukin-1beta and tumor necrosis factor-alpha but not by interleukin-6 or interleukin-8 in a nonhuman primate model. *Am J Obstet Gynecol.* 2006;195:1578–1589.
- 7 DiGiulio DB. Diversity of microbes in amniotic fluid. *Semin Fetal Neonatal Med.* 2012;17:2–11.
- 8 Novy MJ, Duffy L, Axthelm MK, et al. *Ureaplasma parvum* or *Mycoplasma hominis* as sole pathogens cause chorioamnionitis, preterm delivery, and fetal pneumonia in rhesus macaques. *Reprod Sci.* 2009;16:56–70.
- 9 Dando SJ, Nitsos I, Kallapur SG, et al. The role of the multiple banded antigen of *Ureaplasma parvum* in intra-amniotic infection: major virulence factor or decoy? *PLoS One.* 2012;7: e29856.
- 10 Knox CL, Dando SJ, Nitsos I, et al. The severity of chorioamnionitis in pregnant sheep is associated with in vivo variation of the surface-exposed multiple-banded antigen/gene of *Ureaplasma parvum*. *Biol Reprod.* 2010;83:415–426.
- 11 Payne MS, Kemp MW, Kallapur SG, et al. Intrauterine *Candida albicans* infection elicits severe inflammation in fetal sheep. *Pediatr Res.* 2014;75:716–722.
- 12 Kallapur SG, Willet KE, Jobe AH, Ikegami M, Bachurski CJ. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol.* 2001;280:L527–L536.
- 13 Bachurski CJ, Ross GF, Ikegami M, Kramer BW, Jobe AH, Intra-amniotic endotoxin increases pulmonary surfactant proteins and induces SP-B processing in fetal sheep. *Am J Physiol Lung Cell Mol Physiol.* 2001;280:L279–285.
- 14 Kramer BW, Kallapur SG, Moss TJ, et al. Modulation of fetal inflammatory response on exposure to lipopolysaccharide by chorioamnion, lung, or gut in sheep. *Am J Obstet Gynecol.* 2010;202:77 e71–79.
- 15 Kemp MW, Kannan PS, Saito M, et al. Selective exposure of the fetal lung and skin/amnion (but not gastrointestinal tract) to LPS elicits acute systemic inflammation in fetal sheep. *PLoS One.* 2013;8: e63355.
- 16 Moss TJ, Nitsos I, Kramer BW, Ikegami M, Newnham JP, Jobe AH. Intra-amniotic endotoxin induces lung maturation by direct effects on the developing respiratory tract in preterm sheep. *Am J Obstet Gynecol.* 2002;187:1059–1065.
- 17 Sosenko IR, Kallapur SG, Nitsos I, et al. IL-1alpha causes lung inflammation and maturation by direct effects on preterm fetal lamb lungs. *Pediatr Res.* 2006;60:294–298.
- 18 Kallapur SG, Moss TJM, Ikegami M, Jasman RL, Newnham JP, Jobe AH. Recruited inflammatory cells mediate endotoxin induced lung maturation in preterm fetal lambs. *Am J Respir Crit Care Med.* 2005;172:1315–1321.
- 19 Kallapur SG, Nitsos I, Moss TJ, et al. IL-1 mediates pulmonary and systemic inflammatory responses to chorioamnionitis induced by lipopolysaccharide. *Am J Respir Crit Care Med.* 2009;179:955–961.
- 20 Willet K.E., Jobe AH, Ikegami M, Newnham J, Brennan S, Sly PD. Antenatal endotoxin and glucocorticoid effects on lung morphometry in preterm lambs. *Pediatr Res.* 2000;48:782–788.
- 21 Collins JJ, Kuypers E, Nitsos I, et al. LPS-induced chorioamnionitis and antenatal corticosteroids modulate Shh signaling in the ovine fetal lung. *Am J Physiol Lung Cell Mol Physiol.* 2012;303: L778–787.
- 22 Blackwell TS, Hipps AN, Yamamoto Y, et al. NF-kappaB signaling in fetal lung macrophages disrupts airway morphogenesis. *J Immunol.* 2011;187:2740–2747.
- 23 Kallapur SG, Bachurski CJ, Le Cras TD, Joshi SN, Ikegami M, Jobe AH. Vascular changes after intra-amniotic endotoxin in preterm lamb lungs. *Am J Physiol Lung Cell Mol Physiol.* 2004;287: L1178–1185.
- 24 Polglase GR, Hooper SB, Gill AW, et al. Intrauterine inflammation causes pulmonary hypertension and cardiovascular sequelae in preterm lambs. *J Appl Physiol.* 2010;108:1757–1765.
- 25 Kallapur SG, Kramer BW, Jobe AH. *Ureaplasma* and BPD. *Semin Perinatol.* 2013;37:94–101.
- 26 Bry K, Whitsett JA, Lappalainen U. IL-1beta disrupts postnatal lung morphogenesis in the mouse. *Am J Respir Cell Mol Biol.* 2007;36:32–42.
- 27 Kallapur SG, Nitsos I, Moss TJM, et al. Chronic endotoxin exposure does not cause sustained structural abnormalities in the fetal sheep lungs. *Am J Physiol Lung Cell Mol Biol.* 2005;288:L966–L974.
- 28 Kramer BW, Joshi SN, Moss TJ, et al. Endotoxin-induced maturation of monocytes in preterm fetal sheep lung. *Am J Physiol Lung Cell Mol Physiol.* 2007;293: L345–353.

- 29 Kallapur SG, Jobe AH, Ball MK, et al. Pulmonary and systemic endotoxin tolerance in preterm fetal sheep exposed to chorioamnionitis. *J Immunol.* 2007;179:8491–8499.
- 30 Kallapur SG, Kramer BW, Knox CL, et al. Chronic fetal exposure to *Ureaplasma parvum* suppresses innate immune responses in sheep. *J Immunol.* 2011;187:2688–2695.
- 31 Kramer BW, Kallapur SG, Moss TJ, Nitsos I, Newnham JP, Jobe AH. Intra-amniotic LPS modulation of TLR signaling in lung and blood monocytes of fetal sheep. *Innate Immun.* 2009;15:101–107.
- 32 Tang JR, Seedorf GJ, Muehlethaler V, et al. Moderate postnatal hyperoxia accelerates lung growth and attenuates pulmonary hypertension in infant rats after exposure to intra-amniotic endotoxin. *Am J Physiol Lung Cell Mol Physiol.* 2010;299:L735–748.
- 33 Kallapur SG, Presicce P, Rueda CM, Jobe AH, Choungnet CA. Fetal immune response to chorioamnionitis. *Semin Reprod Med.* 2014;32:56–67.
- 34 Kunzmann S, Collins JJ, Yang Y, et al. Antenatal inflammation reduces cav-1 expression and influences multiple signaling pathways in preterm fetal lungs. *Am J Respir Cell Mol Biol.* 2011;45:969–976.
- 35 Kunzmann S, Speer CP, Jobe AH, Kramer BW. Antenatal inflammation induced TGF-beta1 but suppressed CTGF in preterm lungs. *Am J Physiol Lung Cell Mol Physiol.* 2007;292:L223–231.
- 36 Sweet DG, Huggett MT, Warner JA, et al. Maternal betamethasone and chorioamnionitis induce different collagenases during lung maturation in fetal sheep. *Neonatology.* 2008;94:79–86.
- 37 Lappalainen U, Whitsett JA, Wert SE, Tichelaar JW, Bry K. Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung. *Am J Respir Cell Mol Biol.* 2005;32:311–318.
- 38 Benjamin JT, Smith RJ, Halloran BA, Day TJ, Kelly DR, Prince LS. FGF-10 is decreased in bronchopulmonary dysplasia and suppressed by Toll-like receptor activation. *Am J Physiol Lung Cell Mol Physiol.* 2007;292:L550–558.
- 39 Mandell E, Seedorf G, Gien J, Abman SH. Vitamin D treatment improves survival and infant lung structure after intra-amniotic endotoxin exposure in rats: potential role for the prevention of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol.* 2014;306:L420–428.
- 40 Saxena AR, Seely EW, Rich-Edwards JW, Wilkins-Haug LE, Karumanchi SA, McElrath TF. First trimester PAPP-A levels correlate with sFlt-1 levels longitudinally in pregnant women with and without preeclampsia. *BMC Pregnancy Childbirth.* 2013;13:85.
- 41 Tang JR, Karumanchi SA, Seedorf G, Markham N, Abman SH. Excess soluble vascular endothelial growth factor receptor-1 in amniotic fluid impairs lung growth in rats: linking preeclampsia with bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol.* 2012;302:L36–46.
- 42 Liu Y, Cox SR, Morita T, Kourembanas S. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circ Res.* 1995;77:638–643.
- 43 Grover TR, Asikainen TM, Kinsella JP, Abman SH, White CW. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha are decreased in an experimental model of severe respiratory distress syndrome in preterm lambs. *Am J Physiol Lung Cell Mol Physiol.* 2007;292:L1345–1351.
- 44 Asikainen TM, Waleh NS, Schneider BK, Clyman RI, White CW. Enhancement of angiogenic effectors through hypoxia-inducible factor in preterm primate lung in vivo. *Am J Physiol Lung Cell Mol Physiol.* 2006;291:L588–595.
- 45 Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM. Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1 and Tie-2 in human infants dying with bronchopulmonary dysplasia. *Am J Respir Critic Care Med.* 2001;164:1971–1980.
- 46 Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H. Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. *Am J Physiol Lung Cell Mol Physiol.* 2002;282:L811–823.
- 47 Malloy MH. Chorioamnionitis: epidemiology of newborn management and outcome United States 2008. *J Perinatol.* 2014;34(8):611–615.
- 48 Hartling L, Liang Y, Lacaze-Masmonteil T. Chorioamnionitis as a risk factor for bronchopulmonary dysplasia: a systematic review and meta-analysis. *Arch Child Fetal Neonatal Ed.* 2012;97:F8–F17.
- 49 Been JV, Rours IG, Kornelisse RF, et al. Histologic chorioamnionitis, fetal involvement, and antenatal

- steroids: effects on neonatal outcome in preterm infants. *Am J Obstet Gynecol.* 2009;201:587 e581–588.
- 50 Been JV, Rours IG, Kornelisse RF, Jonkers F, de Krijger RR, Zimmermann LJ. Chorioamnionitis alters the response to surfactant in preterm infants. *J Pediatr.* 2010;156:10–15 e11.
- 51 Jones MH, Corso AL, Tepper RS, et al. Chorioamnionitis and subsequent lung function in preterm infants. *PLoS One.* 2013;8:e81193.
- 52 Kumar R, Yu Y, Story RE, et al. Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study. *J Allergy Clin Immunol.* 2008;121:878–884 e876.
- 53 Kenyon SL, Taylor DJ, Tarnow-Mordi W. Broad-spectrum antibiotics for preterm, prelabour rupture of fetal membranes: the ORACLE I randomised trial. ORACLE Collaborative Group. *Lancet.* 2001;357:979–988.
- 54 Kenyon SL, Taylor DJ, Tarnow-Mordi W, Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. ORACLE Collaborative Group. *Lancet.* 2001;357:989–994.
- 55 Wang EE, Ohlsson A, Kellner JD, Association of Ureaplasma urealyticum colonization with chronic lung disease of prematurity: results of a metaanalysis. *J Pediatr.* 1995;127:640–644.
- 56 Schelonka RL, Katz B, Waites KB, Benjamin DK Jr. Critical appraisal of the role of Ureaplasma in the development of bronchopulmonary dysplasia with metaanalytic techniques. *Pediatr Infect Dis J.* 2005;24:1033–1039.
- 57 Lowe J, Watkins WJ, Edwards MO, et al. Association between pulmonary Ureaplasma colonization and bronchopulmonary dysplasia in preterm infants: updated systematic review and meta-analysis. *Pediatr Infect Dis J.* 2014;33:697–702.
- 58 Ballard HO, Anstead MI, Shook LA. Azithromycin in the extremely low birth weight infant for the prevention of bronchopulmonary dysplasia: a pilot study. *Respir Res.* 2007;8:41.
- 59 Ozdemir R, Erdeve O, Dizdar EA, et al. Clarithromycin in preventing bronchopulmonary dysplasia in Ureaplasma urealyticum-positive preterm infants. *Pediatrics.* 2011;128:e1496–1501.
- 60 Ballard HO, ; Shook LA, Bernard P, et al. Use of azithromycin for the prevention of bronchopulmonary dysplasia in preterm infants: a randomized, double-blind, placebo controlled trial. *Pediatr Pulmonol.* 2011;46:111–118.
- 61 Gharehbaghi MM, Peirovifar A, Ghojzadeh M, Mahallei M. Efficacy of azithromycin for prevention of bronchopulmonary dysplasia (BPD). *Turkish J Med Sci.* 2012;42:1070–1075.
- 62 Liggins GC, Howie RN. A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. *Pediatrics.* 1972;50:515–525.
- 63 Crowley PA. Antenatal corticosteroid therapy: a meta-analysis of the randomized trials, 1972 to 1994. *Am J Obstet Gynecol.* 1995;173:322–335.
- 64 Kallapur SG, Kramer BW, Moss TJ, et al. Maternal glucocorticoids increase endotoxin-induced lung inflammation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol.* 2003;284:L633–L642.
- 65 Kuypers E, Collins JJ, Kramer BW, et al. Intra-amniotic LPS and antenatal betamethasone: inflammation and maturation in preterm lamb lungs. *Am J Physiol Lung Cell Mol Physiol.* 2012;302:L380–389.
- 66 Pike K, Pillow JJ, Lucas JS. Long term respiratory consequences of intrauterine growth restriction. *Semin Fetal Neonatal Med.* 2012;17:92–98.
- 67 Bose C, Van Marter LJ, Laughon M, et al. Fetal growth restriction and chronic lung disease among infants born before the 28th week of gestation. *Pediatrics.* 2009;124:e450–458.
- 68 Joss-Moore LA, Wang Y, Baack ML, et al. IUGR decreases PPARgamma and SETD8 expression in neonatal rat lung and these effects are ameliorated by maternal DHA supplementation. *Early Hum Dev.* 2010;86:785–791.
- 69 Massaro D, Massaro GD. Retinoids, alveolus formation, and alveolar deficiency: clinical implications. *Am J Respir Cell Mol Biol.* 2003;28:271–274.
- 70 Londhe VA, Maisonet TM, Lopez B, Shin BC, Huynh J, Devaskar SU. Retinoic acid rescues alveolar hypoplasia in the calorie-restricted developing rat lung. *Am J Respir Cell Mol Biol.* 2013;48:179–187.
- 71 Bland RD, Albertine KH, Pierce RA, Starcher BC, Carlton DP. Impaired alveolar development and abnormal lung elastin in preterm lambs with chronic lung injury: potential benefits of retinol treatment. *Biol Neonate.* 2003;84:101–102.

- 72 Tyson JE, Wright LL, Oh W, et al. Vitamin A supplementation for extremely-low-birth-weight infants. National Institute of Child Health and Human Development Neonatal Research Network. *N Engl J Med.* 1999;340:1962–1968.
- 73 Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet.* 1989;2:577–580.
- 74 Torrance HL, Voorbij HA, Wijnberger LD, van Bel F, Visser GH. Lung maturation in small for gestational age fetuses from pregnancies complicated by placental insufficiency or maternal hypertension. *Early Hum Dev.* 2008;84:465–469.
- 75 Lawlor DA, Ebrahim S, Davey Smith G. Association of birth weight with adult lung function: findings from the British Women's Heart and Health Study and a meta-analysis. *Thorax.* 2005;60:851–858.
- 76 Kotecha SJ, Watkins WJ, Heron J, Henderson J, Dunstan FD, Kotecha S. Spirometric lung function in school-age children: effect of intrauterine growth retardation and catch-up growth. *Am J Respir Crit Care Med.* 2010;181:969–974.
- 77 Lucas JS, Inskip HM, Godfrey KM, et al. Small size at birth and greater postnatal weight gain: relationships to diminished infant lung function. *Am J Respir Crit Care Med.* 2004;170:534–540.
- 78 Ebrahim SH, Floyd RL, Merritt RK II, Decoufle P, Holtzman D. Trends in pregnancy-related smoking rates in the United States, 1987–1996. *JAMA.* 2000;283:361–366.
- 79 Collins MH, Moessinger AC, Kleinerman J, et al. Fetal lung hypoplasia associated with maternal smoking: a morphometric analysis. *Pediatr Res.* 1985;19:408–412.
- 80 Sekhon HS, Jia Y, Raab R, et al. Prenatal nicotine increases pulmonary alpha7 nicotinic receptor expression and alters fetal lung development in monkeys. *J Clin Invest.* 1999;103:637–647.
- 81 Sandberg K, Poole SD, Hamdan A, Arbogast P, Sundell HW. Altered lung development after prenatal nicotine exposure in young lambs. *Pediatr Res.* 2004;56:432–439.
- 82 Sekhon HS, Keller JA, Benowitz NL, Spindel ER. Prenatal nicotine exposure alters pulmonary function in newborn rhesus monkeys. *Am J Respir Crit Care Med.* 2001;164:989–994.
- 83 Wuenschell CW, Zhao J, Tefft JD, Warburton D. Nicotine stimulates branching and expression of SP-A and SP-C mRNAs in embryonic mouse lung culture. *Am J Physiol.* 1998;274:L165–170.
- 84 Lieberman E, Torday J, Barbieri R, Cohen A, Van Vunakis H, Weiss ST. Association of intrauterine cigarette smoke exposure with indices of fetal lung maturation. *Obstet Gynecol.* 1992;79:564–570.
- 85 Elliot J, Vullermin P, Robinson P. Maternal cigarette smoking is associated with increased inner airway wall thickness in children who die from sudden infant death syndrome. *Am J Respir Crit Care Med.* 1998;158:802–806.
- 86 Hoo AF, Henschen M, Dezateux C, Costeloe K, Stocks J. Respiratory function among preterm infants whose mothers smoked during pregnancy. *Am J Respir Crit Care Med.* 1998;158:700–705.
- 87 Milner AD, Marsh MJ, Ingram DM, Fox GF, Susiva C. Effects of smoking in pregnancy on neonatal lung function. *Arch Dis Child Fetal Neonatal Ed.* 1999;80:F8–14.
- 88 Hayatbakhsh MR, Sadasivam S, Mamun AA, Najman JM, Williams GM, O'Callaghan MJ. Maternal smoking during and after pregnancy and lung function in early adulthood: a prospective study. *Thorax.* 2009;64:810–814.
- 89 McEvoy CT, Schilling D, Clay N, et al. Vitamin C supplementation for pregnant smoking women and pulmonary function in their newborn infants: a randomized clinical trial. *JAMA.* 2014;311:2074–2082.
- 90 Grigoriadis S, Vonderporten EH, Mamisashvili L, et al. Prenatal exposure to antidepressants and persistent pulmonary hypertension of the newborn: systematic review and meta-analysis. *BMJ.* 2014;348:f6932.
- 91 Epstein MB, Bates MN, Arora NK, Balakrishnan K, Jack DW, Smith KR. Household fuels, low birth weight, and neonatal death in India: the separate impacts of biomass, kerosene, and coal. *Int J Hyg Environ Health.* 2013;216:523–532.
- 92 Wigglesworth JS, Desai R. Effect on lung growth of cervical cord section in the rabbit fetus. *Early Hum Dev.* 1979;3:51–65.
- 93 Moessinger AC, Harding R, Adamson TM, Singh M, Kiu GT. Role of lung fluid volume in growth and maturation of the fetal sheep lung. *J Clin Invest.* 1990;86:1270–1277.
- 94 Lines A, Hooper SB, Harding R. Lung liquid production rates and volumes do not decrease before labor in

- healthy fetal sheep. *J Appl Physiol.* 1997;82:927–932.
- 95 Hummler E, Barker P, Gatzky J, et al. Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nat Genet.* 1996;12:325–328.
- 96 Benachi A, Chailley-Heu B, Delezoides AL, et al. Lung growth and maturation after tracheal occlusion in diaphragmatic hernia. *Am J Respir Crit Care Med.* 1998;157:921–927.
- 97 Chauhan SP, Doherty DD, Magann EF, Cahanding F, Moreno F, Klausen JH. Amniotic fluid index vs single deepest pocket technique during modified biophysical profile: a randomized clinical trial. *Am J Obstet Gynecol.* 2004;191:661–667.
- 98 Nakamura Y, Funatsu Y, Yamamoto I, et al. Potter's syndrome associated with renal agenesis or dysplasia. Morphological and biochemical study of the lung. *Arch Pathol Lab Med.* 1985;109:441–444.
- 99 Chien LN, Chiou HY, Wang CW, Yeh TF, Chen CM. Oligohydramnios increases the risk of respiratory hospitalization in childhood: a population-based study. *Pediatr Res.* 2014;75: 576–581.
- 100 Thibeault DW, Beatty EC Jr, Hall RT, Bowen SK, O'Neill DH. Neonatal pulmonary hypoplasia with premature rupture of fetal membranes and oligohydramnios. *J Pediatr.* 1985;107:273–277.
- 101 Williams O, Michel B, Hutchings G, Debauche C, Hubinont C. Two-year neonatal outcome following PPRM prior to 25 weeks with a prolonged period of oligohydramnios. *Early Hum Dev.* 2012;88: 657–661.
- 102 Liggins GC. Growth of the fetal lung. *J Dev Physiol.* 1984;6:237–248.
- 103 George DK, Cooney TP, Chiu BK, and Thurlbeck WM. Hypoplasia and immaturity of the terminal lung unit (acinus) in congenital diaphragmatic hernia. *Am Rev Respir Dis.* 1987;136: 947–950.
- 104 Kotecha S. Lung growth for beginners. *Paediatr Respir Rev.* 1:308–313, 2000.

Chronic Neonatal Lung Injury and Care Strategies to Decrease Injury

Robert P. Jankov and A. Keith Tanswell

Abstract

Currently, virtually all cases of bronchopulmonary dysplasia (BPD) occur in infants with birth weights <1250 g. A minority of these infants will develop a severe form of BPD, which evolves into a long-term failure of alveologenesis and vasculogenesis or, in some, a progressive pulmonary hypertension leading to an early death. Effective interventions, based on the mechanisms underlying the lung injury, most need to be developed for this group. We review putative mechanisms of lung and vascular injury, drawn on data from both clinical studies and animal models, then review currently used and potentially promising interventions. Despite numerous initiatives in clinical management, the overall incidence of BPD remains unchanged. Downward or upward manipulation of oxygen saturations is limited by increased risks of neurological impairment or retinopathy. Attempts to minimize ventilator-induced volutrauma have generally been disappointing, though avoidance of early intubation may be beneficial, and a volume-targeted approach to ventilation appears promising. Uncertainties exist about dosing, safety, and efficacy of such therapeutic interventions as high-dose vitamin A and caffeine in the currently most susceptible infant population. Promising approaches based on animal studies, but not yet adequately assessed in human infants, include the use of nonsteroidal antiinflammatory agents, antiprotease therapy and targeting bombesin-like peptides.

Keywords:

BPD, pulmonary hypertension, chronic neonatal lung injury, oxygen toxicity, volutrauma, VILI, therapeutic interventions, clinical management, animal models, potential therapies

Bronchopulmonary Dysplasia as Originally Described

Positive pressure ventilation for the treatment of respiratory distress syndrome (RDS) in preterm infants came into clinical practice in the mid-1960s (1). Shortly thereafter, the chronic neonatal lung injury known as bronchopulmonary dysplasia (BPD) was first described by Northway and colleagues (2). The affected infants, as described, all had severe respiratory failure, were ventilated with high O₂ concentrations, and required high inflation pressures. The clinical course evolved over approximately four weeks from RDS to severe respiratory failure. This was accompanied by a classic sequence of radiological changes. The initial X-ray changes of RDS were replaced by a “whiteout,” which cleared to demonstrate multiple cystic lesions. If infants survived this stage, a streaky pattern consistent with pulmonary fibrosis appeared, though the pattern may actually have been attributable to distended lymphatics.

There was a high mortality rate of approximately 60%. At autopsy there was evidence of both atelectasis and emphysematous changes, pulmonary fibrosis, marked airway injury, and in many, evidence of pulmonary hypertension (PHT).

While the BPD terminology has been preserved over the intervening years the clinical, radiological, and pathological features of chronic neonatal lung injury have changed dramatically. The affected population has changed, the clinical course is generally less severe, the classic sequence of radiological changes is no longer evident, and airway injury and pulmonary fibrosis are no longer common pathological features. The reasons for this evolution must relate to improvements in neonatal care, including the use of antenatal steroids, postnatal surfactant therapy, and improved ventilator design, but their specific contributions are unknown. Indeed, it is not even certain that the disease as originally recognized has the same underlying causes as that observed in the current era (3).

Bronchopulmonary Dysplasia in the Current Era

Advances in neonatal care have had a major impact on the survival of extremely low-birth-weight (ELBW) infants. This has been associated with an increase in the number of ELBW infants surviving BPD. The most commonly used clinical definition of BPD in the current era has been an O₂-dependency at thirty-six weeks postmenstrual age. Virtually all cases of BPD occur in infants with birth weights <1250 gm, with an incidence ranging from 25 to 42% (4), accounting for 10–15,000 new cases per year in the United States (5). In the current era, with the use of antenatal steroids and surfactant replacement therapy, ELBW infants often have a minimal degree of respiratory distress initially, requiring little O₂ supplementation and ventilatory support and frequently can be rapidly extubated to non-invasive modes of respiratory support. Many will subsequently go on to have a progressive deterioration in respiratory function, requiring an increase in inspired O₂ and prolonged invasive respiratory support. Fortunately, the majority will eventually be extubated and be asymptomatic at the time of discharge home.

The use of an O₂-dependency at 36 weeks postmenstrual age as a clinical definition of BPD is limiting, in that it does not differentiate between mildly and severely affected infants. This has led to the development of a classification scheme that differentiates between three levels of severity (6). A minority of ELBW infants will develop severe BPD, sometimes heralded by a limited initial response to surfactant and/or deteriorations associated with development of air leaks, a hemodynamically significant patent ductus arteriosus (PDA), and/or episodes of sepsis. Requirements for O₂ supplementation and invasive ventilation in this population are both significant and prolonged.

Pathological Features of BPD

The major pathological features of severe BPD in the current era are an inhibition, or arrest, of alveolar formation from the in-growth of secondary crests into larger precursor saccules and an associated thickening of the interstitium (7). The inhibited or arrested alveolarization of severe BPD may last many years, even into adult life (8).

BPD-Associated Pulmonary Hypertension

Chronic PHT, characterized by persistently raised pulmonary vascular pressure and resistance, is a common finding in BPD (9). Many of the most severely affected infants with BPD will have associated PHT. Persistent hypoplasia of the pulmonary microvasculature is also evident (10), leading to reduced vascular surface area. Remodeling of pulmonary resistance arteries due to smooth muscle hyperplasia and distal extension of smooth muscle into normally nonmuscular arteries, which are evident at autopsy, is pathognomonic of BPD-associated PHT.

Because PHT may be clinically silent, screening for PHT should be considered in all infants at risk of BPD. Other than risk factors for BPD, additional risk factors for PHT that are evident at birth include maternal pre-eclampsia, prolonged oligohydramnios, and being small for gestational age (11). Available studies estimate the incidence of echocardiographic signs of PHT at between 17 and 43% of BPD cases overall (12–14). The incidence and severity of chronic PHT increases in parallel with the severity of lung disease, being present in as many as 60% of the severest cases (12). Given the retrospective nature of the majority of published data, the true incidence of PHT in BPD may well be greater than currently appreciated. Pathological contributors to PHT include sustained pulmonary vasoconstriction, exaggerated vasoreactivity, vascular hypoplasia, and arterial wall remodeling. The extent to which the latter two structural features contribute to a “fixed” (i.e., irreversible) form of chronic PHT is unknown.

The eventual diagnosis of BPD is frequently heralded by respiratory deterioration during the first two weeks of life, at which time PHT may be apparent. In the vast majority of cases, chronicity of PHT is established by 36 weeks corrected gestational age (14). Although it remains unclear whether chronic PHT is simply a marker of BPD severity or contributes to adverse outcomes in its own right, the diagnosis of chronic PHT imposes a far greater burden of illness, resulting in lengthening of neonatal intensive care unit (NICU) stay, prolongation of need for O₂ therapy, and a fourfold increase in mortality during the NICU stay (13). Comorbidities that

worsen or inhibit recovery of lung function will also exacerbate PHT, including the persistence of shunts that increase pulmonary blood flow (PDA) or large systemic-pulmonary collateral vessels with left to right shunts, airway abnormalities (subglottic stenosis, tracheomalacia, distal airway obstruction), gastroesophageal reflux, and factors contributing to poor growth, such as suboptimal nutrition and prolonged or repeated courses of corticosteroid therapy. The presence of pulmonary vein stenosis is an occasional finding in ex-preterm infants that heralds an extremely poor prognosis, especially in late-onset cases in which PHT is apparent after term-corrected gestation.

Retrospective data suggest that the majority of infants with BPD-associated PHT will demonstrate gradual improvement in hemodynamic parameters during the first year of life, as lung function improves. However, prospective long-term data on these patients is lacking, and there is no knowledge regarding the potential for the reappearance of PHT later in life. For infants with severe BPD, progressive chronic PHT is common, ultimately leading to right ventricular failure and early death, in most cases within one year of diagnosis. Pulmonary hypertensive crises and cardiac arrest are also common, frequently precipitated by worsening hypercapnia and/or systemic hypotension in the settings of improperly applied mechanical ventilation, anesthesia, sedation or intercurrent infection.

Objectives of this Chapter

ELBW infants who develop severe BPD may have a long-term failure of alveolar and vascular growth, with an associated long-term impairment of pulmonary function, as well as an increased incidence of impaired neurodevelopmental outcome. It is this population for which prevention of BPD and PHT is critical. The most effective way of preventing the development of BPD would be to avoid premature delivery. In the absence of any such effective intervention, preventive therapy for BPD and associated PHT must be targeted at the contributing factors that lead to lung injury and their underlying mechanisms of action. In the following pages we will review putative mechanisms of injury in the preterm newborn lung and pulmonary vasculature, drawn from both clinical data and animal

models, then review currently used and potentially promising interventions.

Factors That May Contribute to the Development of BPD and BPD-Associated PHT

Genetic Factors

It has been recognized in recent years that genetic factors may contribute to the development of BPD with, for example, genetic polymorphisms in TNF- α , Toll-like receptor (TLR)-10 and vascular endothelial growth factor (VEGF) being suspected of playing a role (15). Single-nucleotide polymorphisms (SNPs) in, for example, the fibroblast growth factor (FGF) receptor-4 have also been identified as being associated with the development of BPD (16). The enormous complexity of this field is evident from the results of a recent whole genome expression study, which revealed alterations of the expression of nearly 10% of the genome in BPD patients (17). Although genetic factors almost certainly contribute to the development of BPD, no specific loci have yet been consistently associated with increased risk for chronic PHT in this population. There is no question that research in this area could provide us with key mechanistic insights into the development of BPD and BPD-associated PHT. However, lessons learned from such approaches used for diseases such as asthma and cystic fibrosis suggest that translation of such insights into clinically useful interventions may not be rapid.

Oxygen Toxicity

Oxygen toxicity has been recognized as a possible cause of, or contributing factor to, BPD since the time that BPD was first described. That O₂ toxicity is still relevant in the current era has been clearly demonstrated in trials comparing different target O₂ saturations (18) in which the high target group had adverse pulmonary outcomes. Unfortunately, low saturation targets, previously thought to be in a clinically safe range, have been associated with an increased mortality (19), thus markedly limiting the O₂ saturation range available for safe clinical use. A range of 90–95% has been suggested, recognizing that any reduction in mortality may be accompanied by an increase in retinopathy of prematurity.

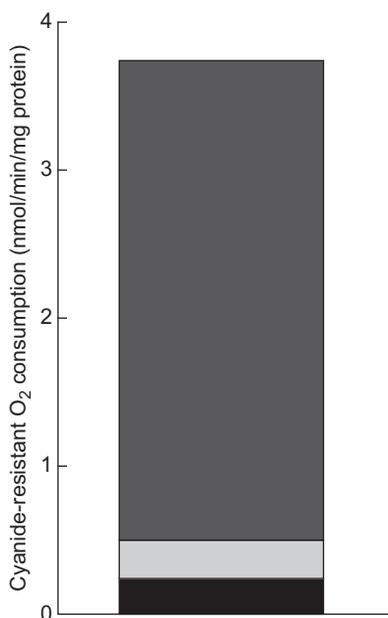


Figure 11-1. As a measure of superoxide production, cyanide-resistant O₂ consumption was measured in lung homogenates from rat pups. Rat pups that had been exposed to 60% O₂ for 14 days (light gray) had double the superoxide production of those exposed to air for 14 days (black), despite both sets of homogenates being equilibrated with 21% O₂, indicative of phagocyte-generated superoxide. To emulate superimposed hyperoxia, homogenates from rat pups that had been exposed to 60% O₂ for 14 days were equilibrated with 100% O₂ (dark gray), resulting in a several-fold increase in superoxide production. Modified from Jankov, Johnstone, Robinson, et al. *Free Rad Biol Med.* 2003;35:200-209.

Oxygen can be toxic by a direct cytotoxic effect of an increased O₂ tension, attributable to reactive oxygen species (ROS). ROS production in lung tissue, in the form of mitochondria-derived superoxide, is directly proportional to the PO₂ to which the lung is exposed (20). Exposure to moderate O₂ concentrations leads to an influx of ROS-generating neutrophils and macrophages (21,22). As shown in Figure 11-1, these effects can be additive. Of note is that extreme hyperoxia generates several-fold more superoxide than that attributed to a phagocyte influx. Saugstad authored an excellent review of the evidence for oxidative stress contributing to the development of BPD (23). Animal data have demonstrated that maturation of enzymatic antioxidants is gestation dependent. It has been thought that the ELBW infant is particularly at risk from ROS-mediated injury at birth, due to a reduced antioxidant defense system being less able to contend with the acute increase in ROS production induced by the acute increase in intrapulmonary O₂

tension that occurs at birth. This is amplified by the use of supplemental O₂ during resuscitation and thereafter. There is ample evidence, from measurement of various markers of oxidative stress, that the ELBW infant has been subjected to oxidative stress prior to the diagnosis of BPD being made, and that oxidative stress is evident in the lungs of infants with BPD. There has been a growing concern that the use of 100% O₂ in the delivery room during resuscitation is such a cause of early oxidative stress, and that effective resuscitation can be achieved with lesser concentrations of O₂. Activation of NF-κB by ROS could certainly link oxidative stress to inhibition of genes regulating lung growth. However, these observations do not prove a cause-and-effect relationship.

We have begun to question a uniformly toxic role for O₂-derived ROS in the development of BPD, based on observations in a rat model of BPD, that have received interventions to limit lung inflammation actually have acceleration alveolar formation and increased estimated alveolar numbers (22,24,25). This would suggest, at least in this model, that it is the inflammatory cells rather than ROS that are important for the development of the lung injury and associated vascular remodeling. The stimulatory effect of 60% O₂ on alveogenesis is likely to be mediated by lipid hydroperoxides, which have been shown to regulate normal alveogenesis in the newborn rat. Rat pups have immature alveolar development at birth, but do have mature antioxidant enzyme development, so this does not completely mimic the human situation with ELBW infants. However, the possibility that ROS are less uniformly toxic than previously thought may help to explain the disappointing results with antioxidant interventions, such as recombinant superoxide dismutase and *N*-acetylcysteine in human trials.

Ventilator-Induced Lung Injury

Ventilator-induced lung injury has also been recognized as a possible cause of, or contributor to, BPD since the time that BPD was first described. There is a plethora of literature to support the induction of lung injury by excessive tidal volumes, but evidence to support lung injury at clinically applicable tidal volumes is more limited. Attempts to minimize volutrauma by the use of

high-frequency ventilation (26) and permissive hypercapnia (27) have shown no benefit. The sustained use of high-frequency ventilation would not seem to be an appropriate clinical option, given the requirement for cyclic lung expansion during fetal breathing movements to sustain lung growth. “Therapeutic” hypercapnia has been extremely effective in animal models of BPD (25) and chronic neonatal PHT (28), but this approach is unlikely to be destined for clinical use given that passive hypercapnia is associated with adverse neurological outcomes (27) and, based on animal data, an increased risk of retinopathy of prematurity. Recent data in adult animals suggests that benefits of therapeutic hypercapnia may be replicated by induction of mild-moderate metabolic (nonhypercapnic) acidosis (29). While requiring confirmation of efficacy in neonatal animal models, “permissive” or “therapeutic” acidosis could represent a potentially more translatable strategy than hypercapnia.

A large before-and-after quality improvement initiative, including delivery room extubation and the decreased use of conventional ventilation in favor of CPAP, actually showed an increase in BPD during the quality improvement cycle (30). However, when assessed by meta-analysis in isolation, there seem to be small, but significant, benefits from avoiding endotracheal intubation (31) and applying nasal CPAP in the delivery room after surfactant therapy in ELBW infants (32). Unfortunately, more than half of these infants will go on to require subsequent intubation and mechanical ventilation. The use of noninvasive modes of intermittent positive pressure ventilation has shown no reduction in the rate of BPD when compared to nasal CPAP alone (33). However, the use of volume-targeted ventilation rather than pressure-limited ventilation has been associated with a reduced combined outcome of death or BPD (34). Induction of pro-inflammatory cytokine release occurs rapidly and at relatively low distending pressures. Any adverse effects of ventilation are likely to be associated with the release of pro-inflammatory cytokines and a resultant phagocyte influx, which would be additive to that caused by hyperoxia and any concurrent sepsis (5).

Hyperbilirubinemia

Given the known antioxidant properties of bilirubin, it might seem that some degree of jaundice

in the early days of life may have evolved as a protective mechanism to assist in adapting to the acute oxidative stress induced by birth, particularly by preterm birth. However, the opposite may in fact be true. In a study comparing aggressive with conservative phototherapy, there were significant beneficial effects of aggressive phototherapy on the development of BPD and the combined outcome of BPD and death (35).

Sepsis

There is very strong evidence to support a role for both early and late bacterial sepsis with the development of BPD (36). This is most likely attributable to the inflammatory response induced by sepsis.

Ureaplasma

There is a clear association between the development of BPD and pulmonary colonization with *Ureaplasma* species. What has been a bone of contention for many years has been whether this is a causal association. This issue has been recently comprehensively reviewed (37). There is a general consensus that *Ureaplasma* from the lower genital tract can ascend during pregnancy to cause chorioamnionitis and result in preterm delivery. Injection of *Ureaplasma* into the amniotic fluid of early-gestation sheep does result in a mild inflammatory response in the fetal sheep lung. There were some transient architectural changes in lung structure. Similar studies in nonhuman primates showed a greater degree of inflammation and significant changes in pulmonary function. There are, therefore, species differences in respect to sensitivity to *Ureaplasma*. With respect to reports from studies with human infants the available literature is confusing and contradictory and provides no clear answers as to any causal role for *Ureaplasma* in the development of BPD. To date, attempts to prevent BPD by treatment with macrolide antibiotics have been disappointing.

Packed Red Blood Cell Transfusion

There is a reported association between packed red blood cell transfusions and the development of BPD (38). Consistent with such a finding has been a single report that there is a decrease in the incidence of BPD with erythropoietin administration, prescribed for treatment of anemia of

prematurity (39). However, there remain significant concerns about the association between erythropoietin administration and increased severity of retinopathy of prematurity (40), as well as there being paradoxical animal data suggesting an increased risk of ventilator-induced lung injury in premature lambs treated with erythropoietin. These latter observations argue for caution in the use of erythropoietin for prevention of BPD until both safety and efficacy have been confirmed.

Patent Ductus Arteriosus and Its Treatments

This issue has been the subject of a recent excellent review (41). In brief, the presence of a persistent left-to-right shunt across a PDA impairs lung mechanics due to an increased rate of hydrostatic fluid filtration into lung tissue, with a resultant prolongation of the need for mechanical ventilation. Use of pharmacological agents to induce an early closure of the PDA does seem to reduce the incidence of both intraventricular and pulmonary hemorrhage, but there is no conclusive evidence to support a role for persistent PDA in the development of BPD. There is, however, convincing evidence that surgical ligation of a PDA is an independent risk factor for the development of BPD. Diuretics have been commonly used in an attempt to improve lung mechanics. Parenteral administration of loop diuretics, such as furosemide, do have a short-term beneficial effect on lung compliance and O₂ requirement, but evidence for long-term benefit is lacking. Similarly, evidence for long-term benefits of thiazide diuretics or spironolactone are also lacking.

Interventions Aimed at Reducing the Incidence/Severity of BPD

Therapies Targeting Lung Growth

As stated earlier, a cardinal feature of BPD in the current era is impaired alveologenesis, angiogenesis, and lung growth. Impaired pulmonary angiogenesis has been suggested to represent a primary event (42). Postnatal treatment with a stimulator of growth related genes, vitamin A, has been shown to reduce the incidence of BPD (43). Despite this, many NICUs do not use vitamin A prophylaxis. This may relate to whether the size of the benefit is seen to justify a

prolonged course of intramuscular injections. As recently reviewed (44), there are also uncertainties about dosing, safety, and efficacy of high-dose vitamin A in the current population of ELBW infants. Widespread acceptance of vitamin A prophylaxis likely depends on the results of current trials assessing an intravenous delivery approach, with ongoing assessments of efficacy and safety.

Alveologenesis begins at approximately 35 weeks of gestation in the human. It is a process in which secondary crests grow into large precursor saccules to subdivide them into smaller alveoli and increase gas-exchange surface area. All appropriately grown ELBW infants are delivered prior to the onset of alveologenesis. Growth factor regulation of alveologenesis, at least in rodents, requires a number of individual growth factors such as VEGF, platelet-derived growth factor (PDGF)-AA and-BB, hepatocyte growth factor (HGF), FGF-7 (also known as KGF), and insulin-like growth factor (IGF)-I (45). That interventions targeted to each of these individual growth factors can arrest/impair alveologenesis (45) suggests that they act in concert to coordinate the process of secondary crest formation during rodent alveologenesis, which is dysregulated during lung injury, leading to impaired alveologenesis. A number of observations suggest that the same pathways may be dysregulated in human infants developing BPD. Serum IGF-I concentrations are reduced in infants developing BPD, as are levels of VEGF in their bronchoalveolar lavage and urine. In premature human neonates, a high concentration of FGF-7 in their airways is associated with a reduced risk for BPD. Reduced concentrations of HGF in tracheal aspirates are also associated with the development of BPD.

If several growth factors act in concert to regulate alveolar development, it seems unlikely that treatment with any one growth factor will restore the impaired alveologenesis induced by neonatal lung injury. Recombinant human FGF-7 (rhFGF-7) protected newborn rats from hyperoxia-induced lethality, but not from hyperoxia-induced inhibition of postnatal alveologenesis (46,47). However, in contrast, rhHGF did partially improve hyperoxia-induced impairment of alveologenesis in neonatal mice (48), and both adenovirus mediated VEGF gene therapy (49) and treatment with rhVEGF (50) preserved

alveolar development in hyperoxia-exposed newborn rats. It is difficult to understand why therapy with a single growth factor, such as rhHGF or rhVEGF, can restore impaired alveologenesis if physiological alveologenesis requires the coordinated expression of several growth factors acting independently. Perhaps their protective effects are mediated by their anti-inflammatory properties, rather than their effect on DNA synthesis. Any consideration of the chronic use of such agents as rhVEGF in human infants must be tempered by observations in transgenic mice. Chronic overexpression of VEGF in the lungs of transgenic mice resulted in increased infant mortality and caused pulmonary hemorrhage, hemosiderosis, alveolar remodeling, and inflammation.

Rather than supplementing positive effectors of lung growth, a more profitable approach may be to use antagonists of growth inhibitors, which are upregulated during lung injury. The obvious candidate is TGF β 1, which may be upregulated by a peroxynitrite-mediated effect on myofibroblasts. Bronchoalveolar lavage fluid of human infants who go on to develop BPD has been reported to have both reduced latent TGF β content and increased TGF β 1 content (51). Increased TGF β immunoreactivity is observed in lung tissue at autopsy of infants with BPD (52). Overexpression of TGF β 1 in mutant mice (53) or neonatal rats overexpressing TGF β due to treatment with an engineered adenovirus (54) have BPD-like lung pathology. Increased TGF β expression has been shown to be induced by hyperoxia in a mouse model of BPD, and we have observed this also to occur in a rat model of BPD. Inhibition of TGF β signaling has been shown to protect against hyperoxia-mediated inhibition of alveologenesis in rodents (55). The peroxisome proliferator-activated receptor gamma (PPAR γ) agonist, rosiglitazone, blocks the increase in TGF β signaling induced in neonatal rat lung by hyperoxia and protects against hyperoxia-mediated inhibition of alveologenesis. Protection is also seen using the PPAR γ regulator, curcumin. One way in which TGF β may act is through a downstream stimulation of CTGF expression, which reversibly stimulates an inflammatory response and an inhibition of alveologenesis.

Optimizing nutrition in ELBW infants through the standardized use of feeding guidelines has a number of advantages, which may impact the risk of BPD (56). Earlier achievement

of full enteral feeds with discontinuation of parenteral nutrition lessens risks of central-line-related sepsis. Earlier achievement of birth weight reflects better nutrition, and undernutrition has been demonstrated in animal models to impair lung growth and enhance lung injury.

Caffeine

A significant reduction in BPD was observed in a large randomized controlled trial (RCT) in which infants were treated with caffeine for apnea of prematurity or to facilitate extubation (57). Concerns were raised about the generalizability of this finding based on the maturity of the population being studied and the fact that the finding was a secondary, rather than a primary, outcome (58). Subsequent large retrospective analyses support a preventive effect of early caffeine therapy against the development of BPD but, concerning, identified increased risks of either death (59) or necrotizing enterocolitis (60) in the caffeine-treated population. The mechanism by which caffeine exerts any beneficial effect on lung injury is unclear. A study in hyperoxic neonatal rats suggested that its protective effects were due to a reduction in pulmonary inflammation, but another study in hyperoxic newborn mice reported that caffeine therapy is associated with increased inflammation, lung cell apoptosis, and impairment of alveolar development.

Steroids and Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

Antenatal steroids are given to reduce the risk of RDS, but have no impact on the incidence of BPD, even when given as multiple courses. Postnatal dexamethasone was shown, many years ago, to improve pulmonary function and facilitate extubation in patients with BPD (61), and its use was widely adopted. This widespread use was curtailed due to its association with adverse neurologic outcomes (62). The pendulum has swung back to a limited extent with the realization that in severe cases of BPD benefits of therapy may outweigh the associated risks. Both the American Academy of Pediatrics and the Canadian Paediatric Society have made position statements supporting the use of a short course of low-dose dexamethasone in selected infants at very high risk of BPD after one to two weeks of age. The effects of dexamethasone

on the lung appear to vary, depending on the duration of exposure and timing in relation to developmental stage. Dexamethasone reverses established chronic PHT in adult rats, yet prolonged treatment of neonatal rats with dexamethasone, during a period (days 3–14) when alveolarization occurs, caused permanent lung hypoplasia and augmented the severity of hypoxia-induced PHT when pups reached maturity.

Alternate strategies, such as the systemic use of a less-potent steroid, hydrocortisone, or inhaled steroids to reduce systemic side effects are as yet of no proven benefit. Results from ongoing RCTs are awaited.

One of the mechanisms by which dexamethasone may have exerted its beneficial effects on the immature lung is through its anti-inflammatory properties. This may be mediated by its highly selective inhibition of the cyclooxygenase-2 (COX-2) isoform. In a neonatal rat model of BPD, exposure to 60% O₂ resulted in an increased lung tissue content of COX-2, but not COX-1. Two recent studies in rodent models of BPD have confirmed that selective COX-2 inhibitors are effective anti-inflammatory agents in hyperoxia-induced lung injury in both rat (24) and mouse (63) models of BPD, suggesting that these agents are worthy of further exploration.

Bronchodilators

Bronchodilators do have a place in the management of bronchospasm observed in some patients with BPD. They improve dynamic compliance by

reducing airway resistance. Their effects are transient, and there is no evidence to date to support any long-term benefits.

Inflammation

There is a widely held view, with which we agree, that BPD is a disease caused by pulmonary inflammatory cell influx and inflammatory cell-mediated tissue injury (5). Both O₂ therapy and ventilation-induced lung injury induce an inflammatory response, whereas the protection against BPD associated with caffeine and some growth factor therapies may be mediated by their anti-inflammatory effects. Whether the prenatal inflammation of chorioamnionitis is a significant contributor to the development of BPD has been an issue of recent vigorous debate (64,65), which appears, as yet, to be unresolved.

Much of our understanding of the role of inflammation in neonatal lung injury comes from animal models of BPD. Depletion of neutrophils in hyperoxia-mediated lung injury in neonatal rodents protects against inhibition of alveologenesis (22, 66). As a start to our discussion of potential targets for intervention, we will use the sequence of events that regulates the BPD-like injury observed in neonatal rats exposed to 60% oxygen for fourteen days (Figure 11-2). The likely initiating event is injury to the alveolar epithelium with the release of proinflammatory cytokines. A critical proinflammatory cytokine in this model is IL-1 because competitive inhibition of its receptor prevents the lung injury (67). IL-1 is an

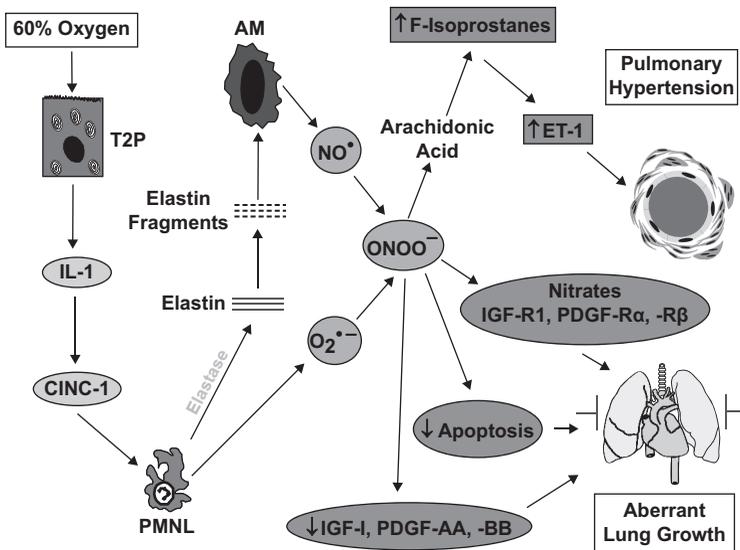


Figure 11-2. Schematic illustrating the sequence of events leading to lung injury in the neonatal rat exposed to 60% O₂ for fourteen days from birth. Abbreviations: T2P = type II pneumocyte; IL-1 = interleukin-1; CINC-1 = cytokine-induced neutrophil chemoattractant-1; PMNL = polymorphonuclear leukocytes/neutrophils; AM = alveolar macrophages; NO• = nitric oxide; O₂•⁻ = superoxide; ONOO• = peroxynitrite; ET-1 = endothelin-1; IGF-R = insulin-like growth factor receptor; PDGF-R = platelet-derived growth factor receptor.

upstream regulator of the neutrophil chemokine, CINC-1, and blocking CINC-1 binding with a CXCR2 antagonist also prevents lung injury (22). Our current studies have shown that neutrophil elastase causes elastin fragmentation, with elastin fragments being the chemotactic agents responsible for the macrophage influx, as previously described by others in a model of adult emphysema. The macrophage influx is essential for the generation of PHT in this model (68) and in another neonatal rat model of BPD-like lung injury induced by systemic bleomycin (28). Macrophage-derived NO combines with superoxide to form peroxynitrite (69), which acts on arachidonic acid to generate F₂-isoprostanes, which bind to thromboxane A₂ receptors to stimulate endothelin-1 generation (70). Peroxynitrite has been shown to play specific roles in the pathogenesis of experimental chronic neonatal PHT, causing pulmonary vasoconstriction (71), vascular remodeling (69,72), and right ventricular dysfunction (73). Treatment with a peroxynitrite decomposition catalyst prevents the PHT, parenchymal thickening, and impairment of alveolarization observed in the 60% O₂ exposure model (69). This is likely to be due to normalization of the expression of critical growth factors, normalization of impaired physiological apoptosis, and as our recent observations suggest, prevention of nitration and inactivation of critical growth factor receptors.

Can any of these observations suggest novel approaches to therapy in BPD? An obvious target is IL-1. Transgenic mice overexpressing IL-1 β develop a lung injury similar to BPD, with lack of alveolar septation and impaired vascular development of the lung, which may be mediated through effects on the retinoic acid pathway. They also have inflammation mediated by the increased expression of neutrophil and macrophage chemokines (74). Antagonism of IL-1 β binding to its receptor protects against neonatal animal models of lung injury induced by hyperoxia (67) but not that induced by excessive ventilation (75). IL-1 β is increased in the lungs and circulations of infants developing BPD, so it does appear to be a rational target for clinical intervention studies.

One of the most interesting, and promising, observations from other animal models relates to gastrin-releasing peptide (GRP), which is homologous to amphibian bombesin. GRP appears to be a potent pro-inflammatory agent. Studies in

neonatal mice demonstrated that exogenous GRP impaired alveolarization. Of great clinical relevance was the observation in the 125-day gestation baboon model of BPD that GRP was elevated, and that an intervention with an antibombesin antibody was protective against impaired alveolarization (76). Elevated urine bombesin-like peptide levels confer a tenfold increased risk of developing BPD in human infants born at or less than 28 weeks gestation (77).

While targeting IL-1 β or bombesin-like peptides in ELBW human infants do seem like reasonable approaches to reducing BPD, it must be remembered that the ELBW population that would be the target for such interventions is already immunodeficient and at high risk of septic episodes, so suppressing their inflammatory response to reduce the risk of BPD could concurrently increase their risk of sepsis.

Inhibition of Neutrophil Elastase

The concept that proteases, such as neutrophil elastase, are critical for the development of chronic neonatal lung injury has been around for many years. An antiprotease intervention with α_1 -antitrypsin protected neonatal rats from O₂-induced chronic neonatal lung injury (78). Unfortunately, a subsequent trial in human infants failed to show benefit, likely due to rapid degradation of intratracheally delivered α_1 -antitrypsin. Interest in inhibition of neutrophil elastase as a means of protecting against neonatal lung injury has been reactivated by two recent publications, in which increased levels of the specific neutrophil elastase inhibitor elafin protected against ventilator-induced lung injury (79,80). The authors attributed the observed protective effects to a preservation of the intact elastin scaffold necessary for alveologenesis. There is ample evidence to support this contention, in that elastin-knockout mice or rats chronically exposed to an inhibitor of lysyl oxidase and elastin cross-linking have impaired alveologenesis. Our recent studies (Figure 11-2) suggest the alternative or supplemental explanation that inhibition of neutrophil elastase limits elastin fragmentation and macrophage recruitment, thus limiting peroxynitrite formation.

Therapies for BPD-Associated PHT

The mainstays of therapy for BPD-associated PHT remain the same as those for BPD, including

supplemental O₂ when necessary, diuretics, adequate nutrition, prevention of infection, and correction of comorbidities that may contribute to lung injury. Unfortunately, therapies specific to pulmonary arterial hypertension employed with success in older children and adults are unsupported by good quality clinical data in patients with BPD and are likely of doubtful value in this context. Ideally, long-term treatment with pulmonary vasodilators should only be instituted following comprehensive evaluation of cardiopulmonary hemodynamics by cardiac catheterization, which allows for accurate determination of PHT severity, evaluation of acute vasodilator responsiveness, and definitive exclusion of major collateral vessels, pulmonary vein stenosis, and left heart disease as contributing factors (81).

Agents Targeting Peroxynitrite

Peroxynitrite is a major reactive nitrogen species formed by the reaction of superoxide with NO. Peroxynitrite can directly injure lung tissue by nitration of proteins and lipids or, following degradation, by generation of the hydroxyl radical (Figure 11-3). NO can act as either a pro-oxidant or an antioxidant, depending on the relative concentration of superoxide. When superoxide is in excess, NO is a pro-oxidant through peroxynitrite formation, whereas when NO is in excess, it is an antioxidant by limiting lipid peroxidation (Figure 11-4). This antioxidant property of NO is not widely appreciated by clinicians but may account for a number of its biological and pharmacological effects. Our data, using a peroxynitrite decomposition catalyst in neonatal rats, suggests a critical role for peroxynitrite in the 60% O₂-induced lung injury (69) and in pulmonary vascular injury secondary to chronic hypoxia exposure (72). The peroxynitrite decomposition catalyst results in peroxynitrite decomposing to nitrate, rather than the toxic hydroxyl radical generated by spontaneous decomposition, and reverses the impaired lung growth and physiological apoptosis observed in this model. Protection has been

observed in other models in which heavy metal-containing synthetic antioxidants that also react with peroxynitrite have been used.

Whether peroxynitrite plays as significant a role in human BPD is unknown. Plasma 3-nitrotyrosine content, a marker for peroxynitrite-mediated reactions, is increased in the first month of life in human infants developing BPD (82), consistent with sustained peroxynitrite-mediated reactions contributing to the development of the lung pathology. However, were peroxynitrite a critical mediator in human BPD, one might have expected a beneficial effect of early treatment with inhaled NO, which could potentially minimize peroxynitrite formation, but no such effect has been observed (83), despite ample experimental data from animal studies to suggest that such an effect would be observed. Possible explanations for the lack of the expected effects of NO therapy in human infants with BPD are discussed later.

Moreover, interventions targeting peroxynitrite, such as the use of peroxynitrite decomposition catalysts, involve the use of heavy metal-containing compounds, which are potentially

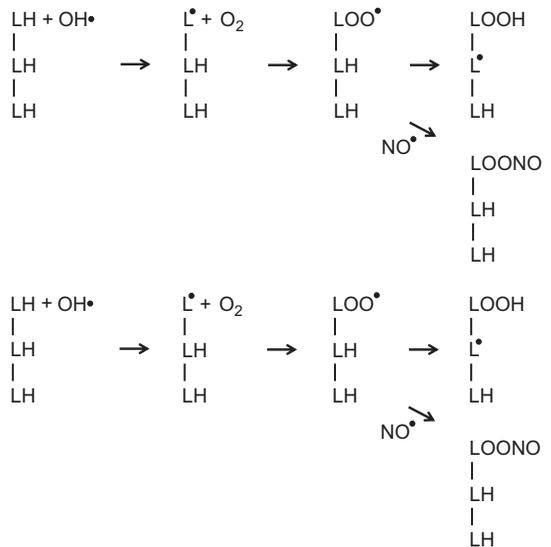


Figure 11-4. The illustration represents three adjacent fatty acids (LH) in a membrane. Exposure to a hydroxyl radical (OH•) results in the formation of a lipid radical (L•). A reaction with O₂ leads to the formation of a lipid peroxyl radical (LOO•). This abstracts a hydrogen from an adjacent fatty acid to form a lipid hydroperoxide (LOOH) and a new lipid radical (L•). The process can then repeat itself sequentially generating lipid hydroperoxides and lipid radicals on adjacent fatty acids in a chain reaction. Nitric oxide (NO•) can terminate this chain reaction by the formation of alkyl peroxynitrite (LOONO) from the lipid peroxyl radical (LOO•).

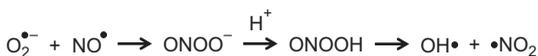


Figure 11-3. Superoxide (O₂^{•-}) reacts with nitric oxide (NO•) to form peroxynitrite (ONOO⁻). This becomes protonated to form peroxynitrous acid (ONOOH), which breaks down to form hydroxyl (OH•) and nitrogen dioxide (•NO₂).

toxic to the immature liver, based on observations in premature baboons delivered at gestational age comparable to ELBW human infants. This would make the testing of the current generation of such compounds in ELBW infants unlikely to meet the required safety standards.

Nitric Oxide

Nitric oxide (NO) is a readily diffusible and highly reactive free radical gas, first identified as the “endothelium-derived relaxing factor” in 1987. NO-mediated vasorelaxation is critical to the rapid decrease in pulmonary vascular resistance following birth and maintenance of normally low pulmonary vascular tone. NO is also critical to alveolar development, in which angiogenesis, mediated by NO downstream of VEGF tyrosine kinase receptor activation plays a critical role. Attenuated vascular NO signaling also contributes to the pathogenesis of acute and chronic PHT in early life. Properties of NO on the lung that are protective of experimental injury also include anti-inflammatory, antioxidant, anti-(smooth muscle) proliferative, and cytoprotective effects. Augmentation of NO signaling has therefore been shown to reverse sustained vasoconstriction, inhibit smooth muscle proliferation, and stimulate angiogenesis and alveolarization in various experimental lung injuries (84). NO signals in the vasculature by binding to soluble guanylate cyclase, thus increasing production of cyclic guanosine monophosphate (cGMP). NO also signals via oxidation to nitrite, or by direct nitrosylation of cysteine thiols to produce *S*-nitrosothiols, both of which mediate the extrapulmonary effects of inhaled NO. However, NO also contributes to disease states via generation of reactive nitrogen species, which causes oxidation and nitration.

Inhaled NO was rapidly adopted into clinical practice in the early 1990s as a short-acting pulmonary-selective vasodilator that remains in common use in neonatal intensive care. Failure of the postnatal transition leading to hypoxemic respiratory failure, known as persistent PHT of the newborn (PPHN), remains the only approved indication for this therapy. An appreciation for a critical role for attenuated lung NO signaling in BPD-like injury derived from animal studies has led to off-label use of inhaled NO, in an effort to

prevent BPD. However, despite promising results in pilot studies, large trials have shown no benefit of early prolonged exposure to iNO as preventive therapy for BPD, on either short- or long-term outcomes (83).

Phosphodiesterase Inhibitors

Neonatal rats exposed to 95% O₂ develop pulmonary hypertension and have arrested alveologenesis. Treatment with Sildenafil, a phosphodiesterase-5 (PDE-5) inhibitor, which, like NO, acts by increasing cGMP signaling, attenuates both the PHT and the impairment of alveologenesis (85). These results have been verified by other groups. Effects of Sildenafil were attributed to stimulated capillary formation with vasculogenesis driving alveologenesis. This is consistent with the protective effect of the angiogenic agent, adrenomedullin, in the same model. The enhanced vasculogenesis may be mediated by an upregulation of HIF-1/2 α and its downstream mediator VEGF. Despite the promising animal studies, a recent pilot study of sildenafil for prevention of BPD was disappointing (86). Inhibition of PDE-4, which increases cyclic adenosine monophosphate (cAMP) signaling, has been reported both to have, and not to have, protective effects against hyperoxia-induced lung injury.

Novel NO-Based Therapies

Assuming that the biological rationale for NO-based therapy in the prevention of neonatal lung and pulmonary vascular injury is sound, there are several possible explanations for the disappointing results of human studies. First, that the beneficial effects of exogenous NO are counterbalanced by adverse ones (e.g., leading to enhanced, rather than diminished, nitration) and/or that inhalation of NO gas is a suboptimal means of providing NO to tissues in which endogenous production is deficient. Circulating and tissue-bound *S*-nitrosothiols contribute importantly to NO-cGMP signaling (87) and cause reversible posttranslational regulation of protein function in a manner akin to phosphorylation. Inhaled NO has been shown inferior as a means of improving tissue NO function in experimental animals, when compared to *S*-nitrosothiol-based (inhaled ethyl nitrite) therapy (88). In pilot human studies, inhaled ethyl nitrite improved oxygenation and hemodynamics in term infants with PPHN (89); however, no studies have been carried out to date

in preterm infants. Another potential means to boost the potential benefits of exogenous NO may be as combination therapy with other agents known to improve lung growth and decrease lung injury, such as vitamin A.

Nitrite was until recently considered a physiologically inert by-product of NO oxidation. It is now apparent that circulating nitrite is recycled in tissues to form NO, thereby acting as a stable endocrine pool for “NO-like” bioactivity that is complementary to endogenous nitric oxide synthase (NOS) (90). Systemic or inhaled inorganic nitrite possesses many theoretical advantages over other forms of NO-based therapy in that tachyphylaxis does not occur with chronic dosing, effects are of relatively rapid onset and last many hours, and (sodium) nitrite is inexpensive and stable. Protective effects of sodium nitrite on adult experimental models of ischemic injury and chronic PHT have been reported (90), but no studies in neonates have been reported to date.

Novel Approaches to Improve Endogenous NO Function

Endogenous NO production by endothelial NOS (eNOS) requires an adequate supply of substrate, L-arginine, and arginine precursors, including L-citrulline. In the absence of sufficient substrate, “uncoupling” of eNOS results in a shift from NO to superoxide production, leading to oxidative and nitrative stress. Upregulation of arginases are an important cause of substrate deficiency directly contributing to inflammation and lung injury, which is preventable by hypercapnic acidosis (91) or by arginase-specific inhibitors (92). Supplementation of L-citrulline has also been shown to inhibit arginase and to prevent hyperoxia-induced lung injury in neonatal rats. Tetrahydrobiopterin (BH4) is an important cofactor for eNOS to remain in a coupled state. Newborn mice haploinsufficient for GTP cyclohydrolase I, a rate-limiting enzyme in BH4 synthesis, spontaneously develop chronic PHT (93). eNOS function may be restored, at least in vitro, by treatment with L-sepiapterin, which serves as a substrate for BH4 synthesis.

Rho-Kinase Inhibition

Activation of the small GTPase, RhoA, and its effector protein, Rho-kinase (ROCK), on

stimulation by G-protein-coupled receptor ligands, including endothelin-1, thromboxane A₂ and 8-isoprostane, is strongly implicated as a key pathway regulating changes in pulmonary vascular tone and smooth muscle phenotype. Therapies that enhance NO-cGMP signaling also suppress RhoA/ROCK activation (94), and beneficial effects of “statins” on pulmonary vascular disease are believed to act through attenuation of this pathway (95). Numerous animal studies and pilot reports using single doses or brief infusions of Fasudil, a ROCK-selective kinase inhibitor, in human adults and children (96) with chronic PHT have confirmed an efficacy that is equal or superior to existing pulmonary vasodilators. In neonatal rats with BPD-like lung injury, ROCK inhibitors prevent and reverse pulmonary vasoconstriction, vascular remodeling, and inhibited pulmonary angiogenesis and alveolarization (97). ROCK inhibitors are also effective when given by inhalation. No studies employing ROCK inhibitors have been conducted to date in human neonates.

Future Directions and Barriers to Progress

Parenchymal Lung Injury in BPD

Sadly, despite numerous initiatives in clinical management, the overall incidence of BPD in ELBW infants has not declined significantly. It may be that there has been some impact on certain subpopulations of affected infants, which are masked within overall data. Use of definitions that allow categorization of mild, moderate, and severely affected infants would seem to be an appropriate first step to overcoming any such hurdle.

There are some approaches to clinical management of ELBW infants for which there is high-quality supportive evidence from the literature:

- (i) Applying nasal CPAP after surfactant therapy in the delivery room, and avoiding endotracheal intubation if possible.
- (ii) Maintaining O₂ saturations in the 90–95% range.
- (iii) Using volume-targeted ventilation if mechanical ventilation becomes necessary.
- (iv) Using aggressive phototherapy to treat hyperbilirubinemia.

- (v) Using pharmacological agents to induce an early closure of the patent ductus arteriosus, in an attempt to avoid surgical ligation.
- (vi) Use of short courses of low-dose dexamethasone in selected infants at very high risk of BPD after one to two weeks of age.

Even assuming that these clinical interventions stand the test of time, their impact is likely to be quite small. If major inroads are to be made, it will likely be through pharmacologic interventions based on mechanistic insights derived from animal models. The two current prophylactic pharmacologic interventions, for which there is evidence of efficacy in the literature, are vitamin A and caffeine. Both interventions are currently subject to uncertainties about dosing, safety, and efficacy in the ELBW population. These issues need to be better resolved before we would feel comfortable in recommending their use, but they certainly may have a place once these uncertainties are resolved. Even then, based on currently available data, their impact is likely to be small.

Given the cumulative evidence to support a critical role for inflammatory cell influx in BPD, the development of effective approaches targeting inflammation would seem to offer the most promise. Perhaps the most promising approach, based on data from extremely premature baboons, would be to target bombesin-like peptides. A small number of rodent studies suggest that the use of NSAIDs should be further studied for their clinical potential.

Suppression of the inflammatory response in ELBW infants, as stated earlier, carries a significant risk of being associated with an increased rate of sepsis in this already immunosuppressed population. Dampening the inflammatory response, without completely suppressing it, may be an option after appropriate dosimetry. Alternatively, being able to target a critical downstream mediator of lung injury due to inflammation, without directly suppressing the inflammatory response, is an exciting possibility raised by the recent observations with neutrophil elastase inhibitors, which certainly seem worthy of further exploration.

BPD-Associated PHT

The current lack of large prospective cohort studies documenting the incidence and clinical course of BPD-associated PHT in prematurely-born

infants is a critical gap in knowledge that must be addressed. Lung or heart–lung transplantation, the only “curative” option for end-stage disease, is rarely feasible in BPD-associated PHT, which contributes to a paucity of high-quality human tissue available for study and a consequently greater reliance on mechanistic and therapeutic insights from preclinical models. A major determinant of long-term survival in progressive BPD-associated PHT is the ability of the right ventricle to maintain adequate output in the face of increased pressure load, yet this aspect of disease has only recently been considered as a distinct therapeutic target (98). Right ventricular adaptation to increased pressure load evolves from a compensated (hypertrophied) state to a decompensated (dilated) state, in which a progressive decline in contractile function heralds imminent death (99). This evolution appears to proceed more rapidly in infants with chronic PHT than in older children and adults. There is currently no knowledge on the pathogenesis of right heart failure in formerly premature infants and currently no published data from relevant animal models.

There are a number of barriers to progress from our currently poor understanding of the pathogenesis and natural history of chronic PHT in BPD. Diagnosis of PHT and right heart dysfunction is problematic in small infants. Clinical signs are unreliable, and catheterization is often not feasible until well after term corrected gestation, leading to a sole reliance on echocardiography, at least for initial diagnosis. Echocardiography can be challenging, especially for evaluation of the right heart, due to the thin chest wall of premature infants and frequent presence of lung hyperinflation. Echocardiography-derived parameters indicating raised pulmonary arterial pressure, such as tricuspid regurgitant jet velocity, are not measurable in all patients, and when present have been shown to correlate poorly with severity as determined by catheter measurement (100). In addition, there are no agreed-upon definitions for echocardiographic diagnosis of PHT in neonates, and certainly none for evaluation of right heart function. Systematic study of echocardiographic parameters of right ventricular function that are useful in newborns is required, incorporating new methodologies, including tissue Doppler and strain imaging, that have shown potential in children and adults. Finally, the effects of therapies designed to ameliorate

injury to the lung and pulmonary vasculature could have unanticipated adverse effects on the right ventricle. Such considerations have

important implications for translation of new therapies and for adoption of existing therapies employed in older children and adults.

References

- Delivoria-Papadopoulos M, Levison H, Swyer PR. Intermittent positive pressure respiration as a treatment in severe respiratory distress syndrome. *Arch Dis Child.* 1965; 40: 474–79.
- Northway WH, Rosan RC, Porter DY. Pulmonary disease following respiratory therapy of hyaline membrane disease. Bronchopulmonary dysplasia. *N Engl J Med* 1967;276:357–368.
- Tanswell AK, Jankov RP. Bronchopulmonary dysplasia: one disease or two? *Am J Respir Crit Care Med.* 2003;167:1–2.
- Jain D, Bancalari E. Bronchopulmonary dysplasia: clinical perspective. *Birth Defects Res A Clin Mol Teratol.* 2014;100:134–144.
- Bhandari V. Postnatal inflammation in the pathogenesis of bronchopulmonary dysplasia. *Birth Defects Research A Clin Mol Teratol.* 2014;100:189–201.
- Jobe AH, Bancalari E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med.* 2001;163:1723–1729.
- Coalson JJ. Pathology of chronic lung disease of early infancy. In: Bland RD, Coalson JJ, eds. *Chronic Lung Disease in Early Infancy.* New York, Dekker; 1999: 85–124.
- Wong PM, Lees AN, Louw J, et al. Emphysema in young adult survivors of moderate-to-severe bronchopulmonary dysplasia. *Eur Respir J.* 2008;32:321–328.
- Mourani PM, Abman SH. Pulmonary vascular disease in bronchopulmonary dysplasia, pulmonary hypertension and beyond. *Curr Opin Pediatr.* 2013;25:329–337.
- Bhatt AJ, Pryhuber GS, Huyck H, et al. Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, flt-1, and tie-2 in human infants dying with bronchopulmonary dysplasia. *Am J Respir Crit Care Med.* 2001;164:1971–1980.
- Kim DH, Kim HS, Choi CW, et al. Risk factors for pulmonary artery hypertension in preterm infants with moderate or severe bronchopulmonary dysplasia. *Neonatology.* 2012;101:40–46.
- An HS, Bae EJ, Kim GB, et al. Pulmonary hypertension in preterm infants with bronchopulmonary dysplasia. *Korean Circ J.* 2010;40:131–136.
- Slaughter JL, Pakrashi T, Jones DE, et al. Echocardiographic detection of pulmonary hypertension in extremely low birth weight infants with bronchopulmonary dysplasia requiring prolonged positive pressure ventilation. *J Perinatol.* 2011;31:635–640.
- Bhat R, Salas AA, Foster C, et al. Prospective analysis of pulmonary hypertension in extremely low birth weight infants. *Pediatrics.* 2012;129: e682–689.
- Mailaparambil B, Krueger M, Heizmann U, et al. Genetic and epidemiological risk factors in the development of bronchopulmonary dysplasia. *Dis Markers.* 2010;29:1–9.
- Rezvani M, Wilde J, Vitt P, et al. Association of a FGFR-4 gene polymorphism with bronchopulmonary dysplasia and neonatal respiratory distress. *Dis Markers.* 2013;35:633–640.
- Pietrzyk JJ, Kwinta P, Wollen EJ, et al. Gene expression profiling in preterm infants: new aspects of bronchopulmonary dysplasia development. *PLoS One.* 2013;8:e78585.
- Askie LM, Henderson-Smart DJ, Irwig L, et al. Oxygen-saturation targets and outcomes in extremely preterm infants. *N Engl J Med.* 2003;349:959–967.
- BOOST II United Kingdom Collaborative Group; BOOST II Australia Collaborative Group; BOOST II New Zealand Collaborative Group, et al. Oxygen saturation and outcomes in preterm infants. *N Engl J Med.* 2013;368:2094–2104.
- Freeman BA, Crapo JD. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J Biol Chem.* 1981;256:10986–10992.
- Jankov RP, Johnstone L, Robinson BH, et al. Macrophages as a major source of oxygen radicals in the hyperoxic newborn rat lung. *Free Rad Biol Med.* 2003;35:200–209.
- Yi M, Jankov RP, Belcastro R, et al. Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat. *Am J Respir Crit Care Med.* 2004;170:1188–1196.
- Saugstad OD. Oxygen and oxidative stress in bronchopulmonary dysplasia. *J Perinat Med.* 2010;38:571–577.
- Masood A, Yi M, Lau M, et al. Cyclooxygenase-2 inhibition

- partially protects against 60% O₂-mediated lung injury in neonatal rats. *Pediatr Pulmonol*. 2014;doi: 10.1002/ppul.22921.
- 25 Masood A, Yi M, Lau M, et al. Therapeutic effects of hypercapnia on chronic lung injury and vascular remodeling in neonatal rats. *Am J Physiol Lung Cell Mol Physiol*. 2009;297:L920–930.
- 26 Soll RF. Elective high-frequency oscillatory ventilation versus conventional ventilation for acute pulmonary dysfunction in preterm infants. *Neonatology*. 2013;103:7–9.
- 27 Thome UH, Carroll W, Wu T-J, et al. Outcome of extremely preterm infants randomized at birth to different PaCO₂ targets during the first seven days of life. *Biol Neonate*. 2006; 90:218–225.
- 28 Sewing AC, Kantores C, Ivanovska J, et al. Therapeutic hypercapnia prevents bleomycin-induced pulmonary hypertension in neonatal rats by limiting macrophage-derived tumor necrosis factor- α . *Am J Physiol Lung Cell Mol Physiol*. 2012;303: L75–87.
- 29 Christou H, Reslan OM, Mam V, et al. Improved pulmonary vascular reactivity and decreased hypertrophic remodeling during nonhypercapnic acidosis in experimental pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2012 302:L875–890.
- 30 Payne NR, Finkelstein MJ, Liu M, et al. NICU practices and outcomes associated with 9 years of quality improvement collaboratives. *Pediatrics*. 2010;125:437–446
- 31 Fischer HS, Bühner C. Avoiding endotracheal ventilation to prevent bronchopulmonary dysplasia: a meta-analysis. *Pediatrics*. 2013;132:e1351–1360.
- 32 Carlo WA. Gentle ventilation: the new evidence from the SUPPORT, COIN, VON, CURPAP, Colombian Network, and Neocosur Network trials. *Early Hum Dev*. 2012;88 (suppl 2):S81–83.
- 33 Kirpalani H, Millar D, Lemyre B, et al. NIPPV Study Group. A trial comparing noninvasive ventilation strategies in preterm infants. *N Engl J Med*. 2013; 369:611–620.
- 34 Wheeler KI, Klingenberg C, Morley CJ, et al. Volume-targeted versus pressure-limited ventilation for preterm infants: a systematic review and meta-analysis. *Neonatology*. 2011;100:219–227.
- 35 Morris BH, Oh W, Tyson JE, et al. Aggressive vs. conservative phototherapy for infants with extremely low birth weight. *N Engl J Med*. 2008;359:1885–1896.
- 36 Wright CJ, Kirpalani H. Targeting inflammation to prevent bronchopulmonary dysplasia: can new insights be translated into therapies? *Pediatrics*. 2011;128:111–126.
- 37 Kallapur SG, Kramer BW, Jobe AH. Ureaplasma and BPD. *Semin Perinatol*. 2013;37:94–101.
- 38 Zhang Z, Huang X, Lu H. Association between red blood cell transfusion and bronchopulmonary dysplasia in preterm infants. *Sci Rep*. 2014;doi: 10.1038/srep04340.
- 39 Rayjada N, Barton L, Chan LS, et al. Decrease in incidence of bronchopulmonary dysplasia with erythropoietin administration in preterm infants: a retrospective study. *Neonatology*. 2012;102:287–292.
- 40 Kandasamy Y, Kumar P, Hartley L. The effect of erythropoietin on the severity of retinopathy of prematurity. *Eye (Lond)*. 2014;doi:10.1038/eye.2014.95.
- 41 Clyman RI. The role of the patent ductus arteriosus and its treatments in the development of bronchopulmonary dysplasia. *Semin Perinatol*. 2013;37:102–107.
- 42 Abman SH. Bronchopulmonary dysplasia. "A vascular hypothesis." *Am J Respir Crit Care Med*. 2001;164:1755–1756.
- 43 Tyson JE, Wright LL, Oh W, et al. Vitamin A supplementation for extremely-low-birth-weight infants. National Institute of Child Health and Human Development Neonatal Research Network. *N Engl J Med*. 1999;340:1962–1968.
- 44 Guimarães H, Guedes MB, Rocha G, et al. Vitamin A in prevention of bronchopulmonary dysplasia. *Curr Pharm Des*. 2012;18:3101–3113.
- 45 Li J, Masood A, Yi M, Lau M, Belcastro R, et al. The IGF-1/IGF-R1 pathway regulates postnatal lung growth and is a nonspecific regulator of alveologenesis in the neonatal rat. *Am J Physiol Lung Cell Mol Physiol*. 2013;304:L626–637.
- 46 Frank L. Protective effect of keratinocyte growth factor against lung abnormalities associated with hyperoxia in prematurely born rats. *Biol Neonate*. 2003;83:263–272.
- 47 Franco-Montoya ML, Bourbon JR, Durrmeyer X, Pulmonary effects of keratinocyte growth factor in newborn rats exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol*. 2009;297: L965–976.
- 48 Ohki Y, Mayuzumi H, Tokuyama K, et al. Hepatocyte growth factor treatment improves alveolarization in a newborn murine model of

- bronchopulmonary dysplasia. *Neonatology*. 2009;95:332–338.
- 49 Thébaud B, Ladha F, Michelakis ED, et al. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. *Circulation*. 2005;112:2477–2486.
- 50 Kunig AM, Balasubramanian V, Markham NE, et al. Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. *Am J Physiol Lung Cell Mol Physiol*. 2005;289:L529–535.
- 51 Liu DY, Wu J, Zhang XY, et al. Expression of IL-8, SP-A and TGF- β 1 in bronchoalveolar lavage fluid of neonates with bronchopulmonary dysplasia. *Zhongguo Dang Dai Er Ke Za Zhi*. 2010;12:444–446.
- 52 Toti P, Buonocore G, Tanganella P, et al. Bronchopulmonary dysplasia of the premature baby: an immunohistochemical study. *Pediatr Pulmonol*. 1997;24:22–28.
- 53 Vicencio AG, Lee CG, Cho SJ, et al. Conditional overexpression of bioactive transforming growth factor- β 1 in neonatal mouse lung – a new model for bronchopulmonary dysplasia? *Am J Respir Cell Mol Biol*. 2004;31:650–656.
- 54 Gauldie J, Galt T, Bonniaud P, et al. Transfer of the active form of transforming growth factor- β 1 gene to newborn rat lung induces changes consistent with bronchopulmonary dysplasia. *Am J Pathol*. 2003;163:2575–2584.
- 55 Nakanishi H, Sugiura T, Streisand JB, et al. TGF- β -neutralizing antibodies improve pulmonary alveologenesis and vasculogenesis in the injured newborn lung. *Am J Physiol Lung Cell Mol Physiol*. 2007;293:L151–161.
- 56 Ehrenkranz RA. Ongoing issues in the intensive care for the periviable infant – nutritional management and prevention of bronchopulmonary dysplasia and nosocomial infections. *Semin Perinatol* 2014;38:25–30.
- 57 Schmidt B, Roberts RS, Davis P, et al. Caffeine for apnea of prematurity trial group. Caffeine therapy for apnea of prematurity. *N Engl J Med*. 2006;354:2112–2121.
- 58 Bancalari E. Caffeine for apnea of prematurity. *N Engl J Med*. 2006;354:2179–2181.
- 59 Dobson NR, Patel RM, Smith PB, et al. Trends in caffeine use and association between clinical outcomes and timing of therapy in very low birth weight infants. *J Pediatr*. 2014;164:992–998.
- 60 Taha D, Kirkby S, Nawab U, et al. Early caffeine therapy for prevention of bronchopulmonary dysplasia in preterm infants. *J Matern Fetal Neonatal Med*. 2014; doi:10.3109/14767058.2014.885941.
- 61 Avery GB, Fletcher AB, Kaplan M, et al. Controlled trial of dexamethasone in respirator-dependent infants with bronchopulmonary dysplasia. *Pediatrics*. 1985;75:106–111.
- 62 Shinwell ES, Karplus M, Reich D, et al. Early postnatal dexamethasone treatment and increased incidence of cerebral palsy. *Arch Dis Child*. 2000;88:F177–181.
- 63 Choo-Wing R, Syed MA, Harijith A, et al. Hyperoxia and interferon- γ -induced injury in developing lungs occur via cyclooxygenase-2 and the endoplasmic reticulum stress-dependent pathway. *Am J Respir Cell Mol Biol*. 2013;48:749–757.
- 64 Thomas W, Speer CP. Chorioamnionitis is essential in the evolution of bronchopulmonary dysplasia – the case in favour. *Paediatr Respir Rev*. 2014;15:49–52.
- 65 Lacaze-Masmonteil T. That chorioamnionitis is a risk factor for bronchopulmonary dysplasia – the case against. *Paediatr Respir Rev*. 2014;15:53–55.
- 66 Auten RL Jr, Mason SN, Tanaka DT, et al. Anti-neutrophil chemokine preserves alveolar development in hyperoxia-exposed newborn rats. *Am J Physiol Lung Cell Mol Physiol*. 2001;281:L336–344.
- 67 Johnson B-H, Yi M, Masood A, et al. A critical role for interleukin-1 receptor in the lung injury induced in neonatal rats by 60% oxygen. *Pediatr Res*. 2009;66:260–265.
- 68 Jankov RP, Luo X, Belcastro R, et al. Gadolinium chloride inhibits pulmonary macrophage influx and prevents O₂-induced pulmonary hypertension in the neonatal rat. *Pediatr Res*. 2001;50:172–183.
- 69 Masood A, Belcastro R, Li J, et al. A peroxytrite decomposition catalyst prevents 60% O₂-mediated rat chronic neonatal lung injury. *Free Rad Biol Med*. 2010;49:1182–1191.
- 70 Jankov RP, Tanswell AK. Pulmonary hypertension and oxidant-induced lung injury in the preterm newborn: new insights into pathogenesis. *Recent Res Dev Physiol*. 2003;1:319–345.
- 71 Belik J, Jankov RP, Pan J, et al. Peroxynitrite inhibits relaxation and induces

- pulmonary artery muscle contraction in the newborn rat. *Free Rad Biol Med.* 2004;37:1384–1392.
- 72 Belik J, Stevens D, Pan J, et al. Pulmonary vascular and cardiac effects of peroxynitrite decomposition in newborn rats. *Free Rad Biol Med.* 2010;49:1306–1314.
- 73 Jankov RP, Lewis P, Kantores C, et al. Peroxynitrite mediates right-ventricular dysfunction in nitric oxide-exposed juvenile rats. *Free Rad Biol Med.* 2010;49:1453–1467.
- 74 Bry K, Whitsett JA, Lappalainen U. IL-1 β disrupts postnatal lung morphogenesis in the mouse. *Am J Respir Cell Mol Biol.* 2007;36:32–42.
- 75 Hillman NH, Kallapur SG, Pillow JJ, et al. Inhibitors of inflammation and endogenous surfactant pool size as modulators of lung injury with initiation of ventilation in preterm sheep. *Respir Res.* 2010;11:151.
- 76 Subramaniam M, Bausch C, Twomey A, et al. Bombesin-like peptides modulate alveolarization and angiogenesis in bronchopulmonary dysplasia. *Am J Respir Crit Care Med.* 2007;176:902–912.
- 77 Sunday ME. Neuropeptides and lung development. In: McDonald JA, ed. *Lung Growth and Development.* New York: Dekker; 1997:401–494.
- 78 Koppel R, Han RNN, Cox D, et al. α_1 -Antitrypsin protects neonatal rats from pulmonary vascular and parenchymal effects of oxygen toxicity. *Pediatr Res.* 1994;36:763–770.
- 79 Hilgendorff A, Parai K, Ertsey R, et al. Inhibiting lung elastase activity enables lung growth in mechanically ventilated newborn mice. *Am J Respir Crit Care Med.* 2011;184:537–546.
- 80 Hilgendorff A, Parai K, Ertsey R, et al. Neonatal mice genetically modified to express the elastase inhibitor elafin are protected against the adverse effects of mechanical ventilation on lung growth. *Am J Physiol Lung Cell Mol Physiol.* 2012;303:L215–227.
- 81 Mourani PM, Ivy DD, Rosenberg AA, et al. Left ventricular diastolic dysfunction in bronchopulmonary dysplasia. *J Pediatr.* 2008;152:291–293.
- 82 Banks BA, Ischiropoulos H, McClelland M, et al. Plasma 3-nitrotyrosine is elevated in premature infants who develop bronchopulmonary dysplasia. *Pediatrics.* 1998;101:870–874.
- 83 Soll RF. Inhaled nitric oxide for respiratory failure in preterm infants. *Neonatology.* 2012;102:251–253.
- 84 Baker CD, Abman SH, Mourani PM. Pulmonary hypertension in preterm infants with bronchopulmonary dysplasia. *Pediatr Allergy Immunol Pulmonol.* 2014;27:8–16.
- 85 Ladha F, Bonnet S, Eaton F, et al. Sildenafil improves alveolar growth and pulmonary hypertension in hyperoxia-induced lung injury. *Am J Respir Crit Care Med.* 2005;172:750–756.
- 86 Konig K, Barfield CP, Guy KJ, et al. The effect of sildenafil on evolving bronchopulmonary dysplasia in extremely preterm infants: a randomised controlled pilot study. *J Mat Fetal Neonat Med.* 2014;27:439–444.
- 87 Raffay TM, Martin RJ, Reynolds JD. Can nitric oxide-based therapy prevent bronchopulmonary dysplasia? *Clin Perinatol.* 2012;39:613–638.
- 88 Auten RL, Mason SN, Whorton MH, et al. Inhaled ethyl nitrite prevents hyperoxia-impaired postnatal alveolar development in newborn rats. *Am J Respir Crit Care Med.* 2007;176:291–299.
- 89 Moya MP, Gow AJ, Califf RM, et al. Inhaled ethyl nitrite gas for persistent pulmonary hypertension of the newborn. *Lancet.* 2002;360:141–143.
- 90 Bueno M, Wang J, Mora AL, et al. Nitrite signaling in pulmonary hypertension: mechanisms of bioactivation, signaling, and therapeutics. *Antioxid Redox Signal.* 2013;18:1797–1809.
- 91 Belik J, Stevens D, Pan J, et al. Chronic hypercapnia downregulates arginase expression and activity and increases pulmonary arterial smooth muscle relaxation in the newborn rat. *Am J Physiol Lung Cell Mol Physiol.* 2009;297:L777–784.
- 92 Pera T, Zuidhof AB, Smit M, et al. Arginase inhibition prevents inflammation and remodeling in a guinea pig model of chronic obstructive pulmonary disease. *J Pharmacol Exp Ther.* 2014;349:229–238.
- 93 Belik J, McIntyre BA, Enomoto M, et al. Pulmonary hypertension in the newborn GTP cyclohydrolase 1-deficient mouse. *Free Radic Biol Med.* 2011;51: 2227–2233.
- 94 Guilluy C, Sauzeau V, Rolli-Derkinderen M, et al. Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol.* 2005;146:1010–1018.
- 95 Rikitake Y, Liao JK. Rho GTPases, statins, and nitric oxide. *Circ Res.* 2005;97:1232–1235.
- 96 Li F, Xia W, Yuan S, et al. Acute inhibition of Rho-kinase attenuates pulmonary

- hypertension in patients with congenital heart disease. *Pediatr Cardiol.* 2009;30:363–366.
- 97 Lee AH, Dhaliwal R, Kantores C, et al. Rho-kinase inhibitor prevents bleomycin-induced injury in neonatal rats independent of effects on lung inflammation. *Am J Respir Cell Mol Biol.* 2014;50:61–73.
98. Bogaard HJ, Abe K, Vonk Noordegraaf A, et al. The right ventricle under pressure: cellular and molecular mechanisms of right-heart failure in pulmonary hypertension. *Chest.* 2009;135:794–804.
- 99 Haddad F, Doyle R, Murphy DJ, et al. Right ventricular function in cardiovascular disease, part ii: pathophysiology, clinical importance, and management of right ventricular failure. *Circulation.* 2008;117:1717–1731.
- 100 Mourani PM, Sontag MK, Younoszai A, et al. Clinical utility of echocardiography for the diagnosis and management of pulmonary vascular disease in young children with chronic lung disease. *Pediatrics.* 2008;121:317–325.

Apnea and Control of Breathing

Christopher C. Stryker, Andrew Dylag, and Richard J. Martin

Abstract

Maturation of neonatal respiratory control is an essential component of the developing respiratory system. Transition from fetal to postnatal life requires continuous versus intermittent respiratory neural output to minimize the risk of apnea with resultant desaturation and bradycardia. Neonatal lung injury is a complication of our therapeutic interventions, many of which may be necessitated by apnea of prematurity. There is, therefore, a need to understand the physiologic maturation of respiratory control, which encompasses both chemo- and mechanoreceptor input to a developing brain stem. A significant contributor to progress has been the widespread use of xanthine (e.g., caffeine) therapy to enhance respiratory neural output. Future studies should identify mechanisms for the ability of such treatment to benefit both respiratory and neurodevelopmental outcomes.

Keywords:

Apnea of prematurity, bradycardia, desaturations, brain stem, chemoreceptors, mechanoreceptors, xanthine therapy

Introduction

Respiratory control and its maturation has been a subject of great interest to physiologists and clinicians since the 1960s. There are several compelling reasons for this continuing interest. First, apnea of prematurity is a significant clinical problem throughout the hospitalization of preterm infants, and the resultant intermittent hypoxia has potential adverse consequences. Second, impaired respiratory control frequently requires ventilatory support, which may be a major contributor to neonatal lung injury. Finally, respiratory control provides a novel link between the immature brain and developing respiratory system, both of which are vulnerable to inflammatory insults. Greater understanding of this linkage may provide new insights into the pathophysiologic mechanisms underlying longer-term respiratory and neurodevelopmental morbidity in this high-risk population.

The Brain stem

Overview of Central Respiratory Control.

Breathing, a life-sustaining homeostatic process that regulates levels of oxygen and carbon dioxide in the blood and tissues, is carried out by the musculature of the upper airways, diaphragm, abdomen, and rib cage, and is under central control by respiratory neurons in the brain stem.

Under normal physiologic conditions, the brain stem motor output pattern that results in breathing movements consists of three phases: inspiration, stage 1 of expiration (also known as postinspiration), and stage 2 of expiration (1). The timing, duration, and magnitude of each phase are determined by the coordinated actions of the various types of respiratory neurons. In early postnatal life when lung volume may be compromised, firing of inspiratory neurons into the expiratory phase may serve to maintain functional residual capacity (FRC) via both diaphragmatic and laryngeal braking (2).

The neural circuitry that generates respiratory rhythm and governs inspiratory and expiratory motor patterns is distributed throughout the pons and medulla (Figure 12-1). The respiratory central pattern generator (CPG), a specialized neural circuit that is intrinsically capable of producing rhythmic activity and motor outputs without sensory feedback, is the fundamental feature of this network that enables breathing to occur automatically and to be sustained continuously throughout life (1). The CPG, however, does not function in isolation. Central and peripheral sensory inputs carry state-characterizing information from multiple sources and influence the activity of the CPG, thereby allowing adjustments to the patterns of inspiratory and expiratory activity in response to changing metabolic conditions. For

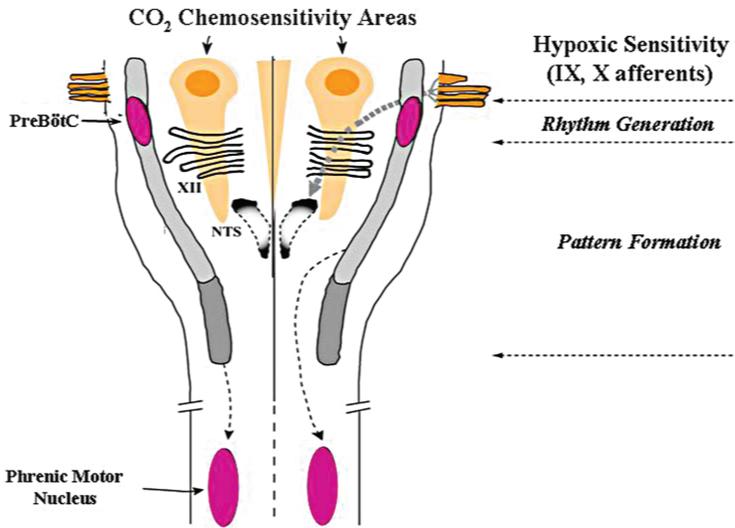


Figure 12-1. Simplified schematic view of the ventral mammalian brain stem. Rhythm generation resides mainly in the pre-Bötzinger (pre-BötC) complex, while pattern formation resides more caudally in the ventral respiratory column. CO₂ chemosensitivity extends within the orange-shaded area beyond the traditionally accepted chemosensitive areas, while the nucleus tractus solitarius (NTS) receives peripheral afferents from IX and X cranial nerve afferents (XII = efferent hypoglossal fibers).

Central	↑ Inhibitory neurotransmission	↓ CO ₂ chemosensitivity	Hypoxic respiratory depression
Peripheral	↑ Inhibition from upper airway afferents	↑ Hypoxic chemosensitivity	↓ Hypoxic chemosensitivity

Figure 12-2. Central and peripheral mechanisms that contribute to instability of respiratory control in fetal and early postnatal life.

example, inhibitory sensory inputs from the upper airway may be particularly prominent in early postnatal life to serve both a protective function and yet to trigger potentially clinically significant apnea (Figure 12-2).

The primary rhythm-generating and pattern-forming network is rostrocaudally organized along the entire length of the ventrolateral medulla, forming bilateral columns of interconnected respiratory nuclei called the ventral respiratory columns (VRC). The pre-Bötzinger complex (pre-BötC) of the ventrolateral medulla is the main source of respiratory rhythm generation (3). The pre-BötC is a core group of synaptically coupled neurons that possess autorhythmic or pacemaker-like activity. Intrinsic cellular properties of the pre-BötC enable the neurons to generate a rudimentary pattern of inspiratory activity when experimentally isolated *in vitro* (4). In the intact brain stem, however, generation of inspiratory rhythm is much more complex. The role of the pacemaker-like cells is diminished by the rhythmic inhibitory, tonic excitatory, and other modulatory inputs that converge on the pre-Bötzinger complex. Unfortunately, there is very limited information regarding maturation of changes in these pacemaker structures and their

influence on formation of the respiratory pattern during early maturation.

Neuronal populations within the nucleus of the solitary tract (NTS), retrotrapezoid nucleus (RTN), and medullary raphé nuclei integrate state-characterizing sensory information and influence the respiratory motor output in response to physiologic stimuli. The NTS is the site of termination of sensory afferents (slowly adapting stretch receptors (SARs), rapidly adapting stretch receptors (RARs), and C-fibers) from the lungs and airways. The NTS serves as the brain's first processing center for sensory input from peripheral arterial chemoreceptors (3). Outputs from the NTS project to other respiratory nuclei and spinal phrenic motor neurons, informing the motor output pattern based on the status of the lungs and airways.

Maturation of Central CO₂/H⁺ Chemosensitivity. Chemosensitive neuronal populations have been identified in a number of brain stem regions (5). The greatest density of CO₂/H⁺ sensitive neurons is in the medullary raphé, ventral medullary surface, and retrotrapezoid nucleus (note that the rat retrotrapezoid nucleus (RTN) is believed to be homologous to the human arcuate nucleus) (6). Many chemosensory signals, transducers, and

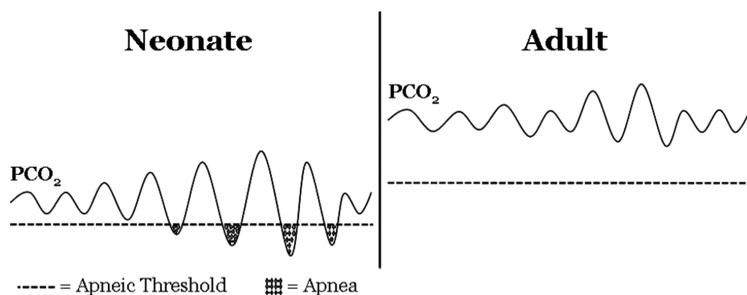


Figure 12-3. Relationship between eupneic PCO_2 and apneic threshold in neonates and adults. Relative to adults, PCO_2 during eupneic breathing is lower in the neonate and closer to the apneic threshold (dashed line). Neonates also have more variability in breathing, causing wider fluctuation in baseline PCO_2 . Together, these factors contribute to more frequent decreases in PCO_2 below the apneic threshold, leading to cessation of breathing. Adapted from Khan et al., *J Appl Physiol*. 2005;98:1171–1176

molecular mechanisms are involved in the process of chemoreception, the complexities of which are only beginning to be elucidated.

Ventilatory responses to CO_2 are present at birth in most mammalian species, including humans. Even prenatally, exposure to CO_2 incites increased depth of fetal breathing movements (7) (see later). In premature infants, CO_2 sensitivity and the hypercapnic ventilatory response are diminished in the early postnatal period (8-10). Increased minute ventilation in response to CO_2 in premature infants is primarily mediated by increases in tidal volume rather than respiratory rate (9). The hypercapnic ventilatory response in these infants is often associated with prolonged expiration characterized by expiratory braking and, in some infants, audible grunting (11). This may serve to preserve FRC. In contrast, older infants and adults, when exposed to CO_2 , have increased respiratory frequency characterized by a shortened expiratory phase. Further, infants with apnea of prematurity have reduced hypercapnic ventilatory responses relative to control infants at the same postconceptional age, suggesting that infants with apnea of prematurity have diminished central respiratory drive (12).

Another curious feature of the premature infant is the relationship between eupneic PaCO_2 levels and the threshold below which decreasing PaCO_2 will result in apnea, the so-called apneic threshold. In premature infants, the apneic threshold is much closer to eupneic levels of PaCO_2 compared to adults (13) (Figure 12-3). The proximity between eupneic CO_2 and the apneic threshold, coupled with baseline respiratory instability (affected by significant peripheral chemoreceptor drive to breathe in the neonatal period, low FRC, and sleep state) results in fluctuations in PaCO_2 that are more likely to cross the

apneic threshold in preterm infants, thus contributing to apnea of prematurity.

Mechanisms accounting for the postnatal maturation of central CO_2 chemosensitivity are unknown. Improved chemoreception may relate to maturation of intrinsic properties of chemosensory neurons themselves or of their synaptic outputs. Alternatively, improved chemosensitivity may relate to changes in the relative importance of different central and peripheral chemosensory sites during development (14).

An important candidate gene presumed to regulate central and peripheral chemoreceptor development is *PHOX2B*, a homeobox gene located on chromosome 4 (15). Its expression is necessary for normal development of the carotid body and NTS. It is also expressed in chemosensitive glutamatergic neurons of the RTN. Mutations in the *PHOX2B* gene lead to congenital hypoventilation syndrome (CCHS), characterized by impaired hypoxic and hypercapnic ventilatory responses and apnea, especially during sleep.

Neurochemistry of Respiratory Control. Excitatory and inhibitory neurotransmitters mediate the rhythmic synaptic communications between neurons of the medullary CPG network. In addition, myriad neuromodulators, although not essential for rhythmogenesis, exert profound effects on respiratory rhythm and inspiratory and expiratory patterns (Figure 12-4). Neuromodulators are substances released by axon terminals within the respiratory network but whose cell bodies are outside the CPG. They include biogenic amines, acetylcholine and other neuropeptides, and defects in their function underlie a number of disease states.

Glutamate, acting on AMPA and NMDA receptors, is the major neurotransmitter mediating excitatory synaptic input to brain stem

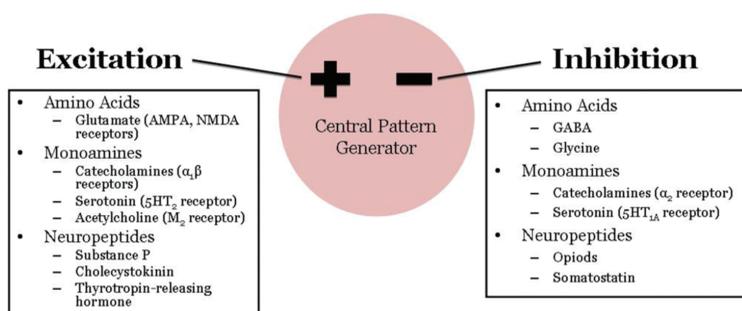


Figure 12-4. Excitatory and inhibitory regulators of respiratory neural output. Effects are dependent on the type of receptor activated and where within the network the mediators are acting.

respiratory neurons and respiratory premotor and motor neurons. Within the CPG, glutamate is involved in generation of augmenting respiratory neuron activity and inspiratory termination (2). Gamma-aminobutyric acid (GABA) and glycine are the two primary inhibitory neurotransmitters in the network, mediating the waves of inhibitory postsynaptic potentials during the silent phase of respiratory neurons. GABA_A receptors and glycine receptors activate ligand-gated chloride channels, resulting in membrane hyperpolarization and fast synaptic inhibition (16). GABA can also mediate slow synaptic transmission via GABA_B receptors, which are G-protein coupled receptors. Interestingly, during late embryonic and postnatal development, GABA and glycine can mediate *excitatory* neurotransmission secondary to changes in the chloride gradient across the membrane. It is unclear how this phenomenon relates to the greater inhibition of respiratory output that characterizes early neonatal life. GABA-mediated inhibitory neurotransmission may reduce CO₂ chemosensitivity exhibited by premature infants. In a rodent model, hypercapnia activates GABAergic neurons and GABA_A receptor blockade eliminates the hypercapnia-induced prolongation of expiratory time in young animals (16).

The ventilatory effects of neuromodulators depend on the type of receptor activated and on the respiratory mechanism controlled by that type of receptor. For example, a neuromodulator may stimulate ventilation when acting in one area of the CPG and depress ventilation when acting elsewhere. Serotonin is of particular importance in the modulation of respiratory function. Serotonergic neurons are located in medullary raphe nuclei and project to the NTS, VRC, RTN, and phrenic motor nuclei. Serotonergic projections may represent the neuroanatomic substrate for

the integration of cardiorespiratory responses (2). Defects in the medullary serotonergic system likely contribute importantly to the pathogenesis of sudden infant death syndrome (SIDS) and in the ventilatory instability observed in Rett syndrome (5,17).

Catecholamines (epinephrine, norepinephrine, and dopamine) elicit varied ventilatory responses depending on the type of receptor activated and the respiratory mechanism involved. Dopamine tends to act peripherally in the carotid bodies, while epinephrine and norepinephrine exert a tonic influence on central respiratory neurons (2). Acetylcholine acting on muscarinic receptors is likely involved in central chemoreception and the ventilatory response to CO₂, while nicotinic receptors are involved at the final stage of respiratory output in effecting acetylcholine transmission at neuromuscular junctions. Other neuroactive peptides are often colocalized with excitatory and inhibitory neurotransmitters and act as auxiliary messengers. For example, opioids and somatostatin depress respiratory neuronal firing, while substance P, cholecystokinin, and thyrotropin-releasing hormone exert excitatory drive. The interactions between classical neurotransmitters and neuromodulatory peptides acting on different receptor types and in different locations within the respiratory network creates nearly endless possibilities for precise shaping and fine-tuning of respiratory output during development.

Peripheral Afferents and Carotid Body Chemosensitivity

The output controlling breathing from the central nervous system is synthesized by end organs, which, in turn, provide feedback via central pathways of pulmonary and lower airway vagal afferents. The sensory receptors of

the lung with afferent fibers coursing in the vagus nerves are separated into three groups: SARs, RARs, and bronchopulmonary C-fibers. SARs and RARs innervate the pulmonary and airway mechanoreceptors via fast-conducting, myelinated fibers, whereas bronchopulmonary C-fibers have slowly conducting axons that lack myelination. These axons terminate in the NTS within the central nervous system. Second-order neurons from the NTS then send projections to phrenic motor neurons in the medulla, pons, and spinal cord. The selective activation and inhibition of SARs, RARs, and C-fibers each affect cardiopulmonary reflexes, collectively influencing the control of breathing.

Slowly Adapting Receptors (SARs). SARs are activated by lung volume and stretch to enhance of inspiratory effort, bronchodilation, and tachycardia (18). They project to ipsilateral subnuclei within the NTS at the rostrocaudal level of the area postrema. Second-order neurons are activated by SAR afferents, which synapse on pump cells (P-cells) and inspiratory- β ($I\beta$) cells. P-cells have phasic activity that tracks lung volume changes and are controlled by glycinergic inhibition during early inspiration and glutamatergic excitation during late inspiration and early expiration. When stimulated, P-cells induce changes that mimic the Breuer-Hering (B-H) reflex, whereas inhibition mimics environments with reductions in SAR input. $I\beta$ cells are inspiratory neurons that, like P-cells, are monosynaptically excited by SARs. They receive input from both the ipsilateral and contralateral vagus nerve and show a ramp-like increase in firing during inspiration while being silent during expiration. These second-order neurons then send projections to the ipsilateral ventral respiratory column (VRC) that are involved in rhythmogenesis.

The reflexes controlled by pulmonary vagal afferents and SARs in humans are similar to other mammals. One example is the Breuer-Hering reflex, which controls the duration of inspiration and expiration in relation to lung inflation. Josef Breuer first showed in adult cats that expansion of the lungs reflexively inhibits inspiration and promotes expiration and deflation of the lungs (19). In this way, the B-H reflex protects against lung overinflation. This reflex is mediated by SARs that act through the NTS, P-cells, and $I\beta$ cells as described earlier. The B-H reflex does not, however, participate in the regulation of fetal

breathing movements because vagotomy in fetal sheep has little effect on the incidence, frequency, or amplitude of these movements (20). However, the B-H reflex is essential in establishing continuous breathing and adequate gas exchange at birth by maintaining functional residual capacity; vagotomy at birth results in respiratory failure and atelectasis (21–22).

In humans, the B-H reflex is elicited by occluding the airway at one of two times in the respiratory cycle: end expiration where the next occluded inspiratory effort is prolonged and expiratory effort shortened, or end inspiration, where the next occluded expiratory effort is prolonged and inspiratory effort is shortened. The inspiratory/expiratory effort after the occlusion maneuver is compared to the nonoccluded breath to calculate a percent change in inspiratory/expiratory time. Using this methodology, the B-H reflex was shown to contribute significantly to tidal breathing in newborns, with decreasing contributions throughout the first year of life (23). The lower lung volumes and higher chest wall compliance in preterm infants and newborns activate the B-H reflex, shortening expiratory time and prolonging inspiratory time. The B-H reflex is also enhanced by premature birth (24), prone sleeping position (25), active sleep (26), and respiratory distress syndrome. The immediate prolongation of expiration and resultant slowing of respiratory rate with CPAP is one presumed manifestation of the B-H reflex.

Rapidly Adapting Receptors. RARs are activated in response to lung deflation, mechanical stimulation, and inhaled irritants and stimulate cough, bronchoconstriction, laryngoconstriction, and airway mucous secretion (18). In contrast to SAR primary afferent fibers, individual RARs project centrally to multiple sites within the NTS, suggesting that each afferent contributes to multiple effects attributed to these receptors. Each RAR synapses on caudal levels of the ipsilateral NTS subnuclei with smaller projections on the contralateral NTS. Excitatory input from RARs is mediated by non-NMDA glutamate receptors while RARs do not express mRNA for GABA or glycine, making it unlikely they have substantial inhibitory activity. When RAR cells receive excitatory input from stimuli such as ammonia, they send projections to second-order inspiratory (I_y) neurons in the NTS and bulbospinal neurons, which stimulate lung inflation (18).

Developmentally, RARs are important for restoring lung inflation in premature and term newborns. They are activated by the low lung volume and tidal breathing environments that are common in newborns. When activated, RARs activate augmented (sigh) breaths, which restore lung volume. The frequency of augmented breaths increases with decreasing gestational and chronologic age, and their description differs between newborns and adults (27). Newborns take two large inspiratory breaths in succession compared to one inspiratory effort in adults. Premature and newborn infants also can hypoventilate after an augmented breath, whereas adults will increase minute ventilation, suggesting that peripheral arterial chemoreceptor inputs may play a larger role in newborns. PaO₂ rapidly increases, and PaCO₂ rapidly decreases during an augmented breath, thereby reducing excitatory input from peripheral arterial chemoreceptors, decreasing respiratory drive, and resulting in apnea. Peripheral arterial chemoreceptors also play a role in initiating augmented breaths as denervation of the carotid sinus decreases the frequency of these maneuvers (28). Therefore, increased RAR activity during lung deflation, coupled with increased sensitivity of peripheral arterial chemoreceptors, contribute to the increased frequency of augmented breaths along with subsequent hypoventilation in premature infants.

C-Fiber Receptors. Bronchopulmonary C-fibers are unmyelinated vagal afferents that are activated by a variety of physical, environmental, and chemical stimuli, mainly capsaicin, carbon dioxide, lung edema, and elevated temperature. These neurons control rapid shallow breathing, cough, apnea, bronchoconstriction, laryngoconstriction, airway mucous secretion, vasodilation, and bradycardia (18). They terminate mainly in the ipsilateral NTS with small projections sent to the contralateral side. Excitatory input is mediated by glutamate acting on non-NMDA receptors in NTS subnuclei. When stimulated, C-fibers release neuropeptides, mainly substance P, which have local direct effects such as bronchoconstriction, increased mucous secretion, and bronchial and nasal vasodilation. The central effects of C-fibers are mediated by second-order interneurons in the central nervous system that have not been identified to date. The end result, however, is reflex apnea characterized by prolonged

expiratory time from excitation of these postinspiratory neurons and continuous firing of central expiratory neurons.

In newborns, stimulation of pulmonary C-fibers causes bronchoconstriction and apnea (29). Capsaicin-induced apnea was most sensitive in newborn rat pups younger than 10 days of age (30). Other stimuli such as acidosis, adenosine, reactive oxygen species, hyperosmotic solutions, and lung edema also stimulate C-fibers and enhance their effects. C-fibers can also be sensitized by inflammatory mediators in the local environment, which may play a role in the apnea typically observed in infants with viral infections such as respiratory syncytial virus (31).

Maturation of Peripheral CO₂/H⁺ and Hypoxic Carotid Body Chemosensitivity. The carotid body is primarily responsible for the control of ventilation in response to arterial oxygen tension. Several histological cell types and their corresponding functions have been identified within the carotid body. Type I, or glomus, cells are the oxygen-sensing cells. Exposure to acute hypoxia results in glomus cell depolarization and neurotransmitter release through voltage-gated calcium channels. Type II cells, similar to glial cells, are not chemosensitive. Postsynaptic afferent nerve fibers oppose the glomus cells and have cell bodies in the petrosal ganglion. The primary target for afferents from peripheral arterial chemoreceptors is the commissural nucleus of the NTS. Glutamate binds to both NMDA and non-NMDA receptors on second-order neurons in the NTS and is responsible for transmission of excitatory inputs from the peripheral arterial chemoreceptors (32). These second-order neurons then send tonic excitatory projections to the RTN, the dorsal respiratory group, and the ventral respiratory group.

The contribution of the peripheral arterial chemoreceptors has been elucidated by separately perfusing the carotid and systemic circulation or measuring direct carotid body output. These studies have determined that peripheral chemoreceptors respond more rapidly to CO₂/H⁺ than central chemoreceptors (33). These chemoreceptors do not appear to influence fetal breathing, but they are necessary for stabilization of breathing patterns because denervation of the carotid body in newborn animals will result in apnea and death (34). The maturation of the carotid body parallels the increased sensitivity of the glomus cell to excitatory and inhibitory

inputs. Newborn animals have increased sensitivity to CO₂ during development, although the contribution of peripheral versus central CO₂ chemosensitivity to this maturational change is unclear (35).

There is a critical period in the first two weeks of postnatal development during which exposure to chronic hypoxia, chronic hyperoxia, and intermittent hypoxia can lead to persistent alterations of chemoreceptor function in animal models (36–37). Similar exposures outside these periods have little to no lasting effect, indicating a level of plasticity in the control of the respiratory system. It remains unclear whether this phenomenon has clinical consequences in humans. At birth, there is an acute insensitivity to changes in oxygen tension that gradually decreases during the first two to three weeks of postnatal development as hypoxic excitation and hyperoxic inhibition of breathing become stronger (38). The current speculation is that the sensitivity of peripheral chemoreceptors to hypoxia is “reset” at birth during the transition from fetal hypoxic to neonatal normoxic life. Exposure to hyperoxia or hypoxia from birth delays the maturation of the oxygen chemoreceptor response in animal models (36). A premature infant is born into a relatively hyperoxic environment, often exacerbated by iatrogenic hyperoxia from respiratory support. Interestingly, this chemoreceptor-resetting phenomenon is also observed in preterm infants who demonstrate enhanced peripheral arterial chemoreceptor influences on breathing after the same critical two- to three-week period. This period coincides with the development of periodic breathing, which is associated with excessive peripheral chemoreceptor activity.

Peripheral arterial chemoreceptors in the carotid body are the primary sites for detection of changes in arterial oxygen tension. Near term, fetal chemoreceptor activity is generally absent and responds poorly to hypoxia. The response to a hypoxic exposure is to increase ventilatory drive and behavioral arousal, and this reflex strengthens with maturation. Exposure to hyperoxia (Dejours test) will reduce ventilation in both animals and humans. Hypoxia induces depolarization of the carotid body glomus cell, activating voltage-dependent calcium channels, which lead to neurotransmitter release. The magnitude of the calcium-dependent response seems to be critical for the maturation of intact hypoxic ventilatory

responses. Studies have shown the calcium-dependent current in rats increases with postnatal age until P14, which is paralleled by development of mature carotid body responses to hypoxia or anoxia (37). Again, we must infer characteristics of the human carotid body chemoreceptors by observing hypoxic ventilatory responses. If human infants are repeatedly exposed to intermittent hypoxia in settings such as prematurity, apnea, and periodic breathing, they demonstrate a greater reduction in minute ventilation when exposed to hyperoxia. This suggests a greater influence of peripheral arterial chemoreceptors on normal breathing in this population.

A normal response to hypoxia in animals with functioning peripheral and central chemoreceptors is an increase in ventilation within thirty seconds of hypoxic gas exposure and decrease after two to three minutes. The trend back to ventilatory baseline is referred to as the hypoxic roll-off, hypoxic ventilatory decline, or hypoxic ventilatory depression. The hypoxic roll-off remains above baseline in mature models but can trend below baseline and result in apnea in newborns. The mechanisms that account for hypoxic roll-off are mediated in the pons and under control of neuromodulators such as norepinephrine, adenosine, GABA, serotonin, opioids, and platelet-derived growth factor. Adenosine has garnered particular attention because during hypoxia, brain adenosine levels can increase over twofold in fetal sheep (39). Blockade of adenosine receptors with caffeine and other methylxanthines can decrease the hypoxic ventilatory depression in newborn infants. This is a mainstay of neonatal therapy in apnea of prematurity (discussed later).

Fetal Breathing

Fetal breathing activity can be identified by the eleventh week of gestation in the human fetus with ultrasound. Although the placenta is the site of gas exchange in utero, fetal breathing movement is important in enhancement of lung growth and development, and decreased diaphragmatic activity is associated with pulmonary hypoplasia. Fetal breathing occurs phasically only during rapid eye movement (REM) sleep with total cessation of breathing during non-REM sleep, possibly secondary to descending inhibitory pontine input to the medullary rhythm-generating center. Various factors may contribute to the inhibition

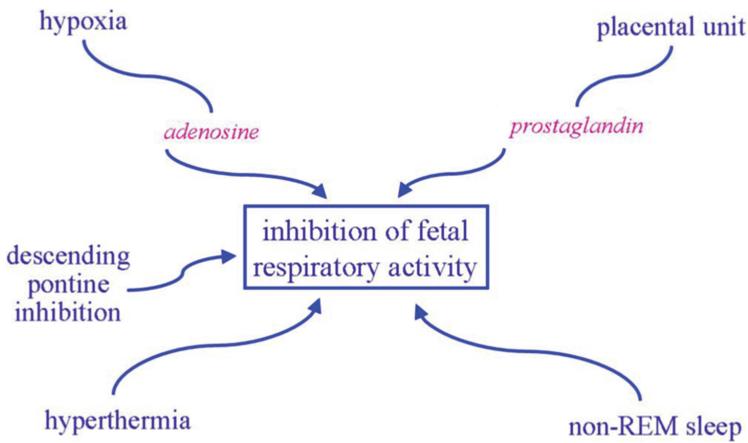


Figure 12-5. Factors contributing to the inhibition of fetal respiratory activity, notably the relatively hypoxic intrauterine environment.

of fetal respiratory activity as summarized in Figure 12-5. Most prominent of these is probably respiratory depression induced by the hypoxic intrauterine environment to which the fetus is so well adjusted. Presumably an increase in fetal breathing movements in response to hypoxia would be counterproductive. In contrast, hypercapnic exposure does increase the incidence and depth of fetal breathing, although only in REM sleep. Ability to generate a ventilatory response to CO_2 , the main chemical stimulus to breathing, is clearly important for a successful fetal to neonatal transition.

Role of a Vulnerable Respiratory System

While immature respiratory control is the major contributor to apneic events in preterm infants, several components of the infants' respiratory system increase vulnerability to cardiorespiratory events. Increased chest wall compliance and low lung volume both contribute to low oxygen reserves, often resulting in low baseline oxygen saturation (Figure 12-6). As a result, even short respiratory pauses may result in desaturation and resultant reflex bradycardia. This is consistent with the observation that episodes of intermittent hypoxia are significantly more frequent when baseline oxygen saturation is targeted at 85–89 versus 90–95% (40).

Although immature central respiratory drive is the major etiology, closure of the upper airway during central apnea may prolong such episodes. It is estimated that a majority of longer apnea is prolonged by an unstable upper airway.

A potential mechanism for this problem is a higher CO_2 threshold for upper airway muscles such as the genioglossus. Delayed activation of these upper airway muscles may then occur, as a rising PaCO_2 triggers diaphragm activation toward the end of a central apnea, resulting in obstructed inspiratory efforts or so-called mixed apnea. This is in contrast with sleep apnea in older populations in whom upper airway obstruction is the primary problem. Purely obstructive apnea is unlikely in preterm infants unless they are inappropriately positioned.

Contribution from Inflammatory Mechanisms

Inflammatory mechanisms contribute to instability of neonatal respiratory control. Clinically, apnea increases in frequency and severity during acute infections in premature infants (41). Although inflammatory cytokines probably do not readily cross the blood–brain barrier, systemic infection does upregulate inflammatory cytokines at the blood–brain barrier, resulting in activation of prostaglandin signaling and resultant inhibition of respiratory neural output (42).

Chorioamnionitis is a major precipitant of preterm birth, definitively associated with neonatal brain injury in the form of periventricular leukomalacia (PVL) and possibly chronic neonatal lung injury in the form of bronchopulmonary dysplasia (BPD). It is possible that antenatal or postnatal exposure of the lung to a proinflammatory stimulus may activate brain circuits via vagally mediated processes. LPS (0.1 mg/kg) instilled into the trachea of newborn rat pups at

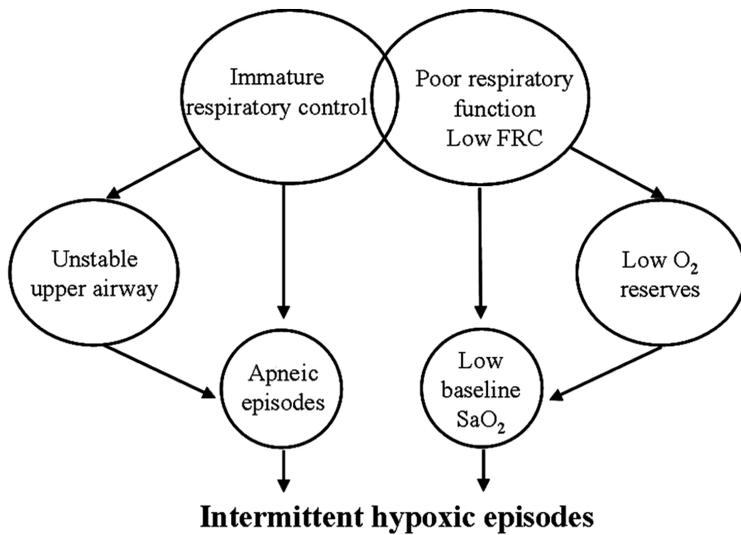


Figure 12-6. The net effect of immature respiratory control superimposed on a low lung volume and low intrapulmonary oxygen reserves is recurrent desaturation in the face of short respiratory pauses.

day of life ten to twelve increases inflammatory cytokine gene expression in the medulla oblongata and attenuated both the immediate and late hypoxic ventilatory response when animals were tested within three hours of treatment (43) (Figure 12-7). This brain stem response to intrapulmonary LPS was diminished after vagotomy, suggesting a lung-to-brain stem communication via vagal afferents. An interesting related line of investigation is the role of intermittent hypoxia and resultant oxidant stress on inflammatory pathways (44). It has been proposed that in the healthy central nervous system with no, or “low-dose,” intermittent hypoxia, microglia are in a surveillance mode that promotes neuronal viability and function by releasing growth/trophic factors that confer neuroprotection and/or increase synaptic strength (i.e., plasticity). In contrast, “high doses,” or chronic intermittent hypoxia, may activate microglia to a toxic, proinflammatory phenotype that triggers neuronal apoptosis and undermines synaptic plasticity.

Diagnostic Challenges in Neonatal Cardiorespiratory Monitoring

Respiration: Cardiorespiratory monitoring is a vital component of clinical care of the neonate. Accurate measurements of respiration, oxygen saturation, and heart rate are imperative to detect clinical apnea during both spontaneous breathing and respiratory support. Although immature

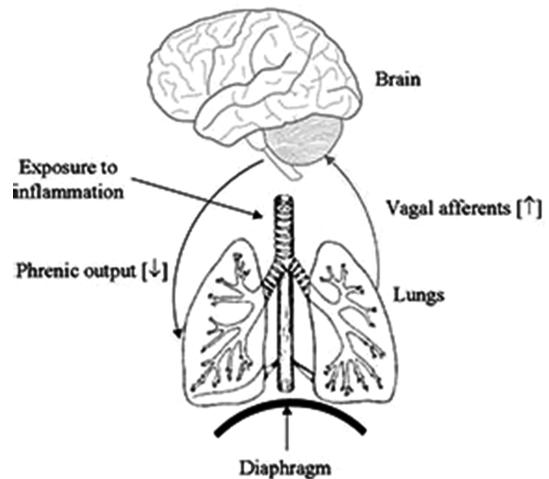


Figure 12-7. Inflammation effects on the respiratory system pre- and postnatal exposure of the respiratory system to endotoxin may elicit a proinflammatory cytokine response in the brain stem via stimulation of vagal afferents. The inflammation may inhibit respiratory neural output by local effects in the brain (adapted from Balan. *Respir Physiol Neurobiol.* 2011;178:458–464).

respiratory control is the major precipitant of cardiorespiratory events in preterm infants, we are dependent on impedance technology to measure respiration (45). The advantage of impedance monitoring is that it can be recorded from ECG electrodes allowing for long-term measurements of respiration (and heart rate) in a noninvasive manner. However, as air moves from one compartment to the other during periods of obstruction, impedance monitoring cannot distinguish obstructed efforts from normal respiration.

Ongoing research is focused on the development of a technology that measures effective airflow without need for an oral/nasal flow sensor. Such a technique has yet to make its way into clinical bedside monitoring.

Heart Rate: Reflex bradycardia is a common accompaniment of apnea of prematurity, and application of additional filters to the EKG waveform allows for more extensive analysis of tracings. An algorithm of heart rate characteristic monitoring, including heart rate variability and decelerations, has generated interesting prognostic data (46). This algorithm may be a predictor of neonatal sepsis prior to routine clinical assessment and may reduce mortality by early prediction of adverse events.

Oxygenation: There is a great interest in the significance of the intermittent hypoxic episodes in preterm infants that result from immature respiratory control and residual lung disease. Pulse oximetry is the most widely used method for continuous noninvasive monitoring of oxygenation, and advances in motion artifact reduction software have improved the false alarm rate. Additional signal processing concerns include the averaging time, which can be modified by the user. Common clinical practice has promoted the use of a long averaging time (16 sec) to reduce false alarms. However, a long averaging time will reduce the detection of short (<2-sec) desaturation events while increasing the number and duration of events >20 seconds. This is most likely due to short desaturation events being averaged into one prolonged event. In contrast, the averaging time had no effect on the time spent in different SpO₂ ranges (45,47). Surprisingly, with a multitude of studies, including recent multicenter trials investigating oxygen saturation ranges in preterm infants, the optimal target range continues to elude us (40, 48–49). Regardless of the chosen oxygen saturation target range, prevention of intermittent hypoxemia continues to be a challenge in patient care, as such events have been associated with morbidity in preterm infants such as retinopathy of prematurity (50). Recent data in a neonatal rodent model indicate that exposure to sustained hypoxia, followed by chronic intermittent hypoxia, has a unique synergistic effect of impairing respiratory control as indicated by vulnerability to a subsequent hypoxic exposure (51).

Biologic Basis for Therapeutic Interventions

The aggressiveness with which therapy is pursued in apneic preterm infants must weigh the potential consequences of apnea and resultant desaturation and bradycardia, with the natural history, which favors spontaneous resolution of these episodes with advancing maturation. For the most widely used therapy, namely methylxanthines, we are still gaining knowledge of the precise mechanisms of action.

Optimization of Mechanosensory Inputs. The respiratory rhythm-generating circuitry within the central nervous system (CNS) depends on intrinsic rhythmic activity and sensory afferent inputs to generate breathing movement. Bloch-Salisbury et al. (52) demonstrated that their novel technique of stochastic mechanosensory stimulation, using a mattress with imbedded actuators, is able to stabilize respiratory patterns in preterm infants as measured by a decrease in apnea and an almost threefold decrease in percentage of time with oxygen saturations < 85%. Interestingly, the level of stimulation employed was below the minimum threshold for behavioral arousal to wakefulness, thus inducing no apparent state change in the infants. The effect could probably not be attributed to the minimal increase in sound level associated with stimulation. Such an approach is clearly worthy of further study. Skin-to-skin care is a highly desirable practice in the NICU to encourage parental attachment. This practice is not only safe, but associated with decreased electrical diaphragm activity, potentially benefiting energy expended on respiratory efforts (53).

Optimization of Gas Exchange and Blood Gas Status. Intermittent hypoxic episodes are almost always the result of respiratory pauses, apnea, or ineffective ventilation. It is unclear whether targeting lower baseline oxygen saturation increases the incidence of apnea with resultant hypoxemia, or whether the incidence of apnea is comparable between oxygen targets. However, lower oxygen saturation baseline predisposes to more frequent or profound intermittent hypoxemia. Similarly, it is unclear whether the beneficial effect of packed red cell transfusion is secondary to improved respiratory control or increased vulnerability to hypoxia in the face of apnea (54–55). However, given the potential oxidative stress associated with clinically significant

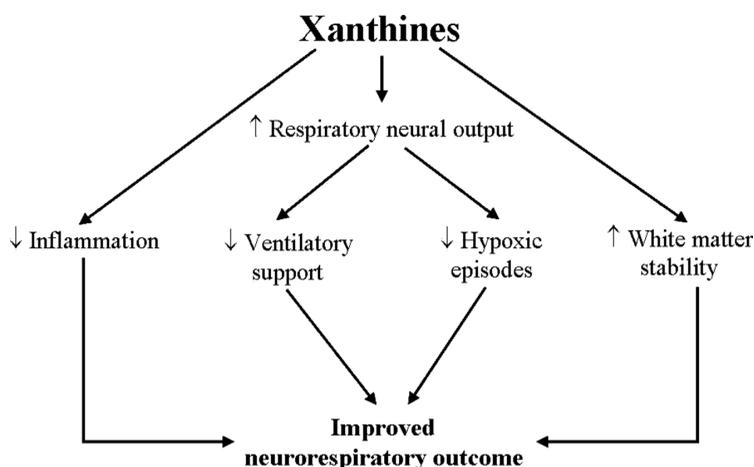


Figure 12-8. Potential pathways for xanthine therapy to improve neurorespiratory outcomes in preterm infants.

intermittent hypoxic episodes, the latter are probably best avoided (56).

Automated control of inspired oxygen is under study. This automated technique has been compared to routine adjustments of inspired oxygen as performed by clinical personnel in infants of twenty-four to twenty-seven weeks gestation (57). During the automated period, time with oxygen saturation within the intended range of 87–93% increased significantly, and times in the hyperoxic range were significantly reduced. This was not associated with a clear benefit for hypoxic episodes; nonetheless, future refinement of this technology may prove useful to minimize intermittent hypoxia. Finally, a novel approach is supplementation of inspired air with a very low concentration of supplemental CO₂ to increase respiratory drive (58). While of interest from a physiologic perspective, and likely to be successful in decreasing apnea, it is doubtful that this would gain widespread clinical acceptance, as most preterm infants have residual lung disease and are prone to baseline hypercapnia, which may make clinicians reluctant to administer supplemental inspired CO₂.

Continuous Positive Airway Pressure (CPAP). CPAP has proven a relatively safe and effective therapy for forty years. It has a dual function to stabilize lung volume and improve airway patency by limiting upper airway closure. Because longer episodes of apnea frequently involve an obstructive component, CPAP appears to be effective by “splinting” the upper airway with positive pressure and to decrease the risk of pharyngeal or

laryngeal obstruction. At the lower functional residual capacity characteristic of many preterm infants with residual lung disease, pulmonary oxygen stores are probably reduced, and there is a very short time from cessation of breathing to onset of desaturation and bradycardia. Therefore, CPAP is likely to reduce this vulnerability to episodic desaturation. Nasal CPAP is well tolerated in most preterm infants. Currently, low- or high-flow nasal cannula therapies are being increasingly used as an equivalent treatment modality that may allow CPAP delivery while enhancing mobility of the infant.

Methylxanthine Therapy. Methylxanthine therapy has been used to prevent and treat apnea of prematurity since the 1970s. Xanthines are nonspecific adenosine receptor inhibitors. Their primary mechanism of action in the perinatal period is thought to be blockade of inhibitory adenosine A₁ receptors with resultant excitation of respiratory neural output (59) (Figure 12-8). An alternative mechanism of caffeine action is blockade of excitatory adenosine A_{2A} receptors at GABAergic neurons and resultant decrease in GABA output, resulting in excitation of respiratory neural output (60).

These complex neurotransmitter interactions elicited by caffeine raised concerns regarding its safety, and a large multicenter trial was undertaken in the 1980s. This study demonstrated that caffeine treatment (used to treat apnea or enhance extubation) effectively decreases the rate of BPD and improves neurodevelopmental outcome at eighteen to twenty-one months, especially in those receiving respiratory support

(61–62). It is possible that this benefit is secondary to decreased apnea and resultant intermittent hypoxic episodes; however, this is speculative.

Recent data in neonatal rodents demonstrate an anti-inflammatory effect of caffeine in proinflammatory states elicited by postnatal hyperoxia or antenatal endotoxin exposure (63–64). Improved lung pathology and respiratory system mechanics were observed after caffeine treatment. In contrast, other data raise concerns about potential adverse effects of neonatal caffeine exposure in various animal models (65–66). The effects of caffeine on the developing brain are also in conflict and include no effect in an ovine model (67), a protective effect in hypoxia-induced perinatal white matter injury (68), and an adverse effect on brain imaging (69).

These conflicting results in the face of clinical benefit suggest that changes in dosing and indications for caffeine that deviate from proven beneficial protocols should proceed with caution. Methylxanthine use is now widespread in a prophylactic mode (54). Initial studies suggest that very early initiation of caffeine therapy results in improved outcome; however, these findings are based on retrospective review with potential confounders (70). Finally, the extended use of caffeine to forty weeks postmenstrual age was associated with a decrease in intermittent hypoxia among a cohort of preterm infants (71). The longer-term effects of this changing therapeutic landscape are unknown.

Future Challenges in Neonatal Respiratory Control. Longer-term respiratory morbidity, including bronchopulmonary dysplasia, remains a major challenge for preterm infants. Much of this morbidity is the result of medical interventions with supplemental oxygen and the various modalities of positive pressure ventilation. Unfortunately, immature respiratory control contributes to the need for these therapeutic approaches. An enhanced understanding of neonatal respiratory control is a high priority for the preterm population. It has also been known for over a decade that preterm birth is associated with later susceptibility to sleep disordered breathing in the pediatric population. However the physiologic or anatomic basis for this prolonged vulnerability is unclear.

Much has been accomplished with xanthine therapy, although more focused adenosine receptor blockade and complementary pharmacotherapy would be useful. In fact, our understanding of the mechanism whereby caffeine enhances respiratory control and improves both respiratory and neurodevelopmental outcomes is very limited.

These questions require translational studies involving neonatal animal models, both large and small, none of which perfectly simulate the human preterm experience. Unfortunately, there is no optimal preterm animal model that simulates apnea of prematurity. Neonatal rodent models are practical, cost effective, and relatively preterm (on a human scale) at birth. Although they do not have spontaneous apnea, traditional physiologic studies such as hypoxic and hypercapnic responses can be performed from an early age under in vivo conditions and in response to pharmacologic interventions. Such models also are currently most suited to knock-in or knock-out genetic manipulation. In vitro rodent models can be focused down on specific neuronal regions and cell groups by employing brain stem slices and brain stem-spinal cord preparations. However, the limitations of such preparations to understanding the normal developmental trajectory should be apparent.

Therefore, there is no substitute for well-designed human studies. We are still in need of practical techniques to noninvasively measure airflow (i.e., effective breathing) in preterm infants, beyond standard impedance monitoring of respiratory motion. Fortunately, techniques such as respiratory inductance plethysmography have advanced the field in differentiating central from obstructive apnea and various combinations thereof. Finally, given that impaired gas exchange is the major consequence in an apneic infant, advances in artifact-free pulse oximetry technology have greatly enhanced our knowledge of the natural history of intermittent hypoxic episodes. We now need to learn whether these episodes matter. Immature respiratory control superimposed on impaired respiratory function is not a good combination. Future models need to incorporate these two adverse phenomena as we seek to improve respiratory outcomes.

References

- 1 Smith JC, Abdala APL, Rybak IA, et al. Structural and functional architecture of respiratory networks in the mammalian brainstem. *Philos Trans R Soc Lond B Biol Sci.* 2009;364:2577–2587.
- 2 Bianchi AL, Denavit-Saubié M, Champagnat J. Central control of breathing in mammals: neuronal circuitry, membrane properties, and neurotransmitters. *Physiol Rev.* 1995;75:1–45.
- 3 Alheid GF, McCrimmon DR. The chemical neuroanatomy of breathing. *Respir Physiol Neurobiol.* 2008b;164:3–11.
- 4 Koshiya N, Smith JC. Neuronal pacemaker for breathing visualized in vitro. *Nature.* 1999;400:360–363.
- 5 Feldman JL, Mitchell GS, Nattie EE. Breathing: rhythmicity, plasticity, chemosensitivity. *Ann Rev Neurosci.* 2003;26:239–266.
- 6 Akilesh MR, Kamper M, Li A, et al. Effects of unilateral lesions of retrotrapezoid nucleus on breathing in awake rats. *J Appl Physiol.* 1997;82:469–479.
- 7 Ritchie JW, Lakhani K. Fetal breathing movements in response to maternal inhalation of 5% carbon dioxide. *Am J Obstet Gynecol.* 1980;136:386–388.
- 8 Krauss AN, Klain DB, Waldman S, et al. Ventilatory responses to carbon dioxide in newborn infants. *Pediatr Res.* 1975;9:46–50.
- 9 Rigatto H, Brady JF, Verduzco RT. Chemoreceptor reflexes in preterm infants: II. The effect of gestational and postnatal age on the ventilatory response to inhaled carbon dioxide. *Pediatrics.* 1975a;55:614.
- 10 Frantz ID, Adler SM, Thach BT, et al. Maturational effects on respiratory responses to carbon dioxide in premature infants. *J Appl Physiol.* 1976;41:41–45.
- 11 Eichenwald EC, Ungarelli RA, Stark AR. Hypercapnia increases expiratory breaking in preterm infants. *J Appl Physiol.* 1993;75:2665–2670.
- 12 Gerhardt T, Bancalari E. Apnea of prematurity. 1. Lung function and regulation of breathing. *Pediatrics.* 1984;74:58–62.
- 13 Khan A, Qurashi M, Kwiatkowski K, et al. Measurement of the CO₂ apneic threshold in newborn infants: possible relevance for periodic breathing and apnea. *J Appl Physiol.* 2005;98:1171–1176.
- 14 Putnam RW, Conrad SC, Gdovin MJ, et al. Neonatal maturation of the hypercapnic ventilatory response and central neural CO₂ chemosensitivity. *Respir Physiol Neurobiol.* 2005;149:165–179.
- 15 Amiel J, Dubreuil V, Ramanantsoa N, et al. PHOX2B in respiratory control: lessons from congenital central hypoventilation syndrome and its mouse models. *Respir Physiol Neurobiol.* 2009;168:125–132.
- 16 Martin RJ, Wilson CG, Abu-Shaweesh JM, et al. Role of inhibitory neurotransmitter interactions in the pathogenesis of neonatal apnea: implications for management. *Semin Perinatol.* 2004;28:273–278.
- 17 Kinney HC, Broadbelt KG, Haynes RL, et al. The serotonergic anatomy of the developing human medulla oblongata: implications for pediatric disorders of homeostasis. *J Chem Neuroanat.* 2011;41:182–199.
- 18 Kubin L, Alheid GF, Zuperku EJ, et al. Central pathways of pulmonary and lower airway vagal afferents. *J Appl Physiol.* 2006;101:618–627.
- 19 Widdicombe J. Reflexes from the lungs and airways: historical perspective. *J Appl Physiol.* 2006;101:628–634.
- 20 Hasan SU, Rigaux A. Effect of bilateral vagotomy on oxygenation, arousal, and breathing movements in fetal sheep. *J Appl Physiol.* 1992;73:1402–1412.
- 21 Lalani S, Remmers JE, Green FH, et al. Effects of vagal denervation on cardiorespiratory and behavioral responses in the newborn lamb. *J Appl Physiol.* 2001;91:2301–2313.
- 22 Wong KA, Bano A, Rigaux A, et al. Pulmonary vagal innervation is required to establish adequate alveolar ventilation in the newborn lamb. *J Appl Physiol.* 1998;85:849–859.
- 23 Rabbette PS, Fletcher ME, Dezateux CA, et al. Hering-Breuer reflex and respiratory system compliance in the first year of life: a longitudinal study. *J Appl Physiol.* 1994;76:650–656.
- 24 De Winter JP, Merth IT, Berkenbosch A, et al. Strength of the Breuer-Hering inflation reflex in term and preterm infants. *J Appl Physiol.* 1995;79:1986–1990.
- 25 Landolfo F, Saiki T, Peacock J, et al. Hering-Breuer reflex, lung volume and position in prematurely born infants. *Pediatr Pulmonol.* 2008;43:767–771.
- 26 Hand IL, Noble L, Wilks M, et al. Hering-Breuer reflex and sleep state in the preterm infant. *Pediatr Pulmonol.* 2004;37:61–64.
- 27 Alvarez JE, Bodani J, Fajardo CA, et al. Sighs and their relationship to apnea in the newborn infant. *Biol Neonate.* 1993;63:139–146.

- 28 Matsumoto S, Takeda M, Saiki C, et al. Effects of vagal and carotid chemoreceptor afferents on the frequency and pattern of spontaneous augmented breaths in rabbits. *Lung*. 1997;175:175–186.
- 29 Frappell PB, MacFarlane PM. Development of mechanics and pulmonary reflexes. *Respir Physiol Neurobiol*. 2005;149:143–154.
- 30 Wang R, Xu F. Postnatal development of right atrial injection of capsaicin-induced apneic response in rats. *J Appl Physiol*. 2006;101:60–67.
- 31 Lee LY, Pisarri TE. Afferent properties and reflex functions of bronchopulmonary C-fibers. *Respir Physiol*. 2001;125:47–65.
- 32 Vardhan A, Kachroo A, Sapru HN. Excitatory amino acid receptors in commissural nucleus of the NTS mediate carotid chemoreceptor responses. *Am J Physiol*. 1993;264:R41–R50.
- 33 Smith CA, Rodman JR, Chenuel BJ, et al. Response time and sensitivity of the ventilatory response to CO₂ in unanesthetized intact dogs: central vs. peripheral chemoreceptors. *J Appl Physiol*. 2006;100:13–19.
- 34 Gauda EB, Lawson EE. Developmental influences on carotid body responses to hypoxia. *Respir Physiol*. 2000;121:199–208.
- 35 Carroll JL, Bamford OS, Fitzgerald RS. Postnatal maturation of carotid chemoreceptor responses to O₂ and CO₂ in the cat. *J Appl Physiol*. 1993;75:2383–2391.
- 36 Carroll JL. Developmental plasticity in respiratory control. *J Appl Physiol*. 2003;94:375–389.
- 37 Gauda EB, Cristofalo E, Nunez J. Peripheral arterial chemoreceptors and sudden infant death syndrome. *Respir Physiol Neurobiol*. 2007;157:162–170.
- 38 Rigatto H, Brady JP, de la Torre Verduzco R. Chemoreceptor reflexes in preterm infants: I. The effect of gestational and postnatal age on the ventilatory response to inhalation of 100% and 15% oxygen. *Pediatrics*. 1975;55:604–613.
- 39 Koos BJ, Mason BA, Punla O, et al. Hypoxic inhibition of breathing in fetal sheep: relationship to brain adenosine concentrations. *J Appl Physiol*. 1994;77:2734–2739.
- 40 SUPPORT Study Group of the Eunice Kennedy Shriver NICHD Neonatal Research Network. Target ranges of oxygen saturation in extremely preterm infants. *N Engl J Med*. 2010;362:1959–1969.
- 41 Hofstetter AO, Saha S, Siljehav V, et al. The induced prostaglandin E2 pathway is a key regulator of the respiratory response to infection and hypoxia in neonates. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104:9894–9899.
- 42 Olsson A, Kayhan G, Lagercrantz H, et al. Il-1 beta depresses respiration and anoxic survival via a prostaglandin-dependent pathway in neonatal rats. *Pediatr Res*. 2003;54:326–331.
- 43 Balan KV, Kc P, Hoxha Z, et al. Vagal afferents modulate cytokine-mediated respiratory control at the neonatal medulla oblongata. *Respir Physiol Neurobiol*. 2011;178:458–464.
- 44 Dale EA, Mabrouk FB, Mitchell GS. Unexpected benefits of intermittent hypoxia: Enhanced respiratory and nonrespiratory motor function. *Physiology*. 2014;29:39–48.
- 45 Di Fiore JM, Martin RJ, Gauda EV. Apnea of prematurity – perfect storm. *Respir Physiol Neurobiol*. 2013;189:213–222.
- 46 Moorman JR, Carlo WA, Kattwinkel J, et al. Mortality reduction by heart rate characteristic monitoring in very low birth weight neonates: a randomized trial. *J Pediatr*. 2011;159:900–906.
- 47 Vagedes J, Poets CF, Dietz K. Averaging time, desaturation level, duration and extent. *Arch Dis Child Fetal Neonat Ed*. 2013;98:F265–266.
- 48 The BOOST II United Kingdom, Australia, and New Zealand Collaborative Groups. Oxygen saturation and outcomes in preterm infants. *N Engl J Med*. 2013;368:2094–2104.
- 49 Schmidt B, Whyte RK, Asztalos EV, et al.; Canadian Oxygen Trial [COT] Group. Effects of targeting higher vs lower arterial oxygen saturations on death or disability in extremely preterm infants: a randomized clinical trial. *JAMA*. 2013;309:2111–2120.
- 50 Di Fiore JM, Bloom JN, Orge F, et al. A higher incidence of intermittent hypoxemic episodes is associated with severe retinopathy of prematurity. *J Pediatr*. 2010b;157:69–73.
- 51 Mayer CA, Ao J, Di Fiore JM, et al. Impaired hypoxic ventilatory response following neonatal sustained and subsequent chronic intermittent hypoxia in rats. *Respir Physiol Neurobiol*. 2013;187:167–175.
- 52 Bloch-Salisbury E, Indic P, Bednarek F, et al. Stabilizing immature breathing patterns of preterm infants using stochastic mechanosensory stimulation. *J Appl Physiol*. 2009;107:1017–1027.
- 53 Soukka H, Grönroos L, Leppäsalo J, et al. The effects of skin-to-skin on the diaphragmatic electrical activity in preterm infants. *Early Hum Develop*. 2014;90(9):531–534.

- 54 Abu Jawdeh EG, O’Riordan M, Limrungsikul A, et al. Methylxanthine use for apnea of prematurity among an international cohort of neonatologists. *J Neonat Perinat Med.* 2013;6:251–256.
- 55 Zagol K, Lake DE, Vergales B, et al. Anemia, apnea of prematurity, and blood transfusions. *J Pediatr.* 2012;161:417–421.
- 56 Martin RJ, Wang K, Koroglu, O, et al. Intermittent hypoxic episodes in preterm infants: Do they matter? *Neonatology.* 2011;100:303–310.
- 57 Claire N, Bancalari E, D’Ugard C, et al. Multicenter crossover study of automated control of inspired oxygen in ventilated preterm infants. *Pediatrics.* 2011;127: e76–83.
- 58 Alvaro RE, Khalil M, Qurashi M, et al. CO₂ inhalation as a treatment for apnea of prematurity: a randomized double-blind controlled trial. *J Pediatr.* 2012;160:252–257.
- 59 Herlenius E, Aden U, Tang LQ, et al. Perinatal respiratory control and its modulation by adenosine and caffeine in the rat. *Pediatr Res.* 2002;51:4–12.
- 60 Mayer CA, Haxhiu MA, Martin RJ, et al. Adenosine A_{2A} receptors mediate GABAergic inhibition of respiration in immature rats. *J Appl Physiol.* 2006;100:91–97.
- 61 Davis PG, Schmidt B, Roberts RS, et al. Caffeine for apnea of prematurity trial: benefits may vary in subgroups. *J Pediatr.* 2010;156:382–387.
- 62 Schmidt B, Roberts RS, Davis P, et al. Long-term effects of caffeine therapy for apnea of prematurity. *N Engl J Med.* 2007;357:1893–1902.
- 63 Koroglu O, MacFarlane PM, Balan KV, et al. Anti-inflammatory effect of caffeine is associated with improved lung function after lipopolysaccharide-induced amnionitis. *Neonatology.* 2014;106:235–240.
- 64 Weichelt U, Cay R, Schmitz T, et al. Prevention of hyperoxia-mediated pulmonary inflammation in neonatal rats by caffeine. *Eur Respir J.* 2013;41:966–973.
- 65 Desfrere L, Olivier P, Schwendimann L, et al. Transient inhibition of astrocytogenesis in developing mouse brain following postnatal caffeine exposure. *Pediatr Res.* 2007;62: 604–609.
- 66 Dayanim S, Lopez B, Maisonet TM, et al. Caffeine induces alveolar apoptosis in the hyperoxia-exposed developing mouse lung. *Pediatr Res.* 2014;75:395–402.
- 67 Atik A, Cheong J, Harding R, et al. Impact of daily high-dose caffeine exposure on developing white matter of the immature ovine brain. *Pediatr Res.* 2014;76 (1):54–63.
- 68 Back SA, Craig A, Luo NL, et al. Protective effects of caffeine on chronic hypoxia-induced perinatal white matter injury. *Ann Neurol.* 2006;60:696–705.
- 69 Doyle LW, Cheong J, Hunt RW, et al. Caffeine and brain development in very preterm infants. *Ann Neurol.* 2010;68:734–742.
- 70 Patel RM, Leong T, Carlton DP, et al. Early caffeine therapy and clinical outcomes in extremely preterm infants. *J Perinatol.* 2013;33: 134–140.
- 71 Rhein LM, Dobson NR, Darnall RA, et al; Caffeine Pilot Study Group. Effects of caffeine on intermittent hypoxia in infants born prematurely. A randomized clinical trial. *JAMA Pediatr.* 2014;168:250–257.

Alveolarization into Adulthood

Manjith Narayanan

Abstract

Alveolarization has been traditionally thought to be complete in the human lung by three to seven years of age. These estimates were based on traditional morphologic measurements from autopsy specimens. Recently new techniques have been used to estimate airway dimensions and alveolar size and numbers with aerosol deposition and hyperpolarized helium in living subjects. Hyperpolarized helium measurements by magnetic resonance demonstrated increases in alveolar numbers until adulthood, a result that changes concepts about lung growth. Further, this technology applied to school-age children who had been premature infants with bronchopulmonary dysplasia demonstrated catch-up alveolarization. Application of new imaging technologies to different patient groups will likely change concepts about how the lung grows, repairs, and remodels with age.

Keywords:

Lung growth, alveolarization, hyperpolarized helium MR, bronchopulmonary dysplasia, remodeling, repair

Significance of Alveolar Structure and Development

Gas exchange in the lung takes place in the peripheral zone of the lung, which consists of functional units called alveoli. While the structure, function, and development of the proximal conducting units of the lung are well established, the peripheral zone is not as accessible to evaluation. Indeed, the peripheral zone was termed as the quiet zone of the lung by Mead in 1970 (1). However, because the functional units are located in the periphery of the lung, it is important to be able to evaluate them. Structural and functional assessment of the lung periphery is an important prerequisite to understand normal growth and development of the lung, to understand the effect of diseases on the lung periphery, and to design diagnostic measures and therapies for these diseases (2). Indeed, the peripheral zone plays a major role in many of the common diseases of the lung such as chronic obstructive pulmonary disease (COPD) (3), asthma (4), and cystic fibrosis (5).

Since the 1970s, great strides have been made toward understanding the structure of the peripheral zones of the lung. The anatomy of the peripheral lung unit has been reasonably well described using *in vitro* techniques such as histology and electron microscopy. Pediatricians have been interested in the development of the

periphery of the lung to understand the pathogenesis and prognosis of developmental disorders such as chronic lung disease of prematurity (CLD) (6) and congenital diaphragmatic hernia (CDH) (7). Awareness of normal and abnormal lung development has become important to adult respiratory physicians, with new evidence suggesting that COPD may have developmental origins, and that multiple fetal and childhood factors may affect its outcome (8).

Development of Alveoli – Difficulties and Current Concepts

Traditionally, the structure of the lung periphery including alveoli was determined by histological methods. However, the structures of interest are complex, with irregular geometry and are three-dimensional with a natural tendency to collapse if taken out of the thorax. Furthermore, unless properly fixed, taking a section of the lung distorts the architecture. Therefore a complete lung or lobe was necessary to perform “morphometric” analysis of peripheral lung structure (i.e., determination of numbers and dimensions of alveoli) (2). This was a major barrier to histological studies of peripheral lung structures in the human.

Studies of normal peripheral lung development, including alveolar development, required

examination of autopsy specimens of lungs of (previously healthy) children at different age groups. Beyond the age of 5 years, it is clear that it is extremely difficult to obtain appropriate specimens. This is aggravated by restrictions from law and ethical principles. Despite this, many pioneering researchers have studied normal human alveolar development (9–12). The concept based on these studies was that pulmonary alveoli stop multiplying by two to three years of age in humans (13,14), although some authors have suggested seven to eight years as the limit (9,10).

Development of Alveoli – Newer Concepts

Newer developments in histology have improved on the techniques available for evaluating the lung (2). However, normal human alveolar development has not been assessed using the newer techniques. In contrast, animal studies conducted using the newer techniques suggest that alveolarization may continue through the period of physical growth (15,16). Also, newer noninvasive methods to probe peripheral lung structures have been recently developed (17,18), which hold promise in resolving the questions raised by the animal studies. The purpose of this chapter is to review the techniques involved in study of alveolarization and the current knowledge regarding the endpoint of alveolarization in human lung development.

Concepts Regarding Alveolarization in Recent Past

Morphometric Studies

Literally, morphometry means the quantitative analysis of form (Greek: morphe-form, metri-measure). Lung morphometry is the science of determining the number and size of components of the three-dimensional lung by counting the number of transections and the fractional area of these components in a random section of this structure. Credit for developing this technique should go to Weibel, who in his seminal work, *Morphometry of the Human Lung* (19), describes the principles of morphometry and the mathematical method of deriving the number and volume of lung structures from measurement of cross sections.

His method for deriving the number of alveoli in the lung is based on the Delesse theorem. The principle derived by Weibel (9) from the theorem states that the number of structures in a given volume (N) bears a relation to the number of transections through these structures in a random section of the volume (n) as $N = k \cdot n^{3/2}$, where k is a constant depending on the shape of these structures. In case of random sections of the periphery of the lung, Weibel derived the specific form of the preceding equation for counting alveoli in a section of the lung periphery: $N = \frac{n^{3/2}}{\beta \cdot \sqrt{\rho}}$

Here, n , the number of transections, is calculated by counting the number of transections of alveoli in a field of known area, and ρ , the volumetric density of alveoli, is computed by measuring linear intercepts or point counting (9). β is the “shape coefficient,” a variable that relates the mean cross-sectional area of a solid to its volume. In case of polyhedral alveoli, β was estimated to be 1.55 by Weibel.

Timeline of Alveolarization by Morphometry

Studies using morphometric techniques to evaluate the timeline of formation of new alveoli in children were conducted three to five decades ago. Weibel (20) estimated the total number of alveoli (N_A) in five subjects (including an 8-year-old boy and a 16-year-old woman) who died of nonrespiratory causes. The estimated N_A in the two young people was nearly the same as that in the adults (296×10^6 as against 294×10^6). Dunnill (10) examined the lungs of ten children (birth to 8 years) who were term born and died of nonrespiratory causes and compared them with adult lungs. He found that N_A increases with age rapidly at first and then gradually up to at least 8 years of age. By eight years of age the average number of alveoli (280 million) approached the average number in an adult (296 million). Davies and Reid (21) counted N_A in lungs of 5 children who died of nonrespiratory causes from birth to eleven years. The alveolar count increased rapidly from birth (17.3×10^6) to three years (196×10^6) and then gradually increased to 303×10^6 at five years and 336×10^6 at eleven years.

Angus and Thurlbeck (22), in their study of forty-six subjects (14 subjects less than 19 years and 32 adults) attempted to determine the endpoint of human alveolarization by morphometry.

They recognized the large scatter of N_A in human lungs. They postulated that the number of alveoli per unit volume (N_A/V) will not change during alveolar multiplication and then decrease rapidly when alveolarization ceases. Using this approach, they could not demonstrate a time point where alveolarization ceased. They noted that alveolar multiplication contributed more to increase in lung volume with growth than alveolar enlargement.

Hislop et al. (23) examined the lungs of twenty-nine infants from twenty-nine weeks of gestation to eighteen weeks of postnatal life. The aim of this study was to determine the early life increase in alveolar number. N_A increased rapidly from about 20 million at twenty-nine weeks of gestation to 288 million at twelve weeks of age. The rate of increase was fastest in fetal life. According to this study, N_A/V increased up to term and then decreased. This implies that alveolarization proceeds more rapidly than lung volume growth up to term and less rapidly after birth.

Thurlbeck (11) did the most influential study regarding human alveolarization to date. He estimated N_A/V and N_A in the lungs of fifty-six children (age: 6 wks–14 y) dying of nonrespiratory causes using Weibel's technique. These morphometric measures were compared with age, body length, and body weight. Results show wide scatter in N_A in different individuals of similar age. Analysis was performed after grouping individuals by age and estimating the average N_A for each age range. Average N_A in the two- to four-year-olds was found to be similar to that of the seven- to eight-year-olds, and therefore, he concluded that alveolarization was complete by two years of age.

Zeltner and Burri (13) examined alveolar microstructure using scanning and transmission electron microscopy in the lungs of seven children dying from nonrespiratory causes. Many alveolar septae in the seventeen-month-old infant's lungs were immature with a double capillary layer, while those in the sixty-four-month-old showed mature septae, which were thinner and contained a single capillary layer. They postulated that microvascular maturation in alveolar septae takes place at the age of two to three years and contributed to the hypothesis that neo-alveolarization was not possible after this process was complete (14,24).

Drawbacks of the Technique of Morphometry

Practical Difficulties

As briefly alluded to in the introduction, the investigator faces a number of difficulties in attempting to count the number of alveoli in the human lung. First of all, by virtue of the elastic nature of the lung, it is prone to collapse when taken out of the thoracic cavity, and thus volumetric information is lost in lung biopsy specimens. Furthermore, unless properly fixed, a thin section of the lung distorts the architecture. To perform structural analysis of the periphery of the lung, the specimen must first be fixed by instillation of fixative through the airways or the blood vessels (2). Therefore a complete lung or lobe is necessary to prepare samples for lung structure by morphometry. This, clearly limits the availability of lung specimen to postmortem specimen.

Attempting to assess alveolar number in childhood using morphometric analysis is problematic because lung specimens from healthy childhood are difficult to acquire, both because of the inherent low mortality rate in children over five years of age and because of the high incidence of respiratory morbidity and mortality associated with available specimens. In addition, changes in law have made it increasingly difficult to access pathologic specimens for research.

Assessment of peripheral lung development ideally requires serial measures in the same lung. The reliance on autopsy specimen means that serial measures of the anatomy of the lung periphery is not possible. Alveolarization was instead studied by calculating the alveolar numbers in different individuals, which introduced the problem of interindividual variability. Also, determining factors influencing growth and development of the lung periphery has only been possible by using animal models and surrogate markers.

Technical Issues

There have been numerous technical advances in the science of morphometry since Weibel's pioneering work (19). These advances have surmounted various technical issues in morphometry. It is beyond the scope of this chapter to explore these in detail, but the reader is referred to the review by Weibel et al. (25), the

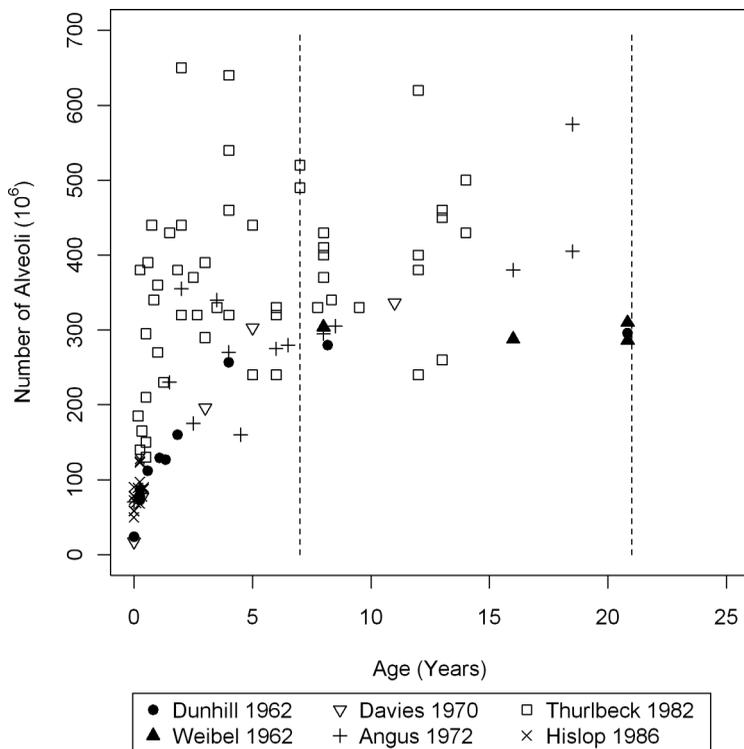


Figure 13-1. Number of alveoli in the developing human lung estimated by morphometry from previously published studies. References: Dunnill 1962(10), Weibel 1962(9), Davies 1970(21), Angus 1972(22), Thurlbeck 1982(12), Hislop 1986(23). Reprinted with permission of the American Thoracic Society. From Narayanan M et al. Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. *Am J Respir Crit Care Med.* 2012 Jan 15;185(2):186–191. Official Journal of the American Thoracic Society. Copyright © 2014 American Thoracic Society.

published scientific debate between experts in the field (26–30), and the official policy statement of American Thoracic Society/European Respiratory Society for quantitative assessment of lung structure (2). I have detailed here some important issues that need to be understood for the evaluation of the published work on human alveolarization.

Compiling the results of the studies exploring alveolarization in children, it is evident that there is a wide variance between studies in both the measured alveolar number and the estimated age of completion of alveolarization (Figure 13-1). The preparation of pathologic specimen is markedly different in different studies. For example, Davies et al. (21) inflated lungs with buffered formalin at 75 cm water pressure, Angus and Thurlbeck (11,22) used inflation pressures of 25 cm water, while Weibel (20) used formalin steam instilled at pressures of 5–10 cm water while the lung was kept inflated by negative pressure. It follows that the degree of alveolar inflation will be different between studies. Despite this, several authors (10,11) have used Weibel's value of 1.55 for the shape coefficient, β (see earlier), despite β being dependent on the relation between surface area and volume.

Another limitation of the studies using morphometric techniques to determine endpoint of alveolarization is that the same inflation pressure was used to inflate lungs of children in different age groups. It has been noted that the range of lung volumes at which fixation occurs is between 50 and 70% of total lung capacity, with the actual value set by individually varying compliance of the thorax (31). This may change the relative degree of inflation of alveoli between the subjects, with a strong likelihood of bias (it is likely that compliance varies with age). The pronounced intersubject scatter (11,22) may also be partially explained by this phenomenon. The assumptions that the shape coefficient, β , and the distribution coefficient of alveoli (i.e., a coefficient relating to standard deviation of the size of individual alveoli) do not vary with age may also be flawed and may lead to a variability of about 20% and 10%, respectively in the results (11).

Measurements by classical morphometry were done on a very small sample (between 1:100000 to 1:1000000) of the alveoli (27). It is essential, in these circumstances, to ensure randomization of sampling. This has been achieved with newer morphometric techniques [e.g.,

design-based stereology as described by Hyde et al. (32,33)]. Unfortunately, the newer techniques (2) have not been used to determine the endpoint of alveolarization in humans.

New Histological Techniques to Determine Alveolar Number

Techniques that surmount the pitfalls mentioned have been developed following the development of the science of stereology and the description of the “disector” by Sterio (34). It is not the remit of this chapter to discuss this technique in detail, but interested readers are referred to excellent reviews on the subject (25,35,36). In brief, the process of counting the number of particles in a specimen starts by successively dividing the fixed specimen into blocks, from which a random number of blocks are selected. The selected blocks are then oriented in a random manner before subdivision into subblocks. A random fraction of these subblocks is then selected, and the process is repeated until sections that can be assessed under a microscope can be prepared. At this stage, random sets of two contiguous sections are assessed, viz., the “sampled section” and the “look-up section” (36). The number of particles that appear in the sampled section, but not the look-up section is calculated, and the number is then summed over all the sampled sections (Q). The number of particles in the whole specimen can then be calculated from Q and the fraction of the specimen assessed under the microscope. In case of counting alveoli in lung specimen, the fact that each alveoli have a single distinct opening is utilized in determining the so-called Euler characteristic of the network of alveolar openings in the periphery of the lung (37,38). It is clear that this technique does not require any assumption of shape or orientation of the alveoli and does not depend on the degree of inflation of the lung (25).

Nonhistologic Techniques to Determine Alveolar Dimensions

³Helium Magnetic Resonance

Over the last decade, tremendous progress has been made to develop safe, noninvasive markers of alveolar structure and/or dimension. One of the main developments is the technique of ³HeMR, which measures the self-diffusion of hyperpolarized

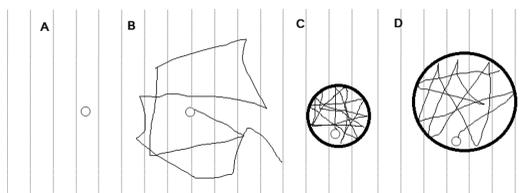


Figure 13–2. Panel A. Hypothetical stationary molecule – Diffusion coefficient $D = 0$. Panel B. Freely diffusing ³He molecule in air, D (free diffusion coefficient) = $0.88 \text{ cm}^2 \cdot \text{sec}^{-1}$. Panel C and D. Diffusion restricted by constraints, that is, alveolar walls in case of inhaled helium. The measured (apparent) diffusion coefficient (ADC) is higher in panel D than panel C and can be used as a marker of alveolar dimension. This representation is a simplification, as in reality, alveoli are not completely spherical, and they are open to alveolar ducts. However, as long as the majority of the constraints on diffusion of ³He are alveolar walls, ADC would represent alveolar dimensions.

³Helium (³He) within the lung during a breath hold using magnetic resonance (MR). A simplified description of the technique is given in the following paragraph. However, readers are referred to the following resources for detailed explanation of the technique (17,18,39,40).

Magnetic resonance (MR) is based on the interaction of atomic nuclei with an unpaired spin (¹Hydrogen in conventional MR; ³Helium and ¹²⁹Xenon in hyperpolarized gas MR) with an external magnetic field (41). The technique utilizes a predetermined “sequence” of radiofrequency pulses to interact with these nuclei. As a result of this interaction, these nuclei emit radiofrequency pulses, which are measured as the “signal”. In diffusion weighted MR, the sequence employed “sensitizes” the nuclei to movement. In other words, the measured signal decreases with the degree of displacement of the nucleus from its original position. The diffusion coefficient of the nucleus (D) can be calculated from the signal. In the case of ³HeMR applied to human lungs, the MR signal is measured during a breath hold after inhalation of a bolus of ³He. The diffusion of ³He in this situation is constrained by alveolar walls, as alveoli are impermeable to the ³He (Figure 13–2). Therefore, the measured value of diffusion of these nuclei, the apparent diffusion coefficient (ADC), is a function of the degree of restriction of diffusion, which in turn depends on the size of the alveoli.

Since the initial description of this technique, many studies have been performed in adults, confirming its utility to detect alveolar damage in emphysema (18,42,43) and in asymptomatic smokers (44). Validation of ADC against

histologic parameters has been carried out in elastase-induced emphysema in rats (45,46) and in rabbits (47) and in explanted COPD affected human lungs (comparing with lungs of donors that were unsuitable for transplant) (48).

Further developments in this field include calculating length scales from the diffusion coefficients of ^3He utilizing differences in the measured signal due to a number of gradient pulses, each of which sensitize ^3He to different degrees of diffusion. Yablonskiy et al. (49,50) used this technique of $^3\text{HeMR}$ and a simplified mathematical model of cylindrical alveolar ducts surrounded by a uniform sleeve of alveoli to estimate the acinar airway diameter (R) and effective alveolar depth (h). These values were validated against histological methods in rats (51) and human lungs (50). Shanbhag described measurements of average displacement (x_{rms}) of the ^3He nuclei using a diffusion probability profile (DPP) calculated from the technique of diffusion-weighted spectroscopy and q-space analysis (52). Two components of these measurements corresponded to diameter of the alveoli and length of airspaces, respectively.

Aerosol-Derived Airway Morphometry (ADAM)

Heyder (53) described a technique of estimating the size of airways using measurement of gravitational deposition of aerosols in airways during a breath-hold following inhalation of boluses of aerosols. This measurement was based on the principle that settling velocity of the aerosol was inversely related to the caliber of the airway in still air (54). Therefore, airway caliber could be estimated (effective airway diameter, EAD) from recovery of aerosol particles on exhalation after a timed breath hold (54,55). The smallest EAD, EAD_{min} , corresponding to the most peripheral zone of the lung, was found to be a reliable estimate of the size of the most distal airspace generation, that is, alveoli (55). Some variables derived from ADAM have been shown to roughly correlate with histologic measures in human and dog lung specimens (56,57).

Relative Merits and Demerits of the Noninvasive Techniques

Both of these noninvasive techniques have the potential to be applied for research into

alveolarization as they do not involve ionizing radiation and are free from other similar ethical concerns. Both these techniques dispense with the problem of sampling, as they effectively sample the whole lung. The respiratory maneuver required for $^3\text{HeMR}$ is easy, as it involves just a short breath hold of three to ten seconds after inhaling the bolus of ^3He from functional residual capacity (FRC). In contrast, ADAM involves breathing in the aerosol to total lung capacity (TLC) followed by a timed breath-hold and exhalation at a constant exhalation velocity.

ADAM measures EAD_{min} , which is suggested to be equivalent to the alveolar diameter. This may be true if the lung is comprised of a perfectly symmetric branching tree, but the airway branching both at conducting level and at acinar level is nonsymmetric, both in terms of angles and relative cross-sectional areas of the daughter segments (58–60). Also, a review of physical principles behind gas mixing and aerosol mixing suggests that aerosol deposition depends on factors other than size of the airspaces (61). These factors are themselves dependent on developmental changes, including increased depth of alveoli and changes in relative flow (61). Therefore, ADAM may not be a suitable tool to evaluate development of alveoli in childhood and adolescence.

The technique of $^3\text{HeMR}$ relies on far fewer assumptions. The extremely high diffusivity of ^3He atoms minimizes problems with nonuniform mixing. Measurements from $^3\text{HeMR}$ have been well validated against histologic measures in both animal and human lungs (see earlier discussion). However, it must be remembered that ADC is not a measure of length but of physical restriction to diffusion. Many calculations of physical dimensions from diffusion restriction of ^3He depend on geometric models and are therefore reliant on the validity of those assumptions (50). x_{rms} does not depend on geometric assumptions (52), but gives a mean displacement of ^3He atoms, which can only be approximated to physical dimensions of alveoli.

Another concept that can affect the interpretation of ADC is “diffusion time”. This is the time available for the ^3He atoms to diffuse in the physically restricted environment of the peripheral lung. If the diffusion time is too short, the diffusion is not restricted, and ADC approximates D , the free diffusion of ^3He atoms in air. If the

diffusion time is too long, the helium atoms undergo minimal net displacement, and ADC tends to zero. Between these extremes (diffusion times of the order of a few milliseconds) the ADC is able to give information about dimensions of alveoli (62). The value of ADC depends on the selection of diffusion time and other factors such as the strength of the external magnetic field and the sequence and magnitude of the gradients employed. Therefore, a control group is necessary in most studies using $^3\text{HeMR}$. As long as the limitations are recognized, $^3\text{HeMR}$ is a potent tool to explore the question of alveolarization in humans.

Early Studies of Alveolar Development Using $^3\text{HeMR}$ and ADAM

Using $^3\text{HeMR}$, Altes et al. (63) measured ADC in twenty-nine healthy subjects ranging from four to thirty years. They reported an increase of ADC with age, which suggests increase in alveolar dimensions with age. Estimation of an expected increase in ADC in the absence of alveolarization was not attempted, and therefore this study did not fully address the question about age of completion of alveolarization.

Zeman et al. (64) used ADAM to determine EAD_{\min} at TLC in fifty-three children and young adults age six to twenty-two years and fifty-nine adults. EAD_{\min} increased with age, and TLC varied as the third power of EAD_{\min} . They postulated that between ages of six and twenty-two, alveoli do not increase in number but expand to cause lung growth. However, the relation of TLC with EAD_{\min} was determined after combining measurements of children and adults. There are also a few reservations with use of this technique to determine alveolar development (see paragraph on relative merits and demerits).

Indirect Measures of Alveolar Dimensions

Measurements of diffusing capacity of carbon monoxide (DL_{CO}), alveolar volume (V_A) and transfer coefficient ($K_{\text{CO}} = \text{DL}_{\text{CO}}/V_A$) are well known physiological measures of alveolar-capillary function. The technique and principles behind these measures are well described in several textbooks and are not elaborated here (65). Some authors have used these measures as surrogates of alveolar growth (66–68). These measures,

however, could be affected by hemoglobin levels, permeability of the alveolar-capillary barrier, ventilation-perfusion mismatch, and differences in pulmonary perfusion (67) and therefore should be interpreted with caution in the context of alveolar development.

Another indirect estimate of average airspace dimension depends on the finding that pressure-volume curves during passive lung deflation were related to the mean size of peripheral airspaces (69). An index of pulmonary distensibility derived from the pressure-volume curves has been shown to be related to morphometrically derived mean linear intercept (70,71). The recoil of thoracic wall has not been taken into account in the derivation, and therefore it is difficult to extrapolate this relationship to measurements in live humans. Also, it is likely that relative contributions to the P-V characteristics may change with age, and therefore, this technique has not been used to evaluate alveolar development.

Alveolarization Until Maturity in Animals

Many recent studies conducted in animals have suggested that new alveoli may continue to form throughout the period of lung growth and even in adulthood. Many of these utilize the new histologic technique mentioned earlier.

Alveolarization Following Pneumonectomy in Adult Animals

Hsia et al. (72) examined peripheral lung structure in the left lungs of five dogs using morphometric techniques five months and sixteen months following right pneumonectomy. Alveolar surface density progressively increases between five and sixteen months postpneumonectomy to reach values approximating the surface density in control dogs. This adaptive response was postulated to be due to initial expansion of alveolar airspaces to fill the thoracic cavity following pneumonectomy followed by septation of enlarged airspaces (i.e. formation of new alveoli). Fehrenbach et al. (73) used the new technique of design-based stereology to assess compensatory lung growth following left pneumonectomy in adult mice. They performed left pneumonectomy in eleven adult mice and determined alveolar numbers in the right lung at day 6 and day 20 postpneumonectomy. By day 20,

the right lung had gained 49% of the total alveoli lost due to removal of the left lung. In the unique situation of pneumonectomy, the residual lung can “regrow” alveoli.

Alveolarization Through the Period of Growth in Rabbits and Rhesus Monkeys

Kovar et al. (16) determined alveolar number by Weibel's morphometric methods in rabbits at various ages from birth to thirty-six weeks of life (adulthood). They found that the alveolar number increases progressively from birth to adulthood, though the rate of new alveolarization decreases with age. Hyde et al. (15) examined the lungs of twenty-six rhesus monkeys at various ages from 4 days to 7.6 years of life (i.e., neonatal period to adulthood: somatic growth complete by about six years) using design-based stereology. The number of alveoli increased significantly with age, throughout the period of somatic growth. Rhesus monkeys are more plausible as models for cessation of alveolarization in humans than rabbits.

Calorie-Related Changes in Alveolar Number

Karlinsky et al. (74) restricted calorie intake by 50% in a group of hamsters for a period of thirty days and compared morphometric data with control hamsters. They found increased mean linear intercept and decreased lung internal surface area in the starved hamsters, suggesting destruction of alveoli related to calorie restriction. Massaro et al. (75) restricted calorie intake in adult mice by 67%. They estimated alveolar number and alveolar dimensions in the calorie-restricted mice using newer histologic techniques (76) and compared alveoli with controls. Alveolar volume increased by 44% within seventy-two hours of calorie restriction. They allowed another group of mice to feed ad-libitum after fifteen days of calorie restriction. Histologic analysis just seventy-two hours after adlibitum feeding showed that alveolar dimensions and number were restored to values prior to calorie restriction. The molecular mechanisms related to these changes were investigated, and the pathway seemed to be associated with mediators associated with apoptosis. A surprising degree of alveolar plasticity in adult mice was revealed – new alveoli could be formed or destroyed based on caloric intake.

Possible Mechanisms of Postmaturity Alveolarization

Schittny et al. (77) determined the progress of alveolarization in Sprague-Dawley rats from four days of life to sixty days (adulthood in these rats) using design-based stereology techniques. They found that new alveolar septae were being formed well into adulthood. Using 3-D synchrotron radiation X-ray tomography, they showed local duplication of single capillary layers in areas of postmaturity septal growth, which indicated a potential mechanism for postmature alveolarization. These studies dispelled the notion that the immature double capillary layers in alveolar walls were a prerequisite for new septation (14,24). Taken together, this information from mammals supports alveolarization beyond early childhood. There is no reason why this could not happen in humans. In their paper, Hyde et al. (15) have called for reconsideration of previous reports of postnatal alveolar development in humans because the previous reports were based on bias-prone techniques. Unfortunately, there have not been any studies on alveolar development in humans using new stereologic techniques. However, recent studies based on noninvasive techniques suggest that new alveoli may continue to form through the period of lung growth in humans.

Alveolarization Until Maturity in Humans

Noninvasive Measurements of Alveolar Dimension During Normal Lung Growth

Narayanan et al. (78) determined ADC and x_{rms} as surrogates of alveolar dimensions using two techniques of $^3\text{HeMR}$ in 109 healthy subjects between seven and twenty-one years. Measurements were also done with different degrees of gentle inflation of the lung in selected subjects. Lung volumes were measured by plethysmography in all subjects. Changes of the $^3\text{HeMR}$ measures during lung growth were compared to changes with lung inflation. Lung inflation was used as a model for lung enlargement without alveolarization, and the comparisons were carried out based on statistical methods. Both variables, ADC and x_{rms} , increased with lung growth, but at a rate significantly less than the expected increase

in the absence of new alveolarization. Based on the statistical model, Narayanan et al. estimated that the observed 3.4-fold increase in FRC between seven and twenty-one years of age was accompanied by a 1.94-fold (95% CI, 1.64–2.30) increase in alveolar number and a 1.75-fold (95% CI, 1.48–2.07) increase in alveolar volume (78). This was the first direct evidence regarding alveolarization to adulthood in humans.

Alveolarization Following Pneumonectomy in Adult Human

Butler et al. (79) assessed measurements derived from $^3\text{HeMR}$ in an adult woman fifteen years after undergoing right pneumonectomy for hilar adenocarcinoma at the age of thirty-three years. The radial dimensions of acinar airways (R) calculated by applying Yablonskiy's model (50) on the $^3\text{HeMR}$ measurements were found to be close to normal ($330 \pm 20 \mu\text{m}$) as against the expected value of $\sim 390 \mu\text{m}$, implying an increase in the number of alveoli. They calculate that there was a 64% increase in the number of alveoli following pneumonectomy.

Recovery of Alveolar Structure Following Preterm Birth

$^3\text{HeMR}$ is an ideal tool to study recovery of lung alveoli following injury. Extreme preterm birth and neonatal chronic lung disease (CLD) are important factors that affect alveolar development. Many studies (80–83) have used histologic techniques to determine peripheral lung structure on lung specimens from children who have died of CLD. Apart from one child who survived up to 7.75 years in Husain's series, all the other human histologic data regarding the lung structure in extreme preterm survivors are from infants who died before forty months of age. Overall, these studies suggest that in children born extremely preterm, disordered peripheral lung development and, consequently, deranged alveolar structure persists until at least three years of age. When considered with the previous concept that human alveolarization was complete by three years, it was assumed that deranged alveolar structure would be a lifelong feature in preterm survivors (84,85).

This reasoning could be challenged for two reasons. First, histologic data are necessarily

limited to the fatal, severe cases of preterm CLD and therefore cannot be generalized to survivors. The second challenge is to the assumption that human alveolarization is complete at three years. Animal models were developed to answer the first challenge. The best known of the animal models was the preterm baboon model developed by Dr. Coalson's team (84,86,87). Despite this pioneering work, there were no data on long-term survivors because the maximum survival reported in the preterm baboons was eight months – a human developmental equivalent of three years. Regarding the second challenge, the assumption has come under increasing scrutiny based on the results with animal models and humans. Narayanan et al. (88) published the first direct proof that alveolar damage sustained due to preterm birth may not be lifelong. They compared measurements related to alveolar dimensions using $^3\text{HeMR}$ in 119 children from ten to fourteen years stratified into four groups (term born, mild preterm, extreme preterm without CLD, and extreme preterm with CLD). ADC was similar in all four groups and was not related to risk factors for CLD, which implied that any derangement in alveolar development due to extreme preterm birth or CLD could be compensated for within the first decade of life in survivors. The intrasubject spread of ADC (SD_{ADC}) was larger in the CLD group, suggesting some subtle residual damage (89). This study had adequate statistical power to detect even small differences in alveolar dimensions in the preterm group. These results strongly support the novel notion that human alveoli have a capacity to regenerate far beyond early childhood.

The Physiologic Rationale for Continued Alveolar Growth

Over the last decade, the possibility that at least some alveolarization occurs beyond early childhood was recognized by authors including Burri (90) and Massaro (91). This concept is supported by many indirect findings from other studies, not necessarily evaluating alveolarization. The findings from animal studies and $^3\text{HeMR}$ discussed earlier reinforce the hypothesis of alveolar development through the period of lung growth. The other indirect findings that support this hypothesis of continued alveolar growth follow.

Metabolic Demand and “the Call for Oxygen”

Tenney et al. (92) measured total alveolar surface area and alveolar diameter in twenty-six representative mammals of diverse body size and metabolic rates. They found that the internal surface area of the lung correlates very well with resting oxygen consumption. Notably, alveolar diameter was found to negatively correlate with body weight-specific oxygen consumption. For example, mammals with high metabolic activity like shrews and bats had the smallest alveoli, and the relatively sluggish mammals like dugong and manatee had the largest alveoli. The relationship was explained by Massaro on the basis of the “call for oxygen” (91). Oxygen delivery can be affected by tidal volume and respiratory rate, but there are physiological limitations to these variables (92).

Sapoval (93) predicted on theoretical considerations that an ideal acinar structure and dimension must exist for ideal gas exchange. It is probable that an ideal alveolar size exists as well, related to metabolic demand. This would make sense from the physical standpoint because oxygen molecules are more likely to encounter alveolar walls during Brownian motion if alveolar walls are closer together (in a smaller alveolus). It is also likely that this ideal dimension is also sensitive to interindividual variations in metabolic demand.

Using design-based stereology in adult human lungs, Ochs et al. (38) showed that alveolar number was closely related to adult lung volume and that mean alveolar size was almost constant between subjects. If alveolarization were completed by two to three years of age, the final number of alveoli, and by extrapolation, the final size of the lung and the final metabolic demand, would have to be set by then, which is implausible.

Alveolar Plasticity

There is evidence from both animal and human studies that alveolar dimensions (and number) may be related to metabolic needs. Massaro et al. (75,94) and Karlinsky et al. (74) showed calorie-related changes in alveolar number in adult mammals. A similar phenomenon has been reported in humans. Coxson (95) compared computed tomographic (CT) images of twenty-one

young adults with anorexia nervosa with images of sixteen age-matched controls (all females). CT measures of attenuation confirmed that the adults with anorexia nervosa had changes similar to emphysematous lungs. Massaro (96) and Coxson (95) hypothesized that these changes were an adaptive response to diminished consumption of oxygen during periods of starvation. Alveolar regeneration after refeeding supports the theory that certain mechanisms must exist for alveolarization to proceed beyond early childhood. Both histologic studies (97) and $^3\text{HeMR}$ studies (98) indicate that alveoli tend to become larger with age in adulthood. This is probably related to declining metabolic needs. Conversely, in a physiological study of elite swimmers, Armour et al. (99) noted increased lung volumes (including vital capacity, total lung capacity, and functional residual capacity) and higher diffusing capacity of carbon monoxide (DL_{CO}) but unchanged diffusion coefficient ($\text{K}_{\text{CO}} = \text{DL}_{\text{CO}}/\text{Alveolar volume}$) and index of pulmonary distensibility compared to controls. Within the limits of the technique, the findings support increased alveolar number in elite swimmers.

Survivors of Preterm Birth

Evidence from $^3\text{HeMR}$ shows that alveolar dimensions in children born very preterm and in survivors of neonatal chronic lung disease are essentially identical to term born children (88). This indicates catch-up of alveolar structure following preterm birth. However, functional respiratory studies in preterm survivors show that preterm infants have lower DL_{CO} at school age (66) and young adulthood (67). DL_{CO} is, however, a test of alveolar–capillary function and is influenced by the permeability of the alveolar–capillary barrier and ventilation–perfusion mismatch, apart from the alveolar surface area (see earlier). Therefore, DL_{CO} can remain abnormal even if alveolar structure has normalized in preterm survivors. This dichotomy between alveolar structure and function is more likely to happen in preterm survivors than in diseases of mature alveolar–capillary units such as COPD. Narang et al. (100) showed that though DL_{CO} is decreased at rest in ex-preterm subjects studied at twenty-one years of age, it normalizes with exercise. Such an improvement with dynamic testing is not plausible with persistent structural damage to

alveoli. This again supports the notion that alveolar structure can recover following preterm birth.

Conclusions and Future Developments

To conclude, it is clear that emerging evidence with newer techniques of measurement of alveoli (both invasive and noninvasive) and other indirect techniques support the theory that new alveoli continue to form through the period of lung growth. It is time for this new paradigm of alveolarization to be disseminated. However, the gold standard “proof” will require replicating histologic studies in normal human lungs using newer stereologic techniques through the period of lung growth. In the absence of such a gold standard study, future studies may utilize the noninvasive

nature of $^3\text{HeMR}$ to conduct longitudinal studies of alveolar dimensions. The theory may also be strengthened if the results described earlier are replicated using measurements of $^3\text{HeMR}$ in growing lungs using various diffusion times. The noninvasive nature of $^3\text{HeMR}$ may also be utilized to test alveolar plasticity by measuring alveolar dimensions before and after influences such as exposure to environmental tobacco smoke or caloric restriction. Measurements from $^3\text{HeMR}$ have potential to be used as noninvasive endpoints if new alveolar therapies are developed in the future. The availability of these new measurement techniques demonstrates the continued growth potential of the healthy alveoli. The critical questions are to determine the mechanisms by which alveolar numbers are regulated and whether knowledge of those mechanisms can be translated to therapies.

References

- Mead J. The lung's “quiet zone.” *N Engl J Med.* 1970 Jun 4;282(23):1318–1319.
- Hsia CC, Hyde DM, Ochs M, Weibel ER. ATS/ERS Joint Task Force on Quantitative Assessment of Lung Structure. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. *Am J Respir Crit Care Med.* 2010 Feb 15;181(4):394–418.
- Calverley PM, Walker P. Chronic obstructive pulmonary disease. *Lancet.* 2003 Sep 27;362(9389):1053–1061.
- Gelfand EW, Kraft M. The importance and features of the distal airways in children and adults. *J Allergy Clin Immunol.* 2009 Dec;124(6 Suppl):S84–87.
- Tiddens HA, Donaldson SH, Rosenfeld M, Pare PD. Cystic fibrosis lung disease starts in the small airways: can we treat it more effectively? *Pediatr Pulmonol.* 2010 Feb;45(2):107–117.
- Baraldi E, Filippone M. Chronic lung disease after premature birth. *N Engl J Med.* 2007 Nov 8;357(19):1946–1955.
- IJsselstijn H, Tibboel D. The lungs in congenital diaphragmatic hernia: do we understand? *Pediatr Pulmonol.* 1998;26(3):204–218.
- Svanes C, Sunyer J, Plana E, Dharmage S, Heinrich J, Jarvis D, et al. Early life origins of chronic obstructive pulmonary disease. *Thorax.* 2010 Jan;65(1):14–20.
- Weibel ER, Gomez DM. A principle for counting tissue structures on random sections. *J Appl Physiol.* 1962 Mar;17:343–348.
- Dunnill MS. Postnatal growth of the lung. *Thorax.* 1962;17:329–333.
- Thurlbeck WM. Postnatal human lung growth. *Thorax.* 1982 Aug;37(8):564–571.
- Thurlbeck WM, Angus GE. Growth and aging of the normal human lung. *Chest.* 1975 Feb;67(2 Suppl):3S–6S.
- Zeltner TB, Burri PH. The postnatal development and growth of the human lung. II. *Morphology-Respir Physiol.* 1987 Mar;67(3):269–82. (0034–5687 (Print)).
- Hislop AA. Airway and blood vessel interaction during lung development. *J Anat.* 2002 Oct;201(4):325–334.
- Hyde DM, Blozis SA, Avdalovic MV, et al. Alveoli increase in number but not size from birth to adulthood in rhesus monkeys. *Am J Physiol Lung Cell Mol Physiol.* 2007 Sep;293(3):L570–579.
- Kovar J, Sly PD, Willet KE. Postnatal alveolar development of the rabbit. *J Appl Physiol.* 2002 Aug;93(2):629–635.
- Mayo JR, Hayden ME. Hyperpolarized helium 3 diffusion imaging of the lung. *Radiology.* 2002 Jan;222(1):8–11.
- Saam BT, Yablonskiy DA, Kodibagkar VD, et al. MR imaging of diffusion of (^3He) gas in healthy and diseased lungs. *Magn Reson Med.* 2000 Aug;44(2):174–179.
- Weibel ER. *Morphometry of the Human Lung.* Berlin: Springer; 1963.
- Weibel ER, Gomez DM. Architecture of the human lung. Use of quantitative methods establishes fundamental relations between size and number of lung

- structures. *Science*. 1962 Aug 24;137:577–585.
- 21 Davies G, Reid L. Growth of the alveoli and pulmonary arteries in childhood. *Thorax*. 1970 Nov;25(6):669–681.
- 22 Angus GE, Thurlbeck WM. Number of alveoli in the human lung. *J Appl Physiol*. 1972 Apr;32(4):483–485.
- 23 Hislop AA, Wigglesworth JS, Desai R. Alveolar development in the human fetus and infant. *Early Hum Dev*. 1986 Feb;13(1):1–11.
- 24 Burri PH. Structural aspects of prenatal and postnatal development and growth of the lung. In: McDonald J, ed. *Lung Growth and Development*. New York: Marcel Dekker; 1997:1–35.
- 25 Weibel ER, Hsia CC, Ochs M. How much is there really? Why stereology is essential in lung morphometry. *J Appl Physiol*. 2007 Jan;102(1):459–467.
- 26 Weibel ER. Morphological quantitation of emphysema: a debate. *J Appl Physiol* (1985). 2006 Apr;100(4):1419–1420; author reply 1420–1421.
- 27 Mitzner W. Morphologic quantification of heterogeneous parenchyma. *J Appl Physiol* (1985). 2006 Apr;100(4):1421–1422.
- 28 Hsia CC. Morphological quantitation of emphysema: a debate. *J Appl Physiol* (1985). 2006 Apr;100(4):1422–1423.
- 29 Fehrenbach H. Morphological quantitation of emphysema: a debate. *J Appl Physiol* (1985). 2006 Apr;100(4):1423–1424.
- 30 Butler JP. Morphological quantitation of emphysema: a debate. *J Appl Physiol* (1985). 2006 Apr;100(4):1424–1425.
- 31 Bachofen M, Bachofen H. Fixation of human lungs. In: Gil J, ed. *Models of Lung Disease: Microscopy and Structural Methods*. New York: Marcel Dekker; 1990:23–36.
- 32 Hyde DM, Tyler NK, Plopper CG. Morphometry of the respiratory tract: avoiding the sampling, size, orientation, and reference traps. *Toxicol Pathol*. 2007;35(1):41–48. (0192–6233 (Print)).
- 33 Hyde DM, Harkema JR, Tyler NK, et al. Design-based sampling and quantitation of the respiratory airways. *Toxicol Pathol*. 2006;34(3):286–295.
- 34 Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. *J Microsc*. 1984 May;134(Pt 2):127–136.
- 35 Mayhew TM, Gundersen HJ. “If you assume, you can make an ass out of u and me”: a decade of the disector for stereological counting of particles in 3D space. *J Anat*. 1996 Feb;188(Pt 1):1–15.
- 36 Kato Y, Takaki R, Toriwaki J, eds. *Stereology of arbitrarily shaped particles: unbiased estimation of number and sizes*. In: *Science on Form: Proceedings of the First International Symposium for Science on Form*. Tokyo: KTK Scientific Publishers; 1986.
- 37 Hyde DM, Tyler NK, Putney LF, Singh P, Gundersen HJ. Total number and mean size of alveoli in mammalian lung estimated using fractionator sampling and unbiased estimates of the Euler characteristic of alveolar openings. *Anat Rec A Discov Mol Cell Evol Biol*. 2004 Mar;277(1):216–26.
- 38 Ochs M, Nyengaard JR, Jung A, et al. The number of alveoli in the human lung. *Am J Respir Crit Care Med*. 2004 Jan 1;169(1):120–124.
- 39 Goodson BM. Nuclear magnetic resonance of laser-polarized noble gases in molecules, materials, and organisms. *J Magn Reson*. 2002 Apr;155(2):157–216.
- 40 Hornak JP. *The Basics of MRI*. Available at: <http://www.cis.rit.edu/htbooks/mri/>. Accessed July 1, 2014.
- 41 Golman K, Olsson LE, Axelsson O, Mansson S, Karlsson M, Petersson JS. Molecular imaging using hyperpolarized ¹³C. *Br J Radiol*. 2003;76(Spec No 2):S118–127.
- 42 Swift AJ, Wild JM, Fichelle S, et al. Emphysematous changes and normal variation in smokers and COPD patients using diffusion ³He MRI. *Eur J Radiol*. 2005 Jun;54(3):352–358.
- 43 Salerno M, de Lange EE, Altes TA, Truwit JD, Brookeman JR, Mugler JP III. Emphysema: hyperpolarized helium ³ diffusion MR imaging of the lungs compared with spirometric indexes—initial experience. *Radiology*. 2002 Jan;222(1):252–260.
- 44 Fain SB, Panth SR, Evans MD, et al. Early emphysematous changes in asymptomatic smokers: detection with ³He MR imaging. *Radiology*. 2006 Jun; 239(3):875–883.
- 45 Chen XJ, Hedlund LW, Moller HE, Chawla MS, Maronpot RR, Johnson GA. Detection of emphysema in rat lungs by using magnetic resonance measurements of ³He diffusion. *Proc Natl Acad Sci U S A*. 2000 Oct 10;97(21):11478–11481.
- 46 Peces-Barba G, Ruiz-Cabello J, Cremillieux Y, et al. Helium-3 MRI diffusion coefficient: correlation to morphometry in a model of mild emphysema. *Eur Respir J*. 2003 Jul;22(1):14–19.
- 47 Mata JF, Altes TA, Cai J, et al. Evaluation of emphysema severity and progression in a rabbit model: comparison of hyperpolarized ³He and ¹²⁹Xe diffusion MRI with lung

- morphometry. *J Appl Physiol*. 2007 Mar;102(3):1273–1280.
- 48 Woods JC, Choong CK, Yablonskiy DA, et al. Hyperpolarized 3He diffusion MRI and histology in pulmonary emphysema. *Magn Reson Med*. 2006 Dec;56(6):1293–1300.
- 49 Yablonskiy DA, Sukstanskii AL, Leawoods JC, et al. Quantitative in vivo assessment of lung microstructure at the alveolar level with hyperpolarized 3He diffusion MRI. *Proc Natl Acad Sci U S A*. 2002 Mar 5;99(5):3111–3116.
- 50 Yablonskiy DA, Sukstanskii AL, Woods JC, et al. Quantification of lung microstructure with hyperpolarized 3He diffusion MRI. *J Appl Physiol*. 2009 Oct;107(4):1258–1265.
- 51 Jacob RE, Minard KR, Laicher G, Timchalk C. 3D 3He diffusion MRI as a local in vivo morphometric tool to evaluate emphysematous rat lungs. *J Appl Physiol* (1985). 2008 Oct;105(4):1291–1300.
- 52 Shanbhag DD, Altes TA, Miller GW, Mata JF, Knight-Scott J. q-Space analysis of lung morphometry in vivo with hyperpolarized 3He spectroscopy. *J Magn Reson Imaging*. 2006 Jul;24(1):84–94.
- 53 Heyder J. Charting human thoracic airways by aerosols. *Clin Phys Physiol Meas*. 1983 Feb;4(1):29–37.
- 54 Brand P, Rieger C, Beinert T, Heyder J. Aerosol derived airway morphometry in healthy subjects. *Eur Respir J*. 1995 Oct;8(10):1639–1646.
- 55 Zeman KL, Bennett WD. Measuring alveolar dimensions at total lung capacity by aerosol-derived airway morphometry. *J Aerosol Med*. 1995 Summer;8(2):135–147.
- 56 Blanchard JD, Heyder J, O'Donnell CR, Brain JD. Aerosol-derived lung morphometry: comparisons with a lung model and lung function indexes. *J Appl Physiol* (1985). 1991 Oct;71(4):1216–1224.
- 57 Nikiforov AI, Lippmann M, Palmes ED. Validation of an in vivo Aerosol Probe Technique by measurements of deposition and morphometry in excised human lungs. *Ann Occup Hyg*. 1988;32(inhaled particles VI):33–39.
- 58 Horsfield K. Diameters, generations, and orders of branches in the bronchial tree. *J Appl Physiol* (1985). 1990 Feb;68(2):457–461.
- 59 Parker H, Horsfield K, Cumming G. Morphology of distal airways in the human lung. *J Appl Physiol*. 1971 Sep;31(3):386–391.
- 60 Horsfield K, Cumming G. Functional consequences of airway morphology. *J Appl Physiol*. 1968 Mar;24(3):384–390.
- 61 Tsuda A, Henry FS, Butler JP. Gas and aerosol mixing in the acinus. *Respir Physiol Neurobiol*. 2008 Nov 30;163(1–3):139–149.
- 62 FICHELE S, PALEY MN, WOODHOUSE N, GRIFFITHS PD, VAN BEEK EJ, WILD JM. Measurements and modeling of long range 3He diffusion in the lung using a “slice-washout” method. *J Magn Reson*. 2005 May;174(1):28–33.
- 63 Altes TA, Mata J, de Lange EE, Brookeman JR, Mugler JP III. Assessment of lung development using hyperpolarized helium-3 diffusion MR imaging. *J Magn Reson Imaging*. 2006 Dec;24(6):1277–1283.
- 64 Zeman KL, Bennett WD. Growth of the small airways and alveoli from childhood to the adult lung measured by aerosol-derived airway morphometry. *J Appl Physiol*. 2006 Mar;100(3):965–971.
- 65 Cotton DJ, Graham BL. Single-breath carbon monoxide diffusing capacity or transfer factor. In: Hamid Q, Shannon J, Martin J, eds. *Physiologic Basis of Respiratory Disease*. Hamilton, Canada: BC Decker Inc.; 2005:659–669.
- 66 Hakulinen AL, Jarvenpaa AL, Turpeinen M, Sovijarvi A. Diffusing capacity of the lung in school-aged children born very preterm, with and without bronchopulmonary dysplasia. *Pediatr Pulmonol*. 1996 Jun;21(6):353–360.
- 67 Vrijlandt EJ, Gerritsen J, Boezen HM, Grevink RG, Duiverman EJ. Lung function and exercise capacity in young adults born prematurely. *Am J Respir Crit Care Med*. 2006 Apr 15;173(8):890–896.
- 68 Balinotti JE, Chakr VC, Tiller C, et al. Growth of lung parenchyma in infants and toddlers with chronic lung disease of infancy. *Am J Respir Crit Care Med*. 2010 May 15;181(10):1093–1097.
- 69 Colebatch HJ, Greaves IA. Exponential analysis of lung elastic behavior. *Am Rev Respir Dis*. 1980 May;121(5):898–899.
- 70 Haber PS, Colebatch HJ, Ng CK, et al. Alveolar size as a determinant of pulmonary distensibility in mammalian lungs. *J Appl Physiol*. 1983 Mar;54(3):837–845.
- 71 Greaves IA, Colebatch HJ. Elastic behavior and structure of normal and emphysematous lungs post mortem. *Am Rev Respir Dis*. 1980 Jan;121(1):127–136.
- 72 Hsia CC, Herazo LF, Fryder-Doffey F, Weibel ER. Compensatory lung growth

- occurs in adult dogs after right pneumonectomy. *J Clin Invest*. 1994 Jul;94(1):405–412.
- 73 Fehrenbach H, Voswinckel R, Michl V, et al. Neoalveolarisation contributes to compensatory lung growth following pneumonectomy in mice. *Eur Respir J*. 2008 Mar;31(3):515–522.
- 74 Karlinsky JB, Goldstein RH, Ojserkis B, Snider GL. Lung mechanics and connective tissue levels in starvation-induced emphysema in hamsters. *Am J Physiol*. 1986 Aug;251(2 Pt 2):R282–288.
- 75 Massaro D, Massaro GD, Baras A, Hoffman EP, Clerch LB. Calorie-related rapid onset of alveolar loss, regeneration, and changes in mouse lung gene expression. *Am J Physiol Lung Cell Mol Physiol*. 2004 May;286(5):L896–906.
- 76 Massaro GD, Massaro D. Formation of alveoli in rats: postnatal effect of prenatal dexamethasone. *Am J Physiol*. 1992 Jul;263(1 Pt 1):L37–41.
- 77 Schittny JC, Mund SI, Stampanoni M. Evidence and structural mechanism for late lung alveolarization. *Am J Physiol Lung Cell Mol Physiol*. 2008 Feb;294(2):L246–254.
- 78 Narayanan M, Owers-Bradley J, Beardsmore CS, et al. Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. *Am J Respir Crit Care Med*. 2012 Jan 15;185(2):186–191.
- 79 Butler JP, Loring SH, Patz S, Tsuda A, Yablonskiy DA, Mentzer SJ. Evidence for adult lung growth in humans. *N Engl J Med*. 2012 Jul 19;367(3):244–247.
- 80 Bonikos DS, Bensch KG, Northway WH Jr, Edwards DK. Bronchopulmonary dysplasia: the pulmonary pathologic sequel of necrotizing bronchiolitis and pulmonary fibrosis. *Hum Pathol*. 1976 Nov;7(6):643–666.
- 81 Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. *Hum Pathol*. 1998 Jul;29(7):710–717.
- 82 Sobonya RE, Logvinoff MM, Taussig LM, Theriault A. Morphometric analysis of the lung in prolonged bronchopulmonary dysplasia. *Pediatr Res*. 1982 Nov;16(11):969–972.
- 83 Hislop AA, Wigglesworth JS, Desai R, Aber V. The effects of preterm delivery and mechanical ventilation on human lung growth. *Early Hum Dev*. 1987 May;15(3):147–164.
- 84 Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. *Am J Respir Crit Care Med*. 1999 Oct;160(4):1333–1346.
- 85 Eber E, Zach MS. Long term sequelae of bronchopulmonary dysplasia (chronic lung disease of infancy). *Thorax*. 2001 Apr;56(4):317–323.
- 86 Coalson JJ, Winter VT, Gerstmann DR, Idell S, King RJ, DeLemos RA. Pathophysiologic, morphometric, and biochemical studies of the premature baboon with bronchopulmonary dysplasia. *Am Rev Respir Dis*. 1992 Apr;145(4 Pt 1):872–881.
- 87 Coalson JJ, Winter V, deLemos RA. Decreased alveolarization in baboon survivors with bronchopulmonary dysplasia. *Am J Respir Crit Care Med*. 1995 Aug;152(2):640–646.
- 88 Narayanan M, Beardsmore CS, Owers-Bradley J, et al. Catch-up alveolarization in ex-preterm children: evidence from (3)He magnetic resonance. *Am J Respir Crit Care Med*. 2013 May 15;187(10):1104–1109.
- 89 Jobe AH. Good news for lung repair in preterm infants. *Am J Respir Crit Care Med*. 2013 May 15;187(10):1043–1044.
- 90 Burri PH. Structural aspects of postnatal lung development-alveolar formation and growth. *Biol Neonate*. 2006;89(4):313–322.
- 91 Massaro D, Massaro GD. Invited Review: pulmonary alveoli: formation, the “call for oxygen,” and other regulators. *Am J Physiol Lung Cell Mol Physiol*. 2002 Mar;282(3):L345–358.
- 92 Tenney SM, Remmers JE. Comparative quantitative morphology of the mammalian lung: diffusing area. *Nature*. 1963 Jan 5;197:54–56.
- 93 Sapoval B, Filoche M, Weibel ER. Smaller is better—but not too small: a physical scale for the design of the mammalian pulmonary acinus. *Proc Natl Acad Sci U S A*. 2002 Aug 6;99(16):10411–10416.
- 94 Massaro GD, Radaeva S, Clerch LB, Massaro D. Lung alveoli: endogenous programmed destruction and regeneration. *Am J Physiol Lung Cell Mol Physiol*. 2002 Aug;283(2):L305–309.
- 95 Coxson HO, Chan IH, Mayo JR, Hlynsky J, Nakano Y, Birmingham CL. Early emphysema in patients with anorexia nervosa. *Am J Respir Crit Care Med*. 2004 Oct 1;170(7):748–752.
- 96 Massaro D, Massaro GD. Hunger disease and pulmonary alveoli. *Am J Respir Crit Care Med*. 2004 Oct 1;170(7):723–724.
- 97 Gillooly M, Lamb D. Airspace size in lungs of lifelong non-

- smokers: effect of age and sex. *Thorax*. 1993 Jan;48(1):39-43.
- 98 Waters B, Owers-Bradley J, Silverman M. Acinar structure in symptom-free adults by Helium-3 magnetic resonance. *Am J Respir Crit Care Med*. 2006 Apr 15;173(8):847-851.
- 99 Armour J, Donnelly PM, Bye PT. The large lungs of elite swimmers: an increased alveolar number? *Eur Respir J*. 1993 Feb;6(2):237-247.
- 100 Narang I, Bush A, Rosenthal M. Gas transfer and pulmonary blood flow at rest and during exercise in adults 21 years after preterm birth. *Am J Respir Crit Care Med*. 2009 Aug 15;180(4):339-345.

Physiologic Assessment of Lung Growth and Development Throughout Infancy and Childhood

Anne-Marie Gibson, Sarath Ranganathan, and Lex W. Doyle

Abstract

Several lung function tests may be used for the physiologic assessment of lung growth and development throughout infancy and childhood. Optimal lung function tests for monitoring cystic fibrosis, bronchopulmonary dysplasia, and recurrent wheezing in children less than 6 years of age have been recently reported, and studies where infant and preschool lung function has been applied in these specific respiratory disorders have been reviewed. Normal reference ranges for older subjects, including into adulthood, have also been reported.

When interpreting physiologic measures of lung growth and development throughout infancy and childhood, it is important to be aware of the influence of growth and maturity, the influence of demographic factors such as sex and ethnicity, the normal intra- and interindividual variability of the parameters at each age, and the diagnostic value of each of the parameters obtained in each test.

Very preterm (< 32 weeks gestational age) or very low birth weight (<1500 g birth weight) survivors, particularly those who had bronchopulmonary dysplasia in the newborn period, have more lung function abnormalities, particularly airway obstruction, than do term-born survivors and are at high risk of adult obstructive lung disease as they grow older.

Keywords:

Airway obstruction, bronchopulmonary dysplasia, lung diffusion capacity, lung function, very preterm, very low birth weight

In this chapter, we describe the major lung function tests that may be used for the physiologic assessment of lung growth and development throughout infancy and childhood. Lung function tests can be used to detect and quantify lung disease, monitor the progress of lung diseases, and assess the effectiveness of any treatments. Some of the lung function tests described in this chapter hail from tests performed in adults that have been adapted for application in infants and small children, but many are specifically created for use in children. We will also describe some of the lung function tests applicable to school-age children and the results for very preterm or low-birth-weight children, particularly those who had bronchopulmonary dysplasia (BPD) in the newborn period.

Respiratory Function in Infants and Small Children

Lung function testing in newborns or other infants in an intensive care setting presents particular

challenges, especially when interpreting the results. These challenges relate to instability of the infants' clinical status, intubation altering the airway mechanics, and air leaks around uncuffed endotracheal tubes. Moreover, the heterogeneous nature of newborn or infant intensive care populations, with large differences in maturity levels and body size, and in indications for requiring respiratory support, all increase the variability in results.

Generally lung function assessments in infants and small children have been restricted to specialist pediatric respiratory units and have used equipment made on site to suit a specific application. Although comparisons of the data from different centers may be difficult, the studies have led to a greater understanding of lung growth and development during infancy and the preschool years. More recently, commercialization of equipment has allowed its use to become more widespread. Over the last decade the American Thoracic Society (ATS) and the European Respiratory Society

(ERS) have published recommendations for lung function testing in infants and preschool children. These guidelines, along with the increasing availability of standardized equipment, will allow for more direct comparisons of research findings to be made and also allow for improved collaboration between centers, both within the same region and internationally.

Lung function assessments in infants are often carried out in a supine position during quiet sleep, sometimes following sedation, such as with chloral hydrate. Therefore testing in infants is inherently different to volitional lung function testing performed in upright, awake preschool and older children.

Lung function measurements in preschool children present a unique set of difficulties compared with testing infants or older children. Preschool children are too old to sedate for lung function and are not generally cooperative during lung function testing. Many of the standard lung function tests such as spirometry require active participation and the ability to follow specific instructions and are therefore limited to children age 5 years and older. There are, however, several studies reporting the use of standard lung

function tests, such as spirometry, in this younger cohort. The success rates tend to be low, and as the complexity of the tests increases, the success rate decreases further. Different strategies have been developed to assess lung function in this age group. Difficulties in testing preschool children have meant that development of lung function tests within this age group has lagged behind that in infants and older children. Recently standardized equipment has become available commercially, and therefore it is expected that research and development with lung function testing within this age group will increase, as was the case following similar developments with infant lung function testing.

The different tests that are possible in preschool children include measurements of tidal breathing, forced expiratory flow-volume, whole body plethysmography, functional residual capacity and ventilation homogeneity by gas dilution techniques, and lung diffusing capacity.

Tidal Breathing Measurements

Measurements of tidal breathing can be made in infants and small children who are not able to actively perform forced expiratory maneuvers. Tidal breathing can be measured directly by a flow meter, such as a pneumotachograph or ultrasonic flow meter, attached to a mask applied to the patient's face, or indirectly by attaching chest and abdominal bands, such as respiratory inductive plethysmography predominantly used during sleep studies (Figure 14-1) (1). Tidal breathing measured by either technique will produce a tidal flow-volume loop (Figure 14-2). The flow-volume loops produced by tidal breathing measures may be useful for recognition of bronchial obstruction that may be seen in preterm infants with BPD, but are not useful for the early diagnosis of respiratory diseases such as cystic fibrosis (2). The most

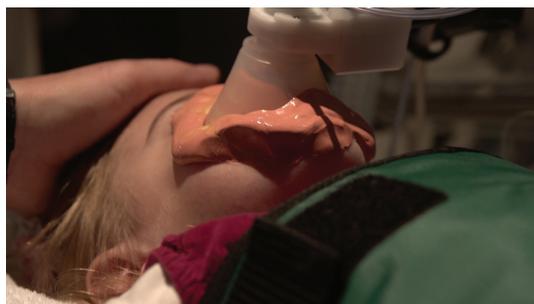


Figure 14-1. Photo of a child having infant lung function testing. The child has been sedated with chloral hydrate and has had a mask with pneumotachograph attached to their face using putty to prevent leaks.

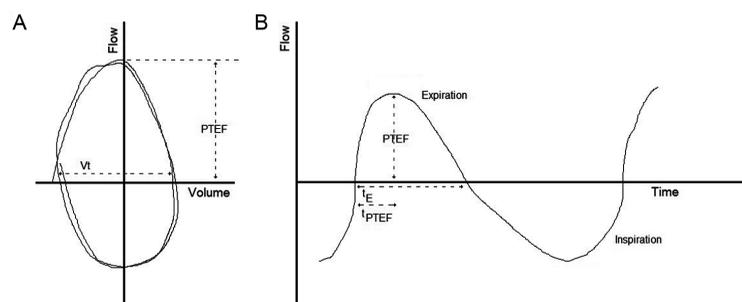


Figure 14-2. Tidal breathing flow-volume traces. Panel (A) shows tidal volume as flow versus volume. Panel (B) represents tidal volume as flow versus time. PTEF: peak tidal expiratory flow, t_E : expiratory time, t_{PTEF} time to reach PTEF.

commonly reported tidal volume variable is the ratio of the time to reach peak flow to total expiratory time (t_{PEF}/t_E) (Figure 14-2), which is reduced in airway obstruction, such as may occur in wheezy infants. Tidal breathing variables are relatively easy to maintain even in an unsedated infant. They may contribute to the overall clinical picture as part of a more complete assessment in an infant with respiratory disease and therefore provide an aid to diagnosis and monitoring, as well as providing an outcome measure for research purposes.

Detailed information on recommendations for tidal breathing testing and analysis are provided in a document published by the ERS/ATS task force on standards for infant respiratory function testing (3). This paper provides recommendations about software and equipment requirements when analyzing tidal breathing measurements in infants. The guidelines also cover terminology and definitions, equipment, data acquisition and analysis, and reporting of tidal breathing results and highlight areas in which further research is needed before consensus can be reached. There have been many efforts to establish normal reference data for tidal volume variables in healthy infants, with varying success, and therefore the physiological implications and the clinical or diagnostic value of most tidal breathing measurements remain limited (1).

Forced Expiratory Flow-Volume Measurements

In older children and adults who are able to actively cooperate, forced expiratory flows, such as those measured by spirometry, may be used for the evaluation, monitoring, and management of respiratory diseases. By altering the techniques and modifying the equipment a version of these tests can be carried out in infants and preschool children. Forced expiratory flows can be measured in infants using a flow meter attached to a face mask and performing either forced deflation or rapid thoracoabdominal compression (RTC; a squeeze) from tidal or raised lung volumes. Forced deflation is relatively invasive and requires the infant to be intubated and a negative pressure applied, which is why it is not routinely used in a clinical setting (4). The RTC technique requires the application of external pressure, or a “squeeze,” to the thoracoabdominal area and is

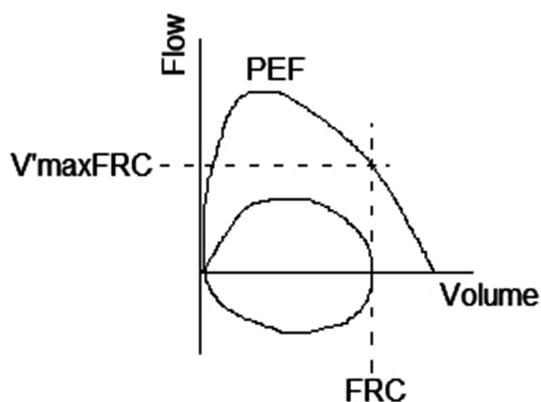


Figure 14-3. $V_{\max FRC}$ flow-volume trace. PEF: Peak expiratory flow; FRC: Functional residual capacity; $V_{\max FRC}$: Maximal flow rate at FRC.

usually accomplished by wrapping the infant with an inflatable jacket or vest. Rapid inflation of the jacket or vest results in a rapid thoracoabdominal squeeze to empty the infant’s lungs. These measurements are taken during the normal tidal breathing cycle and therefore are only able to describe forced expiratory flows during the limited tidal volume.

The most commonly reported measurement from the RTC is maximal expiratory flow at functional residual capacity, $V_{\max FRC}$ (Figure 14-3). The shape of the peak expiratory curve also gives an indication of airway function. As with spirometry, a convex curve generally reflects normal airway caliber whereas a concave curve may indicate airway obstruction. Despite several attempts by different researchers, there are still no widely accepted normal reference data available, which is related to differing equipment, ethnicity, socioeconomic, and age-related factors (4).

Another RTC technique involves augmenting the infant’s breath during inspiration to a set inspiratory inflation pressure, thus increasing the breath above normal tidal volumes prior to the rapid squeeze; this technique is called raised volume rapid thoracoabdominal compression (RVRTC). The RVRTC provides expiratory flow information over a greater volume, from near total lung capacity to near residual volume and for this reason is sometimes referred to as infant spirometry. The jacket pressure, or amount of squeeze applied is incrementally increased to a point where further increases do not result in higher flow rates, that is, flow limitation is achieved.

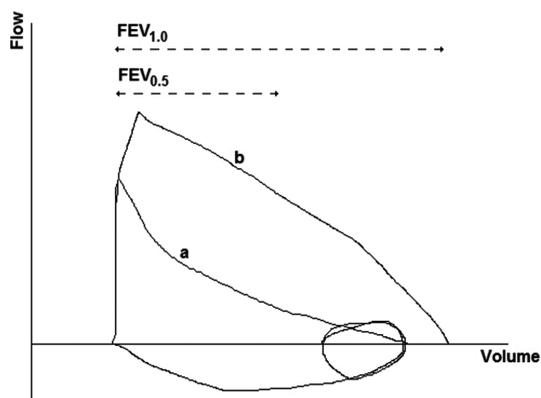


Figure 14-4. RVRTC flow-volume trace. $FEV_{1.0}$: Forced expiratory flow at 1 second during a forced expiration; $FEV_{0.5}$: Forced expiratory flow at 0.5 seconds during a forced expiration. Loop a demonstrates lower inspiratory and jacket (squeeze) pressures; loop b demonstrates higher inspiratory and jacket pressures, nearing flow limitation.

The most commonly reported measurements are similar to those from traditional spirometry and include forced vital capacity (FVC) and forced expiratory volumes at 0.5, 0.75, and 1 s during the forced expiration ($FEV_{0.5}$, $FEV_{0.75}$, and FEV_1 , respectively). Calculation of FEV_1 and FVC are not usually feasible, as expiratory times are too short in the infants. Measurements representing forced expiratory flow are also reported and include forced expiratory flow at 50, 75, and 85% of FVC (FEF_{50} , FEF_{75} , and FEF_{85} , respectively). As with spirometry performed in older children and adults, the volume-time measurements are more reproducible than the flow-volume measurements. As with the tidal volume RTC technique, the shape of the forced expiratory flow-volume curve provides information on potential airway obstruction (Figure 14-4). Detailed information on recommendations for RVRTC measurement and analysis are provided in a document published by the ERS/ATS task force on standards for infant respiratory function testing (5).

Infant Whole-Body Plethysmography

Quantifying lung volume is important for assessing overall lung growth and development throughout infancy and childhood. Most of the other infant lung function tests are volume dependent, for example, if lung volumes are lower, then $FEV_{0.5}$ may be lower. The only static



Figure 14-5. A photo of a child within a whole-body plethysmograph, attached to the pneumotachograph via a face mask.

lung volume measured during infant lung function is FRC. FRC represents the amount of air left within the lungs at the end of a normal tidal breath, and it includes residual volume. FRC can be measured by whole-body plethysmography (FRC_{pleth}) or by an inert gas washout technique. Infant whole-body plethysmography equipment is available commercially, potentially allowing widespread application of the technique. Whole-body plethysmography requires the infant to lie in a closed, rigid clear container; the plethysmograph. The lid of the container is designed so that the infant can be monitored through the test and is easily accessible if necessary. Within the container a flow meter is attached to the infant, usually via face mask (Figure 14-5).

FRC measured in the whole-body plethysmograph is based on Boyle's law, which states that with constant temperature the product of pressure and volume of a fixed mass of gas will be constant (4,6). During testing the flow meter is occluded briefly, during which time the infant makes respiratory efforts against the shutter, which compresses and expands the gas within the remaining lung volume, that is, FRC_{pleth} (4,6). The change in alveolar pressure, measured at the mouth or airway opening, is assumed to represent alveolar volume, which reflects changes in the box pressure within the plethysmograph, and is used to calculate lung volume during the occlusion (4, 6). An abnormally elevated FRC_{pleth} may be due to hyperinflation, and abnormally low values may indicate restriction. Airway resistance (R_{aw}) is also measured during this test and is the pressure difference that must be applied between alveoli and the external atmosphere to produce a gas flow of $1 \text{ L}\cdot\text{s}^{-1}$ at the airway opening (4,6). The advantage of plethysmographic assessment of

R_{aw} is that it can be measured throughout the respiratory cycle and thus reflect dynamic conditions (4,6). Several other variables can be derived from these measurements: airway conductance (G_{aw}) is the reciprocal of R_{aw} ; specific resistance (sR_{aw}) is the product of R_{aw} and FRC ($sR_{aw} = R_{aw} \cdot FRC$) and can be determined from tidal breathing without the need for airway occlusions (4,6). A standardization document discussing plethysmographic measurements of lung volume and airway resistance in infants was published in 2000 as a result of a joint task force of the ERS/ATS and details recommendations relating to equipment requirements, study procedures, and reporting of data for plethysmographic measurements in infants (7).

Measurement of Functional Residual Capacity and Ventilation Inhomogeneity by Gas Dilution Techniques

Measurement of ventilation inhomogeneity is a rapidly growing area in pediatric respiratory physiology. An essential requirement for effective ventilation is the efficient mixing of inspired gas with the resident gas within the lungs. If this gas mixing is inhomogeneous or inefficient, ventilation will change to ensure adequate gas exchange. The airways change with descent through many divisions from the initial, larger, more proximal conducting zones where gas flow is achieved by conduction (airway generations 1–16), down to areas of the lungs where gas movement occurs by diffusion, the smaller more peripheral acinar zone, where gas exchange occurs. Lung structure, especially in the periphery, airway resistance, and lung compliance, all influence gas-mixing efficiency. Even healthy lungs will have ventilation inhomogeneity. In a normal lung, therefore, some degree of ventilation inefficiency will be present, but if there is airway obstruction, either generalized or in a particular region, but especially in the peripheral airways, the distribution of ventilation will be uneven, with a reduction in gas mixing efficiency and gas trapping. This may be the case in infants with cystic fibrosis (2). The multiple breath washout (MBW) tests assess the efficiency of gas distribution and mixing within the lungs.

Commercial systems are available that measure inert gas washout or gas dilution and can be

used in infants. Newer methods are able to measure breath-by-breath gas concentrations and allow for more complex analyses that provide data on overall gas mixing efficiency within the lungs, that is, the inhomogeneity of ventilation (8). The more time it takes to wash-in or wash-out an inert gas, the less efficient is the ventilation. These tests are noninvasive and are performed during tidal breathing.

The most commonly reported variable is the lung clearance index (LCI). The LCI is calculated as the cumulative expired volume needed to lower end-tidal marker gas concentration to 1/40th of the starting concentration divided by FRC, that is, the number of lung volume turnovers needed to clear the marker gas from the lungs. A technique called moment analysis can be used to quantify the degree of inhomogeneity of ventilation distribution as described by the inert gas washout curve. Moment ratios describe the area under the gas washout curve. The higher the moment ratios, the more skewed is the washout curve, which indicates that a greater portion of the lungs is slowly ventilated. Consequently, the LCI will become higher as the lungs must be ventilated for a longer time, and the moment ratios become higher as more of the marker gas leaves the lung late during the washout. Although these simple tests are highly sensitive to airway pathology, particularly obstruction of the peripheral airways, they do not give any information about the mechanisms behind inhomogeneity or where along the airway tree obstruction has occurred.

More complex analysis using the slopes generated during normal tidal breathing suggests that MBW may allow the area of ventilation inefficiency to be localized within the conducting airways (Scond), or within the acinar or gas exchanging zone of the lungs (Sacin). There is a zone within the lungs where ventilation by convection and ventilation by diffusion meet; this zone is known as the diffusion–convection front. Different disease processes occurring within the lungs may move this diffusion–convection front peripherally or proximally and thus alter the Scond and Sacin dependent on where the disease is affecting ventilation efficiency. Phase III slopes are a plot of a single breath measured during the MBW test and show the expired volume of the breath along the x -axis and the concentration of expired gas as the expiration of that breath continues, in this case nitrogen concentration

(Figures 14-6 and 14-7). There are different phases throughout the expired breath; first a very low expired gas concentration (phase I), then a rapid rise (phase II), followed by a plateau in the expired gas concentration (phase III).

Phase I is the apparatus and airway dead space, phase II is the transition, and phase III

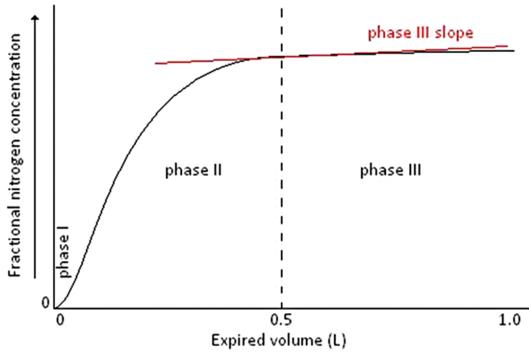


Figure 14-6. A single breath washout curve from the MBW test illustrating phase I, phase II, phase III, and the alveolar phase III slope (red)

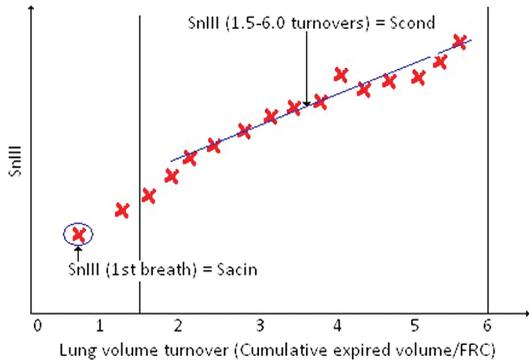


Figure 14-7. Phase III slope analysis illustrating the calculation of Scnd and Sacin.

is known as the alveolar plateau and equates to approximately 65 to 95% of the tidal volume of an expired breath. The phase III slope is calculated by regression over this region of the expired breath and is the change in gas concentration over that tidal volume. To compare these slopes over the entire MBW is not possible as the expired gas concentration of each breath decreases as the MBW progresses; therefore the phase III slope is normalized for gas concentration; this is known as the normalized phase III slope or SnIII. The SnIII values for each breath of the MBW test can then be plotted against the lung volume turnover (cumulative expired volume/FRC; Figure 14-8). Scnd- and Sacin are derived from analysis of concentration SnIII of a multiple breath inert gas washout. The original work by Paiva and Engel provides in-depth description of this theory (9,10). In subjects with marked inhomogeneity occurring as a result of convection-dependent mechanisms the SnIII will increase steadily during the washout, as may be seen in cystic fibrosis (4). This is the result of uneven ventilation among the conducting airways.

Thompson et al. speculated that the Scnd value might be the result of the remodeling process that occurred in chronic asthma that did not respond to treatment, and the functional reflection of airway inflammation, within asthmatic airways (11). Further research is required before such speculative interpretations regarding structure-function relationships in the peripheral airways and acinus can be confirmed.

The ability to measure small airway function is important in a number of disease processes, for example, cystic fibrosis, asthma, and the lungs of preterm infants, all of which may result in

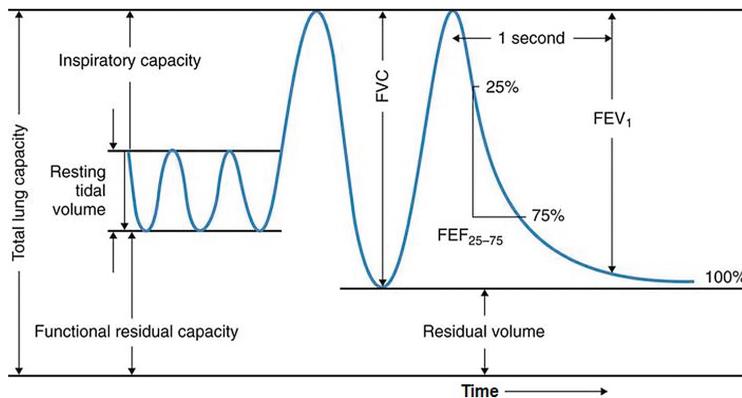


Figure 14-8. Spirogram showing volume on the vertical axis and time on the horizontal axis.

changes within the small airways or acinar air spaces affecting airflow or ventilation efficiency within the airways. The small airway function variables measured in spirometry, such as FEF_{25-75%} or forced expiratory flow at 50% of FVC (FEF_{50%}), may be reduced in the presence of small airways disease, but there is mounting evidence that they are not sensitive or specific to disease processes occurring in small airways or airspaces, especially in the presence of airflow obstruction in the larger conducting airways or gas trapping within the lungs (11–16).

A standardization document published jointly by the ERS and ATS provides guidelines for washout equipment specifications, test performance, and analysis for both infant and adult populations and outlines the important theoretical background and principles essential for understanding gas washout techniques (17).

Lung Diffusing Capacity

Gas exchange is the primary function of the lung, and many lung diseases impair gas exchange. Respiration occurs externally between alveolar gas and pulmonary capillary blood and internally between systemic capillary blood and tissue. Measurements of arterial blood gas reflect the balance between internal and external respiration (6). Ventilation comprises not only gas involved in gas exchange (alveolar ventilation) but also gas that does not take part in gas exchange (dead-space ventilation). The balance between alveolar ventilation and carbon dioxide (CO₂) production is shown in the arterial partial pressure of CO₂ (PCO₂) (6). The arterial partial pressure of oxygen (PO₂) shows the adequacy with which the pulmonary blood flow is oxygenated by the lungs (6).

Diffusion is limited by the surface area over which diffusion occurs, capillary blood volume, hemoglobin concentration, and the properties of the lung parenchyma, for example, alveolar-capillary membrane thickness and/or the presence of excess fluid in the alveoli (6). The total lung volume is not involved in gas exchange. Most gas exchange occurs as a function of diffusion, not bulk flow. The role of ventilation is to provide bulk flow of gas with the ambient air and to provide a constant gradient for oxygen and carbon dioxide (6,18,19). Spirometry measures various parts of bulk flow, whereas diffusing capacity measures

the forces at work in molecular movement with the oxygen concentration gradient from the alveolar surface through to the hemoglobin molecule. The test, diffusing capacity of the lung, commonly uses carbon monoxide as the tracer gas for measurement because carbon monoxide has a high affinity for binding to the hemoglobin molecule (6). This allows a measurement of pure diffusion because the movement of the carbon monoxide only depends on the properties of the diffusion barrier and the amount of hemoglobin (6).

Diffusing capacity of the lung for carbon monoxide (DL_{CO}) is the measure of carbon monoxide transfer (19). In Europe, it is frequently called the transfer factor of carbon monoxide (TL_{CO}). The commonly used clinical tests to measure DL_{CO} are based on a ratio between the uptake of carbon monoxide in milliliters per minute divided by the average alveolar pressure of carbon monoxide at standard temperature and pressure, dry, per minute (STDP) (18).

Measurement of alveolar volume (VA) and pulmonary DL_{CO} can provide a functional assessment of the volume and surface area available for gas exchange, which indirectly estimates the alveolar number and size (20). In subjects from around late school age to adulthood, DL_{CO} and VA increase with somatic growth, such as height. These physiologic results are consistent with morphometric data that parenchymal lung growth occurs in this age range primarily by the increasing size of the existing alveoli (20,21). Factors that influence the diffusing capacity of the lung include increased ventilation-perfusion mismatch, reduction of alveolar surface area for gas exchange, decreased density of pulmonary capillaries, and a reduction in lung capillary blood flow (22). Interstitial lung diseases may result in abnormal DL_{CO} long before spirometry or lung volume abnormalities are evident. Reduced DL_{CO} is not only an abnormality of restrictive interstitial lung disease but also can occur in emphysema. Therefore other obstructive processes that mostly affect the airways can have similar spirometry, but a reduced DL_{CO} implies a loss of alveolar surface area consistent with emphysema.

The most commonly used and standardized technique to measure DL_{CO} is the single-breath breath-holding technique (18). In this test, a subject breathes in a known volume of tracer gas that typically contains 10% helium, 0.3% carbon monoxide, 21% oxygen, and the balance nitrogen (18).

The subject breathes in the tracer gas and holds the breath for 10 seconds. The subject then exhales to wash out the mechanical and anatomic dead space, after which an alveolar sample is collected. DL_{CO} is calculated from the total volume of the lung, breath-hold time, and the initial and final alveolar concentrations of carbon monoxide. The exhaled helium concentration is used to estimate a single-breath estimate of total lung capacity and the initial alveolar concentration of carbon monoxide (6,18). The driving pressure is assumed to be the calculated initial alveolar pressure of carbon monoxide (6). The calculated DL_{CO} is a product of the patient's single-breath estimate of total lung capacity multiplied by the rate of carbon monoxide uptake during the 10-second breath hold (6). This timed breath hold presents problems when attempting the measurement in infants, preschool children, and younger school-age children. A standardization document discussing single-breath determination of carbon monoxide uptake in the lung was published in 2005 as a result of a joint task force of the ERS/ATS and details recommendations relating to equipment requirements, study procedures, and reporting of data for plethysmographic measurements in infants (18).

Techniques for the Measurement of Lung Function in Preschool and School-Age Children

The level of cooperation required for many standard lung function tests means their application within the preschool group is difficult. Recent advances and development of commercially available equipment have led to an increase in the number of centers testing this age group. This will in turn lead to an increased knowledge of the practicalities involved in testing this age group.

Pulmonary function testing plays a key role in the diagnosis and management of chronic pulmonary conditions, such as asthma and cystic fibrosis, in children over 6 years of age.

However, objective physiologic assessments have a limited role in the care of infants and children under 6 years of age, due to the challenges of measuring lung function in these young patients. A number of lung function techniques have been developed and evaluated among

children less than 6 years of age in the research setting and show promise as safe, feasible, and potentially useful clinical tests.

Preschool children present a number of special challenges. The children are generally too old to sedate for pulmonary function testing (PFT), as is done with infants, and measurement of lung function under anesthesia is neither ethically acceptable nor physiologically relevant to clinical management. Children in this age group are not able to voluntarily perform many of the physiological maneuvers required for the pulmonary function tests used in older children and adults. They have a short attention span and are easily distracted. Due to these issues, the children need to be engaged and encouraged by the operator to participate in the test.

Spirometry

Spirometry is commonly used to assess lung function in older children and adults, and there are several reasons why it is an appealing technique to apply to the preschool population. Most patients, especially those age 6 years and older, can easily perform spirometry when coached by an appropriately trained technician. The indications for spirometry are diverse; it can be used for diagnosing and monitoring respiratory symptoms and disease, for preoperative risk stratification, and as a tool in epidemiologic and other research studies. Spirometry is a voluntary maneuver in which a seated patient inhales maximally from tidal breathing to total lung capacity (TLC), and then rapidly exhales to the fullest extent until no further volume is exhaled at residual volume (RV) (Figure 14-8). The maneuver may be performed in a forceful manner to generate FVC.

Preschool children can successfully perform spirometry to identify disease states and track lung function over time. As with other lung function testing, equipment and testing conditions must be appropriate, and coaching must be provided by a skilled and experienced respiratory physiologist. Loeb et al. showed that the percentage of acceptable and repeatable spirometry increased with age, rising above 50% by age six, and reached a plateau with approximately 85% success at age ten (23). They also showed the most common unmet criteria for an unacceptable study among preschool

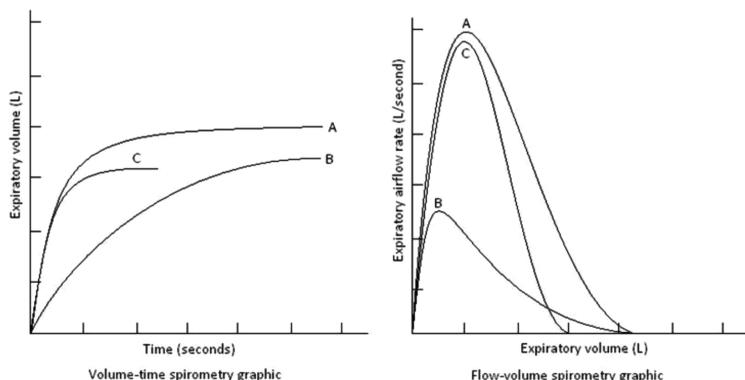


Figure 14-9. Spirometry patterns in various pulmonary disorders compared with normal, displayed as volume-time and flow-volume graphics. A – represents a normal pattern; B – represents an obstructive pattern; C – could either represent the pattern seen from preschool lung function or a restrictive disorder.

children was glottic closure and nonmaximal efforts, while in school-age children it was failure to plateau (23). Recent advances in computer technology have allowed the introduction of visual incentive and interactive computer animation to aid the achievement of reliable and reproducible results in the preschool age group.

A spirogram is a graphic representation of air movement shown as a volume-time tracing or as a flow-volume tracing (Figure 14-9). Values produced in a spirogram provide important graphic and numeric data about the mechanical properties of the lungs, including airflow (FEV_1 , forced expiratory volume in 1 second) and exhaled lung volume. The measurement is normally expressed in liters for volumes or in liters per second for flows and is corrected for body temperature and pressure (BPTS) of gas that is saturated with water vapor. Data from a spirogram show important patterns that help distinguish obstructive pulmonary disorders that typically reduce airflow, such as asthma, from restrictive disorders that typically reduce total lung volumes, for example pulmonary fibrosis.

Abnormal spiograms are usually categorized as restrictive or obstructive impairments. An obstructive component implies airway obstruction, characterized by reduced expiratory flow rates. A restrictive pattern suggests a condition in which vital capacity (volume) is diminished. This must be distinguished from obstructive disease, diagnosed by measurement of normal or increased total lung capacity and decreased flow rates. The variables measured in spirometry are usually used to identify an obstructive pattern; the FEV_1 is the most studied because it is easy to measure, is

reproducible, and is sufficiently sensitive (23–26). This measurement is reduced in obstructive and restrictive disorders. In obstructive diseases, FEV_1 is decreased disproportionately to the FVC, reducing the FEV_1/FVC ratio indicating airflow limitation. In restrictive disorders, the FEV_1 , FVC, and total lung capacity are all decreased, and the FEV_1/FVC ratio is normal or even high (27,28). FVC is a measure of lung volume and is typically reduced in diseases that cause the lungs to be smaller or reduce the amount of air a subject can inhale. These processes are generally termed restrictive and can include disorders of the lung parenchyma, such as pulmonary fibrosis, or of the ability to inhale, for example muscular weakness.

However, a reduced FVC is not always due to reduced total volumes and can be due to severe airflow obstruction and air trapping in large hyperinflated lungs, as in emphysema. When this occurs, the FVC is reduced because of reduced airflow, air trapping, and increased residual volume. Reduced FVC can occur despite a normal or increased total lung volume. Therefore, FVC is not a reliable indicator of total lung capacity or restriction, especially in the setting of airflow obstruction.

The shape of the flow-volume curve can indicate the location of airflow limitation, for example, the large upper airways or smaller airways (Figure 14-9). With common obstructive airflow disorders, such as asthma, the disease usually affects the expiratory loop and can reduce the effort-dependent peak expiratory flow as well as subsequent airflows that are independent of effort. The expiratory loop is typically concave in this instance. In contrast, several anatomic disorders that narrow the large airways can produce a variety

of patterns of shortening or flattening of either the expiratory or inspiratory loop of the curve (variable upper airway obstruction) or both loops of the curve (fixed upper airway obstruction). Other measurements reflect small airways, such as measures of flow from a spirogram, like the maximal midexpiratory flow (MMEF) or forced expiratory flow at 25% to 75% vital capacity ($FEF_{25-75\%}$). The $FEF_{25-75\%}$ is the slope of the spirogram when between 25% and 75% of the FVC has been expired. The $FEF_{25-75\%}$ is a more sensitive early indicator of airway obstruction than FEV_1 , but it is less reproducible (25). The ATS/ERS have produced guidelines that outline acceptability and reproducibility criteria, although based mostly on spirometry when performed in adults (25) and a modification specific to spirometry measured in preschool children (29).

Reference Equations for Ventilatory Function Measurements in Children

Reference Data in Infants and Preschool Children

The lack of availability of reference data is a significant limitation when interpreting infant lung function. Acquiring measurements from healthy individuals is problematic due to the usual requirement for sedation. Where reference data exist, they have often become obsolete as commercial systems are developed and the measurements have changed. Currently, no appropriate reference data are available for the RTC or RVRTC technique or plethysmographic measurements. In contrast, reference data for the inert gas multiple breath washout techniques are imminent. In preschool children, reference data exist for gas-mixing techniques, but may be device specific (30).

In contrast to other lung function tests, excellent reference data are available for spirometry as a result of the superlative work of the Global Lungs Initiative. Results were collated from over 57,000 individuals from 72 centers in 33 countries spanning the age range from 3 to 95 years (31). These reference data are cross-sectional in nature so still remain problematic for interpretation of longitudinal results but still provide a de facto reference population for spirometry and will enhance interpretation of spirometry measurements at all ages and in individuals of various ethnicities.

Clinical Application of Pulmonary Function Testing in Preterm Children

Differences in FEV_1 Between Preterm and Control Subjects

From the limited data available for very preterm preschool children, those with BPD have persistent reductions in airflow and higher airway resistance compared with those who did not have BPD and compared with control subjects (32–34). Figure 14-10 shows some of the studies with values for FEV_1 reported from early childhood through to adulthood for preterm survivors compared with controls, usually of normal birth weight or born at term (35–54). Some studies in Figure 14-10 predate the widespread use of surfactant into clinical practice, with cohorts born before 1990, whereas other studies comprise births from more recent years, when surfactant was freely available. The ages of the subjects range from 7 to 8 years through to the late twenties. Some are regional cohorts, with the intent being to assess as many survivors as possible from a defined geographical region, which reduces selection biases and hence makes any results more widely applicable. Other studies are highly selective, sharing characteristics such as having been ventilated or having had BPD in the newborn period. The applicability of the results from these latter studies to all preterm survivors is less clear than for complete geographical cohorts. Other characteristics of those selected for study, such as birth weight or gestational age, also vary widely.

The numbers of preterm subjects in individual studies vary from as few as 29 to as high as 240. Some have reported results as percent predicted for age, height, and sex; others have reported results as *z*-scores for age, height, and sex. Regardless of the basis for the results, if mean, SD, and sample size have been reported for both groups, it is possible to pool the data and calculate overall standardized mean differences and 95% confidence intervals, contrasting preterm survivors with controls.

Most individual studies have reported significant reductions in FEV_1 in preterm subjects compared with controls (Figure 14-10). The results are mostly consistent, regardless of the differences in the demographic characteristics and other features between the various studies, including whether the cohorts were born before or after surfactant was available. In the pooled analysis, the mean

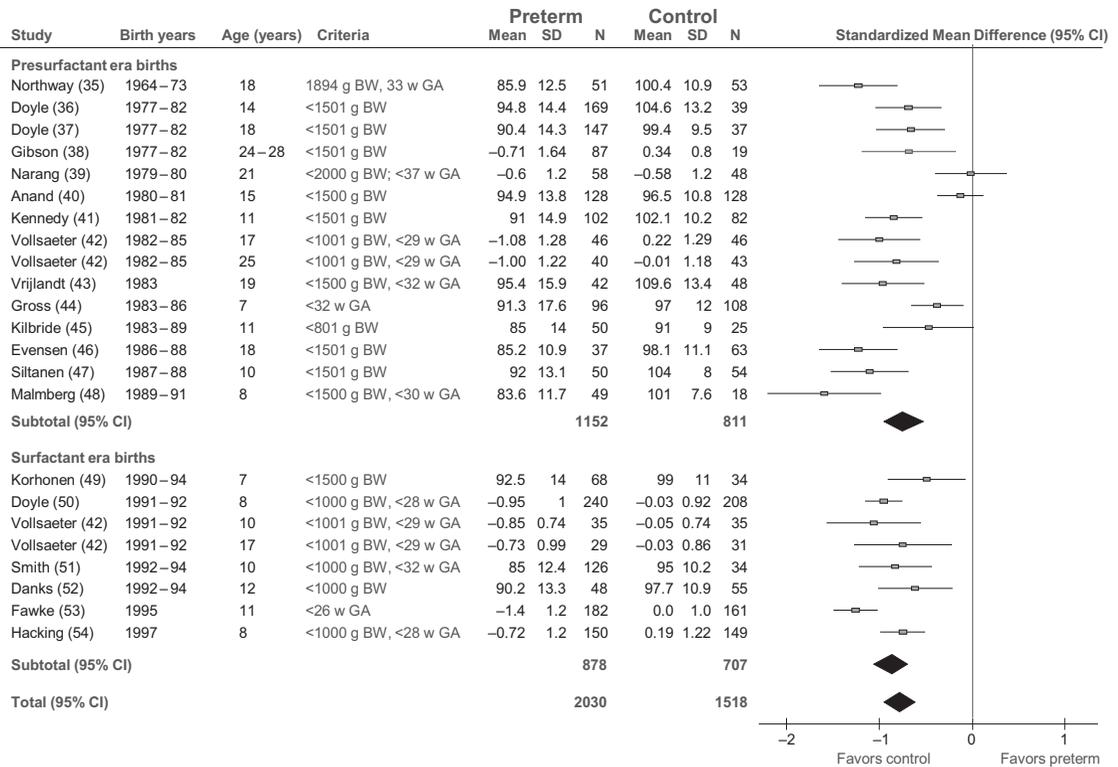


Figure 14-10. Standardized mean differences for FEV₁ compared between preterm groups and controls, contrasting births before and after surfactant was available. Original values for individual studies are either percent predicted or Z-scores. Studies are ordered by year of birth of the participants, and are referenced.

reduction in standardized mean difference for the FEV₁ in the preterm groups compared with controls was -0.79 (95% CI -0.94, -0.63; 23 studies; 3,548 participants). The pooled mean differences between preterm and control groups were not significantly different for studies reporting outcomes in the presurfactant era (-0.76 (95% CI -0.97, -0.54; 15 studies; 1,963 participants) compared with the surfactant era (-0.87 (95% CI -1.05, -0.68; 8 studies; 1,585 participants). The largest difference between preterm and term-born subjects from the surfactant era was from the regional study that included only survivors born <26 weeks gestation, with the standardized mean difference of -1.26 SD between groups (53). There were no obvious trends over time within either subgroup, with the studies ordered by increasing years of birth of the subjects.

Changes with Increasing Age of the Subjects

Several of the studies reported results from the same cohort, but at different ages. In one of these studies, from survivors of birth weight <1501 g

born in 1977–1982 from a single hospital, the mean standardized differences between preterm subjects and controls at 14, 18, and 25 to 28 years of age were almost the same (-0.69 (36), -0.66 (37), and -0.68 (38) SD, respectively). In contrast, in another study that reported on survivors <1001 g birth weight or <29 weeks gestational age from a regional cohort in Norway, results between 17 and 25 years for subjects born in 1982–85, and between 10 and 17 years for subjects born in 1991–92 showed an improvement over time, with the mean standardized difference between preterm subjects and controls falling from -1.00 to -0.82 for those born in 1982–85, and from -1.06 to -0.75 for those born in 1991–92 (42).

Changes between Eras Within the Same Region

Several of the studies reported results from similar subjects born within the same region, but in different eras, with little evidence for a substantial change over time. In one of these studies, from survivors <1000 g birth weight or <28 weeks' gestation born in the state of Victoria at 8 years

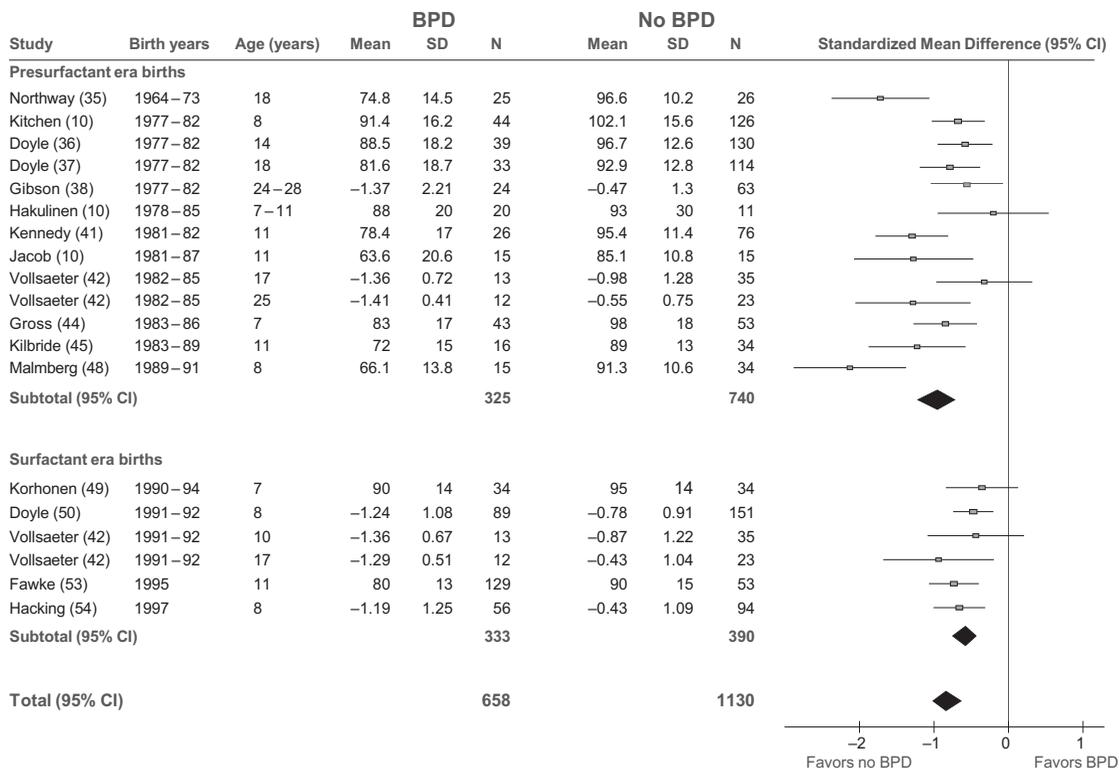


Figure 14-11. Standardized mean differences for FEV₁ within preterm survivors comparing BPD with no BPD, contrasting births before and after surfactant was available. Values for individual studies are either percent predicted or Z-scores. Studies are ordered by year of birth of the participants and are referenced.

of age, the mean standardized differences between preterm subjects and controls were -0.95 for those born in 1991–92 (50) and -0.75 for those born in 1997 (54). In the other study of survivors <1001 g birth weight or <29 weeks gestational age from a regional cohort in Norway, mean standardized differences between preterm subjects and controls for subjects born in 1982–85 were -1.00 for 17-year-olds and -0.82 for 25-year-olds, and for subjects born in 1991–92 were -1.06 for 10-year-olds and -0.75 for 17-year-olds, respectively (42).

Differences in FEV₁ Between BPD and no BPD Groups Among Preterm Survivors

Within preterm groups, those who had BPD in the newborn period had even more reductions in FEV₁ compared with those who did not have BPD in most individual studies (35–38,41,42,44,45,48–50,53–57), with an overall standardized mean difference of -0.81 (95% CI -0.99, -0.63; 19 studies, 1,788 participants)

between groups (Figure 14-11). The reductions with BPD were larger in the presurfactant era (standardized mean difference -0.95 [95% CI -1.20, -0.69; 13 studies, 1,065 participants]) than in the surfactant era (standardized mean difference -0.58 [95% CI -0.73, -0.42; 6 studies, 723 participants]). There were no obvious trends with time in either subgroup.

Changes with Increasing Age of the Subjects

Results from one study suggest that airway obstruction may increase as survivors with BPD grow older (42). In that study of survivors <1001 g birth weight or <29 weeks gestational age from a regional cohort in Norway, mean standardized differences between BPD and no BPD preterm subjects became larger, from -0.32 for 17-year-olds to -1.28 for 25-year-olds born in 1982–85, and from -0.44 for 10-year-olds to -0.93 for 17-year-olds born in 1991–92 (42). However, in another study from survivors of birth weight <1501 g born in 1977–1982 from a single hospital, the mean standardized differences between BPD and no

BPD preterm subjects were more stable over a longer period of time with similar mean differences at 8 (−0.68) (55), 14 (−0.58) (36), 18 (−0.79) (37), and 25–28 (−0.56 SD) (38) years of age.

The linear relationships between FEV₁ measurements at 7 years of age and those at 21 years of age were reported to be strong, with 34% of variance explained, in one cohort of 58 subjects born <2000 g and <37 weeks gestation (39). In this study the relationships were not reported separately for those who did and those who did not have BPD in the newborn period. In another study of survivors born <1501 g, the relationships between FEV₁ measurements obtained at 24 to 28 years of age compared with earlier ages became stronger as the subjects aged and were even stronger in those who had BPD in the newborn period compared with those who did not (38). In this latter study, for those without BPD, the percent of variance explained in FEV₁ measurements at 24 to 28 years with those made earlier was 34% at 8 years, 43% at 11 years, 61% at 14 years, and 77% at 18 years. For those with BPD, the percent of variance explained in FEV₁ measurements at 24 to 28 years with FEV₁ measurements collected earlier was 58% at 8 years, 98% at 11 years, 83% at 14 years, and 82% at 18 years (38).

Changes Between Eras Within the Same Region

Several of the studies reported FEV₁ results from similar subjects born within the same region, but in different eras, with some evidence of change over time. The first study from Northway reported differences between BPD and non-BPD 17-old survivors of −0.32 SD for those born in 1982–85, the presurfactant era, compared with −0.93 SD for those born in 1991–92, the surfactant era (42). In the other study from survivors <1000 g birth weight or <28 weeks gestation born in the state of Victoria at 8 years of age, the mean standardized differences between BPD and no BPD preterm subjects were −0.47 for those born in 1991–92 (50) and −0.66 for those born in 1997 (54). Both of these latter cohorts were born during the surfactant era.

Other Differences in Lung Function Between Preterm Survivors and Controls

In addition to changes in the FEV₁, there are several studies that report changes in other variables consistent with airflow obstruction in the

smaller airways (reduced FEF_{25–75%}) and air trapping (raised RV/TLC) in very preterm survivors, especially in those who had BPD (38,42,53,58,59). In addition to overall reductions in airflow variables, very preterm survivors have higher proportions with values in clinically important ranges; Fawke and colleagues reported that 32% of the subjects born < 26 weeks gestational age and 66% of those with BPD had results that were clinically important (53). Despite having more abnormal lung function on average and higher proportions with values in clinically important ranges, most very preterm survivors are asymptomatic. However with the expected decline in lung function with aging from the midtwenties onward, it is anticipated that disproportionately more preterm survivors than controls will present with symptoms of chronic obstructive airways disease as they grow older, particularly those who survived with BPD.

Several studies have shown preterm subjects; especially those with BPD have reduced diffusion capacity within their lungs from infancy (20) through childhood (49,57,60,61) and young adulthood (43). There is evidence that preterm subjects have increased airway reactivity compared to controls, in addition to obstruction. In these studies preterm subjects showed increased bronchoconstriction when exposed to methacholine and histamine airway challenges and increased bronchodilator response, especially in those with BPD (39,53,62,63). Airflow impairment seen in preterm cohorts may be associated with this increased airway reactivity.

Impaired diffusion and air trapping within the lungs also occur more frequently in preterm survivors compared with controls, and the impairment continues into early adulthood. Northway et al. reported increased airway resistance, increased air trapping, and reduced ventilation efficiency in a preterm cohort with BPD at 18 years of age compared with controls (35). Vrijlandt et al. demonstrated persistent airflow obstruction, air trapping, and reduced lung diffusion in 19-year-old preterm survivors (43).

Conclusions and Future Directions

In this chapter we have described several lung function tests that may be used for the physiologic assessment of lung growth and development throughout infancy and childhood. In 2013 the

ATS published a workshop report that addresses the optimal lung function tests for monitoring cystic fibrosis, BPD, and recurrent wheezing in children less than 6 years of age and reviews recent studies where infant and preschool lung function has been applied in these specific respiratory disorders (2). Normal reference ranges for older subjects, including into adulthood, have also been reported (31). When interpreting physiologic measures of lung growth and development throughout infancy and childhood, it is important to be aware of the influence of growth and maturity, the influence of demographic factors, such as sex and ethnicity, the normal intra- and interindividual variability of the measurements at each age, and the diagnostic value of each of the variables for each test (3).

Very preterm or very low-birth-weight survivors, particularly those who had BPD in the

newborn period, have more lung function abnormalities, particularly airway obstruction, than do term-born survivors and are at high-risk of adult obstructive lung disease with aging. More studies are needed to determine whether early studies of lung imaging and pulmonary function tests can better identify infants who are at risk for poor respiratory outcomes throughout the life span. Further development of noninvasive methods to assess lung growth throughout the life cycle will provide greater opportunities to develop insights into factors that adversely or favorably modulate long-term respiratory outcomes. These physiological measurements need to be performed in parallel with the new noninvasive imaging techniques to better understand lung growth and function during childhood.

References

- Baldwin DN, Pillow JJ, Stocks J, Frey U. Lung-function tests in neonates and infants with chronic lung disease: tidal breathing and respiratory control. *Pediatr Pulmonol.* 2006;41:391–419.
- Rosenfeld M, Allen J, Arets BH, et al. An official American Thoracic Society workshop report: optimal lung function tests for monitoring cystic fibrosis, bronchopulmonary dysplasia, and recurrent wheezing in children less than 6 years of age. *Ann Am Thorac Soc.* 2013;10:S1–S11.
- Bates JH, Schmalisch G, Filbrun D, Stocks J. Tidal breath analysis for infant pulmonary function testing. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/American Thoracic Society. *Eur Respir J.* 2000;16:1180–1192.
- Hammer J, Eber E. *Paediatric Pulmonary Function Testing. Progress in Respiratory Research.* Bolliger CT, ed. Basel, Switzerland: Karger; 2005.
- American Thoracic S, European Respiratory S. ATS/ERS statement: raised volume forced expirations in infants: guidelines for current practice. *Am J Respir Crit Care Med.* 2005;172:1463–1471.
- West JB. *Respiratory Physiology: The Essentials.* 9th ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2012.
- Stocks J, Godfrey S, Beardsmore C, Bar-Yishay E, Castile R; ERS ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/American Thoracic Society. Plethysmographic measurements of lung volume and airway resistance. *Eur Respir J.* 2001;17:302–312.
- Robinson PD, Latzin P, Gustafsson PM. *Multiple-breath washout.* Sheffield, UK: European Respiratory Society Journals Ltd; 2010.
- Paiva M, Engel LA. Theoretical studies of gas mixing and ventilation distribution in the lung. *Physiol Rev.* 1987;67:750–796.
- Paiva M, Engel LA. The anatomical basis for the sloping N2 plateau. *Respir Physiol.* 1981;44:325–337.
- Thompson BR, Douglass JA, Ellis MJ, et al. Peripheral lung function in patients with stable and unstable asthma. *J Allergy Clin Immunol.* 2013;131:1322–1328.
- Hasegawa M, Nasuhara Y, Onodera Y, et al. Airflow limitation and airway dimensions in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2006;173:1309–1315.
- Corsico A, Milanese M, Baraldo S, et al. Small airway morphology and lung function in the transition from normality to chronic airway obstruction. *J App Physiol.* 2003;95:441–447.
- Hansen JE, Sun XG, Wasserman K. Discriminating measures and normal values for expiratory obstruction. *Chest.* 2006;129:369–377.
- Verbanck S, Paiva M, Schuermans D, Hanon S, Vincken W, Van Muylem A. Relationships between the lung clearance index and conductive

- and acinar ventilation heterogeneity. *J App Physiol*. 2012;112:782–790.
- 16 Verbanck S, Thompson BR, Schuermans D, et al. Ventilation heterogeneity in the acinar and conductive zones of the normal ageing lung. *Thorax*. 2012;67:789–795.
- 17 Robinson PD, Latzin P, Verbanck S, et al. Consensus statement for inert gas washout measurement using multiple- and single-breath tests. *Eur Respir J*. 2013;41:507–522.
- 18 Macintyre N, Crapo RO, Viegi G, et al. Standardisation of the single-breath determination of carbon monoxide uptake in the lung. *Eur Respir J*. 2005;26:720–735.
- 19 Crapo RO, Forster RE II. Carbon monoxide diffusing capacity. *Clin Chest Med*. 1989;10:187–198.
- 20 Balinotti JE, Chakr VC, Tiller C, et al. Growth of lung parenchyma in infants and toddlers with chronic lung disease of infancy. *Am J Respir Crit Care Med*. 2010;181:1093–1097.
- 21 Hislop AA. Airway and blood vessel interaction during lung development. *J Anatomy*. 2002;201:325–334.
- 22 Carlsen KC, Haland G, Carlsen KH. Natural history of lung function in health and diseases. *Curr Opin Allergy Clin Immunol*. 2009;9:146–150.
- 23 Loeb JS, Blower WC, Feldstein JF, Koch BA, Munlin AL, Hardie WD. Acceptability and repeatability of spirometry in children using updated ATS/ERS criteria. *Pediatr Pulmonol*. 2008;43:1020–1024.
- 24 Piatti G, Fasano V, Cantarella G, Tarantola C. Body plethysmographic study of specific airway resistance in a sample of healthy adults. *Respirology*. 2012;17:976–983.
- 25 Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. *Eur Respir J*. 2005;26:319–338.
- 26 Nielsen KG, Bisgaard H. Discriminative capacity of bronchodilator response measured with three different lung function techniques in asthmatic and healthy children aged 2 to 5 years. *Am J Respir Crit Care Med*. 2001;164:554–559.
- 27 Pellegrino R, Viegi G, Brusasco V, et al. Interpretative strategies for lung function tests. *Eur Respir J*. 2005;26:948–968.
- 28 Miller A. Lung function testing: selection of reference values and interpretative strategies. *Am Rev Respir Dis*. 1992;146:1368–1369.
- 29 Beydon N, Davis SD, Lombardi E, et al. An official American Thoracic Society/European Respiratory Society statement: pulmonary function testing in preschool children. *Am J Respir Crit Care Med*. 2007;175:1304–1345.
- 30 Lum S, Stocks J, Stanojevic S, et al. Age and height dependence of lung clearance index and functional residual capacity. *Eur Respir J*. 2013;41:1371–1377.
- 31 Quanjer PH, Stanojevic S, Cole TJ, et al. Multi-ethnic reference values for spirometry for the 3–95-yr age range: the global lung function 2012 equations. *Eur Respir J*. 2012;40:1324–1343.
- 32 Talmaciu I, Ren CL, Kolb SM, Hickey E, Panitch HB. Pulmonary function in technology-dependent children 2 years and older with bronchopulmonary dysplasia. *Pediatr Pulmonol*. 2002;33:181–188.
- 33 Kairamkonda VR, Richardson J, Subhedar N, Bridge PD, Shaw NJ. Lung function measurement in prematurely born preschool children with and without chronic lung disease. *J Perinatol*. 2008;28:199–204.
- 34 Vrijlandt EJ, Boezen HM, Gerritsen J, Stremmelaar EF, Duiverman EJ. Respiratory health in prematurely born preschool children with and without bronchopulmonary dysplasia. *J Pediatr*. 2007;150:256–261.
- 35 Northway WH Jr, Moss RB, Carlisle KB, et al. Late pulmonary sequelae of bronchopulmonary dysplasia. *N Engl J Med*. 1990;323:1793–1799.
- 36 Doyle LW, Cheung MM, Ford GW, Olinsky A, Davis NM, Callanan C. Birth weight <1501 g and respiratory health at age 14. *Arch Dis Child*. 2001;84:40–44.
- 37 Doyle LW, Faber B, Callanan C, Freezer N, Ford GW, Davis NM. Bronchopulmonary dysplasia in very low birth weight subjects and lung function in late adolescence. *Pediatrics*. 2006;118:108–113.
- 38 Gibson AM, Reddington C, McBride L, Callanan C, Robertson C, Doyle LW. Lung function in adult survivors of very low birth weight, with and without bronchopulmonary dysplasia. *Pediatr Pulmonol*. 2015;50:987–994.
- 39 Narang I, Rosenthal M, Cremonesini D, Silverman M, Bush A. Longitudinal evaluation of airway function 21 years after preterm birth. *Am J Respir Crit Care Med*. 2008;178:74–80.
- 40 Anand D, Stevenson CJ, West CR, Pharoah PO. Lung function and respiratory health in adolescents of very low birth weight. *Arch Dis Child*. 2003;88:135–138.
- 41 Kennedy JD, Edward LJ, Bates DJ, et al. Effects of birthweight and oxygen supplementation on lung function in late childhood in children of very

- low birth weight. *Pediatr Pulmonol.* 2000;30:32–40.
- 42 Vollsaeter M, Roksund OD, Eide GE, Markestad T, Halvorsen T. Lung function after preterm birth: development from mid-childhood to adulthood. *Thorax.* 2013;68:767–776.
- 43 Vrijlandt EJ, Gerritsen J, Boezen HM, Grevink RG, Duiverman EJ. Lung function and exercise capacity in young adults born prematurely. *Am J Respir Crit Care Med.* 2006;173:890–896.
- 44 Gross SJ, Iannuzzi DM, Kveselis DA, Anbar RD. Effect of preterm birth on pulmonary function at school age: a prospective controlled study. *J Pediatr.* 1998;133:188–192.
- 45 Kilbride HW, Gelatt MC, Sabath RJ. Pulmonary function and exercise capacity for ELBW survivors in preadolescence: effect of neonatal chronic lung disease. *J Pediatr.* 2003;143:488–493.
- 46 Evensen KA, Steinshamn S, Tjonna AE, et al. Effects of preterm birth and fetal growth retardation on cardiovascular risk factors in young adulthood. *Early Hum Dev.* 2009;85:239–245.
- 47 Siltanen M, Savilahti E, Pohjavuori M, Kajosaari M. Respiratory symptoms and lung function in relation to atopy in children born preterm. *Pediatr Pulmonol.* 2004;37:43–49.
- 48 Malmberg LP, Mieskonen S, Pelkonen A, Kari A, Sovijarvi AR, Turpeinen M. Lung function measured by the oscillometric method in prematurely born children with chronic lung disease. *Eur Respir J.* 2000;16:598–603.
- 49 Korhonen P, Laitinen J, Hyodynmaa E, Tammela O. Respiratory outcome in school-aged, very-low-birth-weight children in the surfactant era. *Acta Paediatr.* 2004;93:316–321.
- 50 Doyle LW; the Victorian Infant Collaborative Study Group. Respiratory function at age 8–9 years in extremely low birthweight/very preterm children born in Victoria in 1991–92. *Pediatr Pulmonol.* 2006;41:570–576.
- 51 Smith LJ, van Asperen PP, McKay KO, Selvadurai H, Fitzgerald DA. Reduced exercise capacity in children born very preterm. *Pediatrics.* 2008;122:e287–293.
- 52 Danks M, Burns YR, Gibbons K, et al. Fitness limitations in non-disabled extremely low birthweight adolescents. *J Paediatr Child Health.* 2013;49:548–553.
- 53 Fawke J, Lum S, Kirkby J, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. *Am J Respir Crit Care Med.* 2010;182:237–245.
- 54 Hacking DF, Gibson AM, Robertson C, Doyle LW. Respiratory function at age 8–9 after extremely low birthweight or preterm birth in Victoria in 1997. *Pediatr Pulmonol.* 2013;48:449–455.
- 55 Kitchen WH, Olinsky A, Doyle LW, et al. Respiratory health and lung function in 8-year-old children of very low birth weight: a cohort study. *Pediatrics.* 1992;89:1151–1158.
- 56 Hakulinen AL, Heinonen K, Lansimies E, Kiekara O. Pulmonary function and respiratory morbidity in school-age children born prematurely and ventilated for neonatal respiratory insufficiency. *Pediatr Pulmonol.* 1990;8:226–232.
- 57 Jacob SV, Coates AL, Lands LC, et al. Long-term pulmonary sequelae of severe bronchopulmonary dysplasia. *J Pediatr.* 1998;133:193–200.
- 58 Gough A, Linden M, Spence D, Patterson CC, Halliday HL, McGarvey LP. Impaired lung function and health status in adult survivors of bronchopulmonary dysplasia. *Eur Resp J.* 2014;43:808–816.
- 59 Cazzato S, Ridolfi L, Bernardi F, Faldella G, Bertelli L. Lung function outcome at school age in very low birth weight children. *Pediatr Pulmonol.* 2013;48:830–837.
- 60 Mitchell SH, Teague WG. Reduced gas transfer at rest and during exercise in school-age survivors of bronchopulmonary dysplasia. *Am J Respir Crit Care Med.* 1998;157:1406–1412.
- 61 Hakulinen AL, Jarvenpaa AL, Turpeinen M, Sovijarvi A. Diffusing capacity of the lung in school-aged children born very preterm, with and without bronchopulmonary dysplasia. *Pediatr Pulmonol.* 1996;21:353–360.
- 62 Halvorsen T, Skadberg BT, Eide GE, Roksund OD, Carlsen KH, Bakke P. Pulmonary outcome in adolescents of extreme preterm birth: a regional cohort study. *Acta Paediatr.* 2004;93:1294–1300.
- 63 Pianosi PT, Fisk M. High frequency ventilation trial. Nine year follow up of lung function. *Early Hum Dev.* 2000;57:225–234.

Perinatal Disruptions of Lung Development:

Mechanisms and Implications for Chronic Lung Diseases

Michael A. O'Reilly

Abstract

The lung evolved to efficiently exchange oxygen and carbon dioxide between the external environment and circulating blood. Because it is an open conduit to the environment, the lung must also maintain tight barrier function, defend against inhaled toxins, and have tremendous repair capacity when injured. Creating, maintaining, and repairing the remarkably complex structure of the lung is controlled by genes that regulate cell autonomous functions and cell–cell interactions. However, many studies have now shown how perinatal environmental factors can also permanently modify lung development, thereby influencing long-term lung function and respiratory health. Environmental factors include oxygen at birth, nutrition and intrauterine growth restriction, prenatal and postnatal infections, ionizing radiation, ozone, tobacco smoke, and chemical toxins such as bisphenol A. Although perinatal influences are often considered detrimental to long-term respiratory health, how the lung responds to different levels of oxygen at birth suggests that its response to other influences may represent developmental changes in an organ evolutionarily designed to functionally adapt to its environment. Improving respiratory health as people age may therefore require a better understanding of how the perinatal environment plays a role in generating biologic fit and novelty.

Keywords:

Adaptation, birth, developmental plasticity, evolution, gene–environment interactions, oxygen

Introduction

The lung evolved in mammals, birds, reptiles, amphibians, and some fish to allow greater aerobic abilities to sustain increased metabolic activity. Airflow moves unidirectionally in alligators and birds and bidirectionally in mammals and most vertebrates (1). One-way flow may be advantageous to creatures under high strenuous activity, such as birds flying at high altitude or alligators diving under water because it ensures that the lung is uniformly oxygenated under conditions where the partial pressures of oxygen are low (2). Although differences in the number and symmetry of lobes exist in mammals and between species, the position of the lung along the foregut is essentially conserved among all species. It is located ventral to the esophagus and between the thyroid and stomach. The bird and alligator lung

contains two lobes with tubular parabronchi, while the amphibian lung is a pair of two thin-walled sacs containing inner partitions that increase the surface area for absorbing oxygen. In contrast, mammalian lungs are asymmetrically organized, such that the human and rat lung contain three right and two left lobes, whereas the mouse lung has four right and one left lobe. Airway caliper gradually decreases as airways lengthen and branch until the airways terminate in saccular-alveolar units designed to efficiently exchange oxidant gases between a thin squamous epithelial surface and an underlying capillary network.

Mammalian lung development is typically subdivided into five stages beginning with the embryonic, followed by the pseudoglandular, canalicular, saccular, and alveolar stages (3). The

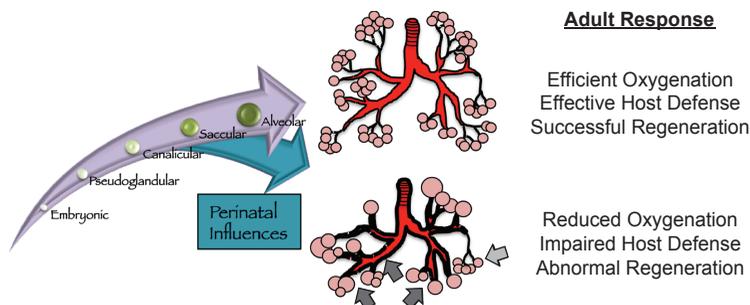


Figure 15-1. The perinatal environment influences saccular and alveolar lung development. Mammalian lung development proceeds through five stages that culminate in the formation of a dichotomously branched organ with many uniformly sized alveoli. Perinatal influences (blue arrows) alter bronchoalveolar development, often resulting in shorter, muscularized airways that terminate in heterogeneously sized alveoli. Such changes perturb efficiency of gas exchange, ability to defend against inhaled toxins and microorganisms, and regeneration of injured regions of the adult lung.

first four stages occur prenatally, while the last alveolar stage begins prenatally and continues postnatally for ten to fourteen days in mice and two to three years or more in humans. Lung development begins as an outgrowth of the embryonic foregut endoderm in humans around twenty-two days and in mice around embryonic (e) 9.5 days following fertilization. In humans, the lung undergoes twenty-three airway generations and when mature produces approximately 54,000 branches and 300 million alveoli (4,5). The mouse lung contains approximately 5,000 branches that terminate in approximately 2 million alveoli (6,7).

The primitive endoderm specifies the respiratory epithelium, while the mesoderm specifies all other cell types, including cartilaginous rings of the trachea, airway and vascular smooth muscle, vascular endothelium, mesothelium, pericytes, fibroblasts, and lipofibroblasts. Complex cell–cell interactions between the endoderm and the mesoderm are required for both initiating lung bud formation and progression through each stage [for review, see (8,9)]. Reciprocal transplantation of embryonic mouse bronchial mesoderm with distal bud endoderm stimulates branching morphogenesis, while bronchial endoderm stimulates lung bud formation (10). Heterologous transplantation studies suggest mesoderm influences branching morphogenesis but not the differentiation state of the alveolar epithelium (11). On the other hand, distal bud mesoderm can induce alveolar epithelial cell differentiation in tracheal bud epithelium (12). Recent three-dimensional imaging studies using cell-restricted fluorescently labeled reporter mice suggest that the developing

respiratory epithelium undergoes two nested waves of development (13). The first wave occurs throughout lung development and defines the airway and alveolus. A second wave that follows and terminates before birth defines the proximal airway and the bronchoalveolar junction (BADJ).

Each of the five distinct anatomical phases of lung development requires the orderly expression of genes that specify cell-specific phenotypes and cell–cell interactions. Interference during the early stages (embryonic, pseudoglandular, canalicular) of lung development are likely to be perinatal lethal because they will severely compromise lung development and hence the ability to efficiently exchange oxygen at birth. Interference during later stages (saccular, alveolar) is more likely to affect the ability to exchange oxidant gases, defend against inhaled pollutants, and general respiratory health later in life (Figure 15-1). Indeed, numerous review articles have been written on how maternal and newborn exposures to oxygen, ozone, infection, tobacco smoke, and nutritional status can profoundly and permanently influence lung development and respiratory health later in life (14–21). Early-life influences have been associated with increased airway hyperactivity, reduced lung function, and greater respiratory morbidity following inhaled pollutants or infections later in life. Consistent with the developmental window in which these exposures take place, perinatal influences often disrupt bronchoalveolar development as defined by changes in the number and size of distal airways and alveoli. A healthy functioning lung is therefore a product of perinatal interactions between cells and genes specifying the general structure of

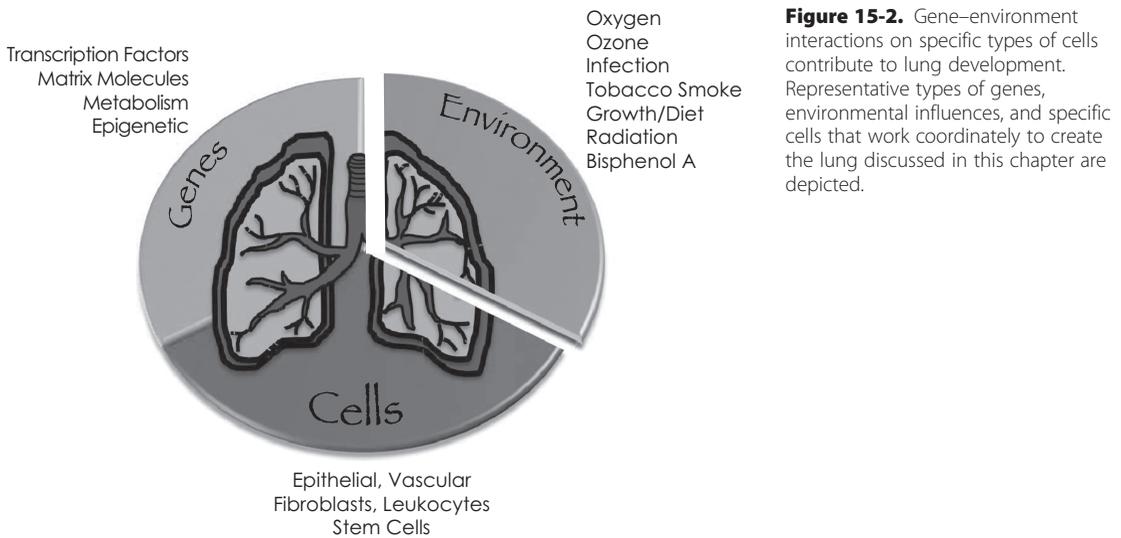


Figure 15-2. Gene–environment interactions on specific types of cells contribute to lung development. Representative types of genes, environmental influences, and specific cells that work coordinately to create the lung discussed in this chapter are depicted.

the lung (Figure 15-2). Rather than writing another review on the subject, we have chosen to examine how the oxygen environment at birth influences postnatal lung development. Using the influence of the oxygen environment at birth as an example, we then postulate how other perinatal exposures influence cellular plasticity designed to promote evolutionary fit and novelty. Perinatal influences that cause respiratory morbidity may therefore represent a maladaptive or failed response of the lung seeking to adapt to its environment. Framing perinatal influences as evolutionary adaptation rather than a toxic or stochastic process offers a new way to look at age-related lung diseases whose origins began early in life. Doing so may provide new opportunities for research and development of therapies designed to treat chronic lung disease.

Perinatal Influences on Lung Development

Unlike organs such as the heart and brain, the lung is unique in that it is not required for fetal development or survival until birth, when it is called on to replace the placenta's role in gas exchange. But unlike the placenta that is protected within the mother, the lung is exposed to the external environment. The newborn lung must therefore defend itself and other internal organs against inhaled toxins and pathogens. It does so using anatomical and physiological barriers, innate and adaptive immunity, antioxidant

defenses, and a high repair/regenerative capacity (22–24). Anatomical and physiological barriers serve to physically impede entry and enhance removal of harmful agents from the lung. This includes airway constriction, mucociliary clearance, and cough. Innate and adaptive immunity act when the anatomical and physiological barriers fail. Innate defenses are an ancestral form of host defense that uses a limited set of cell surface receptors, including Toll receptors and complement, to detect invading pathogens. Adaptive immunity uses an abundant and diverse set of randomly generated receptors, such as immunoglobulins to detect pathogens and activate cell-mediated killing of infected or damaged cells. Antioxidant defenses mature during the last 15% of lung development and are thought to protect the lung from oxidative damage formed as it transitions out of a low hypoxic in utero environment to air at birth (24). The ability to properly regenerate injured cells and restore normal lung function is perhaps the final level of protection. Accordingly, the same environmental exposures during critical developmental windows may affect proliferation, differentiation, and survival of progenitor/stem cells, thereby altering the balance of cells used to build the lung (Figure 15-3). Successful transition to air at birth therefore requires that the newborn lung is capable of exchanging oxidant gases, defending against oxidative stress and inhaled microbes, and repairing cell injury or regenerating tissue when damaged.

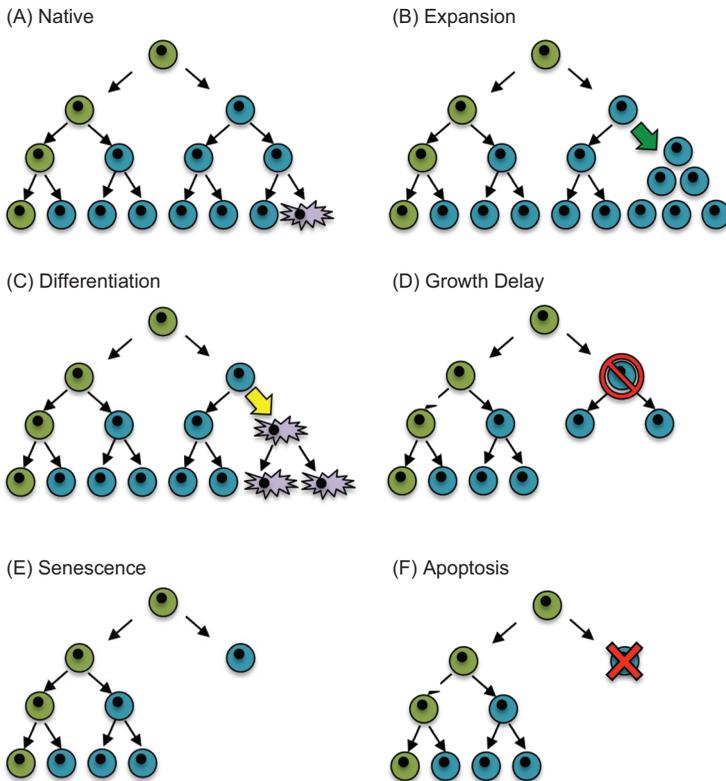


Figure 15-3. Hypothetical model of how perinatal stress might influence the balance of cells in a tissue. (A) A putative stem cell (green) divides asymmetrically to produce itself and a committed daughter cell (blue) that subsequently divides symmetrically or differentiates into a mature cell (purple). Perinatal stresses may alter the balance of cells in a tissue by (B) stimulating expansion of differentiated cells (green arrow), (C) stimulating differentiation of a committed cell (yellow arrow), (D) transiently inhibiting proliferation (red slashed circle), (E) activating senescence programs that terminate cellular expansion, or (F) inducing cell death (red X).

Oxygen Environment at birth

The transition to breathing oxygen is perhaps one of the most profound environmental changes one will ever experience. At term, the human fetal lung is bathed in amniotic fluid with a pO_2 of about 11 mm Hg or less than 1% oxygen (25). Focal hypoxia created by the lower oxygen levels in amniotic fluid relative to arterial levels may stimulate hypoxic signaling of hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF) necessary for promoting vascular growth and lung development (26). Indeed, expression of the transcription factor hypoxia inducible factor (HIF)-1 α , HIF-1 β , or HIF-2 are required for proper maturation of surfactant production and ability to survive at birth (27,28). Hydroxylation of prolines on HIF by HIF-dependent prolyl hydroxylases stimulates HIF degradation. Inhibition of HIF prolyl hydroxylases in preterm baboons increases HIF expression and improves lung growth and function (29, 30). Birth rapidly creates a hyperoxic environment in the airspace that may influence lung development by terminating HIF

signaling. Maturation expression of antioxidant enzymes supports the idea that birth is an oxidizing event that requires protection against reactive oxygen species (ROS) produced during the transition to air (31). Although this implies the oxygen environment at birth is toxic, it may also serve to regulate normal postnatal lung growth. However, there are few studies suggesting that ambient levels of oxygen affect lung development. Perhaps the best example that birth may influence genes controlling postnatal lung development comes from a study showing how expression of the transcription factor Klf4 increases in mice two hours after birth (32). Mice lacking Klf4 display excessive proliferation and apoptosis in the respiratory epithelium and loss of α -smooth muscle actin in septal tips. This implies that the elevated expression of Klf4 at birth is required for the proper transition to air. Another example comes for a recent paper showing how oxygen-induced DNA damage signaling may influence proliferation of cardiomyocytes in the newborn mouse (33). Whether this is directly related to the oxygen environment in the lung or some form of oxidative stress created in

the heart as the pAO_2 changes remains to be determined.

Although it remains unclear whether the transition to air regulates proper lung development, excessive levels clearly disrupt lung development. Excess levels are often given to preterm infants whose lungs are not adequately prepared to breathe air. Such infants often also have immature antioxidant defense that make them hypersensitive to oxygen-induced injury (34–36). Prolonged oxygen exposure can contribute to bronchopulmonary dysplasia (BPD), a chronic form of lung disease characterized by restrictive airways, inflammation, mild fibrosis, and reduced angiogenesis, increased elastogenesis, and alveolar simplification (34,37). Treatment strategies are generally supportive and designed to enhance oxygen delivery, improve monitoring of oxygen saturations, minimize tissue injury, and stimulate lung growth (for review see (38)). This includes the use of exogenous surfactant, milder ventilation strategies, inhaled nitric oxide, and retinoic acid. Such treatments have reduced infant mortality as well as increase survival of preterm infants born as young as twenty-three to twenty-four weeks gestation. However, these advances have changed the pathology of BPD from a scarring disease seen radiographically to one that requires continuous ventilation and supplemental oxygen treatment. This new BPD appears to be a form of disrupted lung development and impaired lung function that can be recapitulated in newborn animals exposed to high oxygen. Hence, there has been an increasing interest in understanding how oxygen influences lung development with a focus on the potential use of antioxidants and anti-inflammatory agents as therapeutic agents (24,39). But, recombinant human CuZn superoxide dismutase delivered intratracheally to very low-birth-weight preterm infants did not influence the incidence of BPD (40,41). It did, however, improve respiratory health at one year of corrected age and the incidence of retinopathy in preterm infants born at extremely low gestational age. It also improved oxygenation and development of the pulmonary vasculature in a preterm sheep model of prematurity (42). Unfortunately, additional studies testing the efficacy of antioxidant strategies have not gone forward, perhaps because there is some concern that antioxidants may adversely influence signaling pathways required for development of other organs such as brain.

Inflammation created by oxygen damage to the lung or by maternal and newborn infections may also contribute to BPD. But, a study in fetal sheep infected with *Ureaplasma parvum*, a common microorganism found in women with chorioamnionitis, showed that the inflammatory response to infection alone did not disrupt lung airspace or vascular development (43). On the other hand, maternal exposure of mice to lipopolysaccharide (LPS) and neonatal hyperoxia disrupts cardiac structure and function to the extent that it causes cardiac failure in adults (44). These studies suggest inflammation alone may not be sufficient to promote BPD. Inflammation may enhance oxygen-dependent changes in lung development and hence the pathogenesis of BPD. So, although there has been great interest in the role of oxidants and inflammation to mediate high oxygen-dependent changes in lung development, current antioxidant and anti-inflammatory strategies have yet to prove effective. Perhaps a combination therapy is needed.

Despite reduced mortality of preterm infants, survivors develop persistent pulmonary disease (PPD) later in life, and antioxidant therapy can improve some of these adverse outcomes. Children born preterm often display reduced lung function, are often rehospitalized following respiratory viral infections, and have increased risk for developing asthma (45–49). There is also a growing concern that premature infants will have neurodevelopmental delay and may be at risk for high blood pressure and heart disease as adults (50,51). These observations suggest that many infants born prematurely will require costly lifelong health care. Indeed, according to the National Heart Lung and Blood Institute (NHLBI) web site (<http://www.nhlbi.nih.gov/new/press/06-07-26.htm>), the annual costs of treating children born prematurely in 2005 were \$26.2 billion dollars, of which 10% was just for treating infants with BPD. The high cost of treating prematurity is second to the treatment of asthma and far exceeds costs of treating cystic fibrosis. Hence, there is an urgent need to better understand how prematurity, and more specifically, how early-life exposure to oxygen, behaves as an environmental risk factor for promoting disease later in life (52).

A number of clinical trials have attempted to define an acceptable level of oxygen use in preterm infants that minimizes harm. Early clinical

trials showed an increase in the incidence of retinopathy of prematurity (ROP) with the unrestricted use of oxygen (53). However, the Supplemental Therapeutic Oxygen for Prethreshold Retinopathy of Prematurity (STOP-ROP) trial found high oxygen saturation levels did not increase the severity of ROP in those infants with prethreshold ROP (54). Subsequently, the Benefits of Oxygen Saturation Targeting (BOOST) trial showed how a higher oxygen-saturation range prolonged oxygen dependence (55). These and other studies identified the need to know whether the incidence of ROP was less in infants treated with a lower oxygen saturation target. The Surfactant, Positive Pressure, and Pulse Oximetry Randomized Trial (SUPPORT) compared the incidence of ROP in 1300 infants born between twenty-four and twenty-eight weeks gestation who were treated with the use of a low (85–89%) or high (91–95%) oxygen saturation target (56). While infants treated with a low oxygen saturation target had reduced ROP, mortality was increased when compared to infants treated with a high oxygen saturation target. Similar findings were recently reported in the BOOST II trial, which enrolled 2,400 infants at fifty-four hospitals in the United Kingdom, Australia, and New Zealand (57). While none of these clinical trials established an acceptable level of oxygen use in preterm infants, they collectively suggest preterm infants should be treated with oxygen saturations in the 90–95% range despite the increased risk for developing ROP. An alternative approach has been to define oxygen exposure as a cumulative dose over the first three days of life (58). Infants whose cumulative oxygen exposure was in the highest quartile were two to three times more likely to experience symptomatic airway dysfunction than infants in the lowest quartile. This implies the environmental load of inhaled oxygen is sufficient to predict risk of oxygen-related respiratory morbidity.

Because we recently wrote a review on animal models of oxygen-induced BPD (16), we will briefly summarize some of the main points on how high oxygen exposure at birth influences lung development in animal models. High oxygen exposure in newborn rodents promotes alveolar simplification and abnormal vascular development (59,60). Failure to produce vessels has been attributed to the oxygen-dependent loss of VEGF

(61–63). Inhibiting VEGF signaling disrupts postnatal alveolar development (64–66), while overexpression of VEGF can partially protect the developing rat lung exposed to neonatal hyperoxia (67,68). In addition, neonatal hyperoxia suppresses the number of circulating endothelial cell precursors detected in blood and lung (69) and when restored can attenuate murine models of BPD (70,71). These changes may contribute to pulmonary hypertension and cardiovascular disease observed in human adolescents born preterm (72) and in adult rodents exposed to hyperoxia as neonates (73,74).

The effect of hyperoxia on the respiratory epithelium is equally complex. Alveolar epithelial type II cells express VEGF. Hyperoxia may directly inhibit expression of VEGF and indirectly by affecting the expansion of type II cells (59,75,76). Exposure of newborn rodents to hyperoxia for several days inhibits proliferation of type II cells (75). Growth arrest may be mediated by cell cycle checkpoints activated in response to oxidative DNA damage (77). The observation that overexpression of extracellular superoxide dismutase in type II cells attenuates oxygen-dependent changes in newborn lung development and DNA strand breaks supports this idea (78,79). Additional DNA damage may occur when leukocytes are recruited to the injured lung (80). Cell cycle checkpoints are thought to prevent replication of damaged DNA and also allow time for repair to take place. As seen in adult mice lacking the cell cycle inhibitor p21 (cdkn1), cell cycle arrest may limit the extent of oxidative stress and damage created by early-life oxygen exposure (81). On the other hand, we recently showed how short-term hyperoxia stimulates expansion of type II cells in newborn mice, which are subsequently growth arrested with continued exposure (82). Hyperplasia of type II cells has also been observed in a preterm baboon model of BPD and in adult rats exposed to sublethal doses of oxygen (4, 27). Although the relevance to lung disease remains to be determined, excessively expanded type II cells produced during neonatal hyperoxia are slowly pruned or depleted over several weeks when mice are returned to room air (82). Because type II cells produce surfactant necessary for reducing alveolar surface tension, express innate immune and other inflammatory genes, and function as transient amplifying precursors for type I cells, their depletion in adult mice exposed to neonatal

hyperoxia may contribute to the altered lung function and host response to respiratory viral infection observed later in life (83,84).

Although early-life high oxygen exposure clearly perturbs normal lung development, sadly, a codified model of oxygen exposure in animal models has yet to be defined or accepted by the research community. This makes it difficult to compare outcomes between investigators who have used different doses and durations of hyperoxia on different developmental windows (16). Sex and strain of the animal can also influence how the lung responds to hyperoxia (85,86). We have taken two approaches to address this issue. First, preterm human infants are often born and exposed to excess oxygen during the sacular stage of lung. They typically go home breathing room air. Because the sacular stage of lung development in mice is between e17.5 and pnd 4, we have exposed mice to hyperoxia between birth and pnd4. The oxygen-exposed mice are then returned to room air so that we can study the long-term effects of oxygen exposure during the sacular phase of lung development. We found that exposure to 100% oxygen alters the balance of alveolar epithelial type I and II cells, promotes alveolar simplification, and alters the host response to influenza A virus infection (87). Exposure to 60% oxygen for four days altered lung development but not the host response to infection. Second, by exposing newborn mice to different levels of oxygen that produced the same cumulative dose, we were able to confirm the idea that quantifying neonatal cumulative excess oxygen can predict risk for respiratory morbidity later in life (88). Quantifying oxygen exposure as cumulative dose over different developmental windows may therefore help interpret findings from other studies that used different doses and durations of exposure. For example, adult mice or rats exposed to 100% oxygen as neonates develop cardiovascular disease (73,74). Perhaps the cumulative oxygen model can predict risk for high blood pressure such as seen in adolescent humans who were born preterm (72).

While preterm infants are often exposed to high oxygen at birth, people born at high altitude are born into low partial pressures of oxygen or hypoxia. It has been known for several decades that high altitude native Tibetans have a depressed response to hypoxia when at sea level

(89). Whereas hypoxia stimulates erythropoietin and hemoglobin concentrations, natives born at high altitude develop larger lungs with increased functional capacity that is better suited for breathing lower partial pressures of oxygen (90). Hypoxia-induced lung growth is not seen after the age of ten, suggesting that the low oxygen-environment at birth affects postnatal lung development. Lower levels of hemoglobin have also been reported in high-altitude Tibetans and Bolivian Aymara people (91). Although lower levels of hemoglobin may seem counterproductive under high altitude conditions, it may be protective because elevated hemoglobin concentrations typically observed in hikers at high altitude are associated with mountain sickness and cardiovascular disease. In 2010, several studies were published showing how lower hemoglobin levels correlated positively with haplotypes in the HIF prolyl hydroxylase EGLN1, HIF-2 α , and PPARA (92–95). Interestingly, the pattern of variation in EGLN1 differed between Tibetans and Andean natives (95). The Tibetan PHD2 haplotype (D4E/C127S) diminishes interaction of PHD2 with the heat shock protein chaperone p23, thereby reducing hydroxylation activity and downregulation of HIF pathway (96). Higher blood flow and circulating nitric oxide products may also serve to protect against high-altitude hypoxia (97). Exposure of newborn mice to low oxygen has revealed other pathways that may help clarify how a low oxygen environment influences development of the lung and other organs. For example, exposure of newborn mice to 12% oxygen causes lung simplification and intriguingly alters many of the same signaling pathways (such as TGF- β , PPAR- γ) typically activated by high oxygen exposure (98,99). As defined by elevated tumor necrosis factor- α and interleukin-6, neonatal hypoxia promotes an inflammatory state in rat heart and skeletal muscle (100). Thus, the low oxygen environment, such as seen in people living at high altitude, permanently influences postnatal lung development.

The successful adaptation of the lung to hypoxia at birth may come at an increased cost of altered long-term health of the high altitude native (for review see (90)). When compared to lowlanders, high-altitude natives have increased risk for cardiovascular disease particularly related to cardiac hypertrophy. A zip code study of children born at high altitude in Colorado suggests

that birth at high altitude increases rehospitalization following infection with respiratory syncytial virus (RSV) (101). There is also some evidence that living at high altitude reduces brain activity (102). High-altitude natives may have lower rates of obesity (103), but are often born small for gestational age and have transient growth delay with compensatory catchup growth (90, 104). Rats living at high altitude also develop hypertension and have a shortened life span that can be reversed when birthed into sea-level amounts of oxygen (105). Not only do these data imply birth into low oxygen can be an early antecedent of disease later in life, but they also surprisingly sound like diseases attributed to prematurity and exposure to too much oxygen at birth. In other words, being born too early and exposed to too much oxygen or being born at high altitude and thus less oxygen alters lung development, host response to respiratory viral infections, cognition, and overall growth. This implies that complete development of the lung and perhaps other organs is heavily influenced by the amount of oxygen present in the environment at birth.

So, why would the lung and other organs respond to different levels of oxygen at birth? The geological record suggests that the oxygen environment ranged from as low as 15% to as high as 35% over the past 300 million years (106). These fluctuating changes in oxygen levels may have substantially influenced development and evolution of the mammalian lung and perhaps other organs (106,107). In fact, the transition from aquatic to terrestrial habitation by vertebrates likely occurred during a time when atmospheric levels of oxygen were rising (108). The cutaneous respiration and inadequate removal of carbon dioxide by aquatic species was incompatible with life on land, leading to the evolution of a more sophisticated vascular and respiratory tree (108). As organismal size and complexity increased over time, these two systems became critical for the efficient uptake and transport of oxygen to tissues and organs, thus increasing chances for survival (109). In *Drosophila melanogaster*, high oxygen increases body weight and wing size, whereas low oxygen promotes smaller body weight and wing size (106). Changes in oxygen concentrations negatively correlate with tracheal diameter and cell size in these insects (106, 110). Hence, the oxygen environment at

birth is a modifier of evolutionary plasticity for the developing lung and other organs.

Additional Old and New World Perinatal Influences to Lung Development

The developing lung is constantly exposed to other environmental influences, both as a fetus and as a newborn. If the modern lung evolved in response to a changing oxygen environment, perhaps it evolved in response to other ancient environmental influences as well. Ozone, radiation, diet, and pathogens like viruses and bacteria are additional examples of old world perinatal influences. Because the mammalian lung evolved in an environment containing these agents, it presumably is programmed to respond to them. Consistent with this argument, expression profiling studies have defined a pattern of gene expression that defines a “time-to-birth” program wherein developmental genes are expressed first and genes involved in oxygen transport, protection against reactive oxygen species, and host defense are expressed near birth (111). This type of genetic programming ensures that the lung is ready to breathe air and defend against environmental toxins and pathogens at birth. Cigarette smoke and bisphenol A are examples of new world pollutants because they are products whose abundance in the environment is caused by human activity. The response to these agents may be more primitive or less programmed because the lung did not evolve when they were present in the environment. The following briefly describes the pulmonary response to an old world pollutant, ozone, and the new world pollutants, tobacco smoke and bisphenol A. Regardless of the exposure material, the net outcome is an effort to develop a lung that is best suited to function in an environment containing that material.

The photolysis of oxygen into ozone in the upper atmosphere by UV radiation allowed for the massive expansion of life. Normally there is little ozone in the lower atmosphere because UV radiation is weak. However, ozone has emerged as an urban pollutant created by chemical reactions between nitrogen oxides and volatile organic compounds typically produced from industries producing electricity, motor vehicle exhaust, gasoline vapors, and chemical solvents. Epidemiologic evidence suggests that ozone is an airway irritant that provokes asthma in both adults and

children (18). Children may be at greatest risk because their lungs are still developing and likely to have greater exposure to ozone and other air pollutants as they play outdoors in polluted air. Indeed, early-life episodic ozone exposures of 0.5 parts per millions (ppm) in nonhuman primates and rats shorten growth of distal airways (112,113). A single acute exposure of 1 ppm for three hours is sufficient to inhibit alveolar cell proliferation in three-day-old mice (114). But, it is important to remember that ozone is not the only component of air pollution and that pollutant effects can originate from maternal exposures. For example, maternal diesel exhaust exposure enhances ozone-dependent airway hyperreactivity in newborn mice (115). Nonetheless, ozone is an oxidant gas that may influence distal airway and alveolar structure through similar mechanisms as hyperoxia, namely oxidant injury and inflammation (17). By shortening the airway and increasing alveolar size, the lung may be attempting to limit the amount of surface exposed to a highly reactive oxidant gas and yet still be able to efficiently exchange oxygen and carbon dioxide.

Tobacco smoke exposure is a significant cause of lung disease in the elderly population and is the primary cause of COPD and lung cancer. It has also been linked to airway hyperreactivity and asthma in children (18). Studies in animal models reveal maternal and neonatal exposures are sufficient to permanently affect host defense and lung development. Prenatal nicotine exposure reduces lung function in newborn rhesus monkeys presumably via the binding of nicotine to the nicotinic acetylcholine receptor (116). Neonatal mice exposed to cigarette smoke have reduced innate immunity and slight deficits in alveolar development as adults (117). Interestingly, high oxygen exposure in newborn mice enhances COPD in adult mice exposed to tobacco smoke products (118). Given the large number of pollutants present in tobacco smoke, it will be challenging to figure out which ones are responsible for altering lung development and how this happens. However, changes in innate immunity and increased airway hyperreactivity may represent an attempt to build a lung that is best suited to exclude particulate matter whose origins may be less molecularly defined than oxidant gases (i.e., anti-oxidants) or pathogens (i.e., toll-receptors).

Bisphenol A (BPA) is an organic chemical used to make polycarbonate plastics and epoxy

resins. Because it is used to produce materials for packaging and storing food, its effects on children's health are a concern. Fetal exposures in rhesus macaques stimulate expression of Scgb1a1 and MUC5AC/5B in the proximal conducting airways (119). Increased expression of these proteins suggests BPA may be inducing airway mucin defenses. Fetal exposure of pregnant mice to BPA reduced the extent of influenza A virus-associated pulmonary inflammation and antiviral gene expression in the offspring (120). This implies maternal BPA slightly modulates innate immunity. Similarly, maternal administration of BPA enhanced the response to ovalbumin challenge in offspring as defined by increased serum IgE, airway eosinophilia, and airway hyper-responsiveness (121). But those findings were not confirmed in a related study, which actually observed a subtle suppression of response to ovalbumin (122). This may reflect differences in experimental design and the developmental window when BPA was administered. Nonetheless, the observation that the developing lung is permanently affected by maternal exposure to an organic compound that is unlikely to reach the fetus is provocative. It suggests that the fetal lung is sampling what the mother is being exposed to and using that information to modify lung development. Once again, the response seems to be to build a lung designed to exclude materials that would negatively impact its ability to efficiently exchange oxidant gases. But, this example also raises the question of why the developing lung and not every organ is so heavily influenced by perinatal exposures.

Environmental Influences on Developmental Plasticity as an Antecedent of Evolutionary Novelty

By now it should be apparent that prenatal and postnatal environmental influences on genes and cells contribute greatly to proper lung health and disease later in life. Environmental influences are often thought of as being bad because they change lung function often for the worse. Asthma in children of mothers who smoked tobacco during pregnancy is a classic example of how perinatal exposures cause respiratory morbidity later in life (18). Similarly, neonatal ozone exposure inhibits distal branching and promotes alveolar

simplification (112,113). Likewise, exposure to hyperoxia causes airway hyperreactivity and alveolar simplification. On the other hand, increased lung development seen in children born at high altitude or under low levels of oxygen is another example of how the environment can influence lung development and health (90). Birth into low oxygen environment stimulates lung growth in order to increase alveolar surface area and hence capture more oxygen under conditions of limiting levels of oxygen. In this example, the developing lung is “functionally adapting” to a low oxygen environment (90). This raises the question of whether the developing lung is trying to functionally adapt to other environmental agents like tobacco smoke and bisphenol A. Hypothetically, some forms of asthma that are linked to an early-life environmental influence may be considered an adaptive response to an airway irritant. For example, childhood asthma caused by mothers who smoked tobacco products may represent a developmental response by an organ expecting to function in a dirty air environment. Increased mucous production and airway smooth muscle reflects the lungs’ first line of defense, namely to exclude toxins via mucociliary clearance, cough, and airway closure. Although this biology is considered productive against respiratory irritants, it is interpreted as morbid when observed in a clean air environment. Exposure to high oxygen at birth inhibits angiogenesis, which could be beneficial when the lung is saturated by oxygen and extensive vasculature is not needed. However, this becomes maladaptive when the lung returns to room air and requires an extensive microvascular network to efficiently capture oxygen at lower partial pressures. Long-term consequences of impaired vascular development include pulmonary hypertension and cardiac hypertrophy with risk for cardiac failure.

Environmental influences on the developing lung may therefore represent a process by which the lung is functionally adapting to that environment. Some changes may provide a selective advantage to an adverse environment and may be evolutionarily beneficial if inherited. Tibetans and Andean natives born at high altitude build larger lungs. Interestingly, Tibetans build larger lungs even if they are born at low altitude. Because Tibetans lived at high altitude for 60,000 years longer than Andeans, they may have evolved heritable changes that drive lung

development independent of the oxygen environment. The idea that environmental pressures influence organ development in an evolutionary manner is well rooted in simpler models. The spadefoot toad is one example where the environment can profoundly influence organ development (123). Larvae typically live in small ponds with limited food resources. They develop small jaw muscles, smooth keratinized mouth parts, and an elongated gut. However, when ponds are larger and support growth of shrimp, larvae become carnivores and develop large jaw muscles, notched and keratinized parts of the mouth, and a shorter gut better suited for eating shrimp. The peppered moth in England is an example of how the environment influences survival of two highly related subspecies (124). The light-colored species was protected from predators because it could hide in light-colored trees and lichen. Air pollution produced during the Industrial Revolution killed lichen, and the trees became black from the soot. The light-colored subspecies became susceptible to predators, while the dark-colored species was better able to hide on trees and therefore flourished. As air quality improved over the past decade, the light-colored species has become more common. Although this serves as an example of how the environment affects two different subspecies, it represents how the environment influences the fitness of a species.

Environmental influences on developmental plasticity should therefore be considered an antecedent of evolutionary novelty. In other words, the developing lung is responding to environmental influences so that it is best suited to function in that environment. Rather than considering perinatal influences as disruptive to lung development, perhaps we should consider them as an integration of ecological and evolutionary developmental biology (eco-evo-devo) designed to generate novelty. Those environmental influences that are beneficial will become heritable over time (125). Others may be maladaptive, impede normal lung development, and increase risk for respiratory morbidity later in life. Such changes would not be considered evolutionarily beneficial to the species and would therefore not be inherited. Assuming this concept has some validity, it becomes less surprising that the research literature is replete with papers documenting how the environment alters expression of virtually every molecular pathway known to be involved

in saccular and alveolar development. In other words, use all the available tools necessary to functionally adapt to the environment.

Research Opportunities

The widespread acceptance that gene-environment interactions during critical developmental windows profoundly alter lung development and respiratory health over a lifetime provides the foundation to define new questions and research opportunities. Although the following examples reflect those by the author, they hopefully will stimulate additional ideas that will help clarify how early-life environmental exposures affect respiratory health later in life.

1. In the early sixteenth century, Paracelsus proclaimed, "All things are poison and nothing is without poison; only the dose permits something not to be poisonous." When studying how a single agent or mixture of environmental factors influences lung development, the dose and duration of exposure needs to be considered. A super physiologic or prolonged dose may generate experimental data, but whether those outcomes are grounded in reality of how lower real-world doses affect the lung needs to be considered when designing experiments.
2. Pediatricians often say, "Children are not little adults" because children respond to drugs and disease differently than adults. But even children are not fetuses. Future studies therefore need to define prenatal and postnatal developmental windows within which specific environmental factors influence lung development. Identifying developmental windows of sensitivity or vulnerability will help clarify mechanisms of action and potentially guide development of age-specific interventions.
3. It is often written in review articles that the normal adult mammalian lung contains approximately forty different cell types, yet the origin of this statement seems to have disappeared in the historical literature. However, it should not be surprising to find that this is a gross underestimation when one considers how expression of cell surface receptors has markedly increased the diversity of leukocytes present in the lung (126). The emerging use of microfluidic single-cell RNA sequencing is also uncovering an equally rich diversity among the nonhematopoietic population of cells (127,128). Pulse-chase labeling with ^3H -thymidine or cell-restricted fluorescent reporter genes and cell-specific ablation with toxins has identified region-specific niches containing stem cells required for proper lung development and repair (129). Having more than one stem cell niche may seem excessive or redundant until one appreciates that deposition of pollutants (particles and gases) in the airspace varies according to size, shape, and chemical reactivity (130). Unique specific stem cell niches may therefore have evolved to facilitate repair of specific areas of the lung damaged by region-specific pollutants. The molecular and cellular tools being used to identify and characterize the "omics" of stem cells need to be used to understand how the environment influences cell-specific pathways and processes controlling lung development.
4. It may equally prudent to identify hallmarks of perinatal influences to lung development much like the cancer field has identified hallmarks of cancer progression. This includes changes in proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, increased angiogenesis, enhanced cell invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction (131). Underlying these changes is genomic instability and inflammation. Interestingly, the same environmental stressors and chemicals that promote cancer also affect postnatal lung development (132,133). Except for genomic instability, some of the hallmarks defined in cancer progression may therefore also be involved in reprogramming cell fate and hence organ development.
5. Environmental factors alter development by epigenetically altering cells toward different fates. Because epigenetics reflects heritable genetic changes that are not caused by changes in the DNA sequence, more research is needed to know whether perinatal influences on lung development can be reversed and whether they are heritable.
6. As an open conduit to the environment, the lung is uniquely poised to act as a sensor of the

environment and transfer that information to other organs. Because perinatal influences often affect development of other organs, future studies should also consider the lung as a portal for how other organs respond to a changing environment.

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The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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References

- Farmer CG, Sanders K. Unidirectional airflow in the lungs of alligators. *Science*. 2010;327(5963):338–340.
- Farmer CG. The provenance of alveolar and parabronchial lungs: insights from paleoecology and the discovery of cardiogenic, unidirectional airflow in the American alligator (*Alligator mississippiensis*). *Physiol Biochem Zool*. 2010;83(4):561–575.
- Maeda Y, Dave V, Whitsett JA. Transcriptional control of lung morphogenesis. *Physiol Rev*. 2007;87(1):219–244.
- Ochs M, Nyengaard JR, Jung A, et al. The number of alveoli in the human lung. *Am J Respir Crit Care Med*. 2004;169(1):120–124.
- Kitaoka H, Takaki R, Suki B. A three-dimensional model of the human airway tree. *J Appl Physiol* (1985). 1999;87(6):2207–2217.
- Knust J, Ochs M, Gundersen HJ, Nyengaard JR. Stereological estimates of alveolar number and size and capillary length and surface area in mice lungs. *Anat Rec (Hoboken)*. 2009;292(1):113–122.
- Metzger RJ, Klein OD, Martin GR, Krasnow MA. The branching programme of mouse lung development. *Nature*. 2008;453(7196):745–750.
- Morrissey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell*. 2010;18(1):8–23.
- Herriges M, Morrissey EE. Lung development: orchestrating the generation and regeneration of a complex organ. *Development*. 2014;141(3):502–513.
- Spooner BS, Wessells NK. Mammalian lung development: interactions in primordium formation and bronchial morphogenesis. *J Exp Zool*. 1970;175(4):445–454.
- Hilfer SR, Rayner RM, Brown JW. Mesenchymal control of branching pattern in the fetal mouse lung. *Tissue Cell*. 1985;17(4):523–538.
- Shannon JM. Induction of alveolar type II cell differentiation in fetal tracheal epithelium by grafted distal lung mesenchyme. *Development*. 1994;166(2):600–614.
- Alanis DM, Chang DR, Akiyama H, Krasnow MA, Chen J. Two nested developmental waves demarcate a compartment boundary in the mouse lung. *Nat Commun*. 2014;5:3923.
- Kajekar R. Environmental factors and developmental outcomes in the lung. *Pharmacol Ther*. 2007;114(2):129–145.
- Madurga A, Mizikova I, Ruiz-Camp J, Morty RE. Recent advances in late lung development and the pathogenesis of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol*. 2013;305(12):L893–905.
- Buczynski BW, Maduekwe ET, O'Reilly MA. The role of hyperoxia in the pathogenesis of experimental BPD. *Semin Perinatol*. 2013;37(2):69–78.
- Auten RL, Foster WM. Biochemical effects of ozone on asthma during postnatal development. *Biochim Biophys Acta*. 2011;1810(11):1114–1119.
- Wang L, Pinkerton KE. Air pollutant effects on fetal and early postnatal development. *Birth Defects Res C Embryo Today*. 2007;81(3):144–154.
- Schwartz DA. Gene-environment interactions and airway disease in children. *Pediatrics*. 2009;123 Suppl 3: S151–159.
- Harding R, Maritz G. Maternal and fetal origins of lung disease in adulthood. *Semin Fetal Neonatal Med*. 2012;17(2):67–72.
- Rehan VK, Asotra K, Torday JS. The effects of smoking on the developing lung: insights from a biologic model for lung development, homeostasis,

- and repair. *Lung*. 2009;187(5):281–289.
- 22 Turvey SE, Bonilla FA, Junker AK. Primary immunodeficiency diseases: a practical guide for clinicians. *Postgrad Med J*. 2009;85(1010):660–666.
- 23 Beers MF, Morrisey EE. The three R's of lung health and disease: repair, remodeling, and regeneration. *J Clin Invest*. 2011;121(6):2065–2073.
- 24 Davis JM, Auten RL. Maturation of the antioxidant system and the effects on preterm birth. *Semin Fetal Neonatal Med*. 2010;15(4):191–195.
- 25 Sjostedt S, Rooth G, Caligara F. The oxygen tension of the amniotic fluid. *Am J Obstet Gynecol*. 1958;76(6):1226–1230.
- 26 Lee YM, Jeong CH, Koo SY, et al. Determination of hypoxic region by hypoxia marker in developing mouse embryos in vivo: a possible signal for vessel development. *Dev Dyn*. 2001;220(2):175–186.
- 27 Saini Y, Harkema JR, LaPres JJ. HIF1 α is essential for normal intrauterine differentiation of alveolar epithelium and surfactant production in the newborn lung of mice. *J Biol Chem*. 2008;283(48):33650–33657.
- 28 Compennolle V, Brusselmans K, Acker T, et al. Loss of HIF-2 α and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat Med*. 2002;8(7):702–710.
- 29 Asikainen TM, Chang LY, Coalson JJ, et al. Improved lung growth and function through hypoxia-inducible factor in primate chronic lung disease of prematurity. *FASEB J*. 2006;20(10):1698–1700.
- 30 Asikainen TM, Waleh NS, Schneider BK, Clyman RI, White CW. Enhancement of angiogenic effectors through hypoxia-inducible factor in preterm primate lung in vivo. *Am J Physiol Lung Cell Mol Physiol*. 2006;291(4):L588–595.
- 31 Clerch LB, Massaro D. Tolerance of rats to hyperoxia. Lung antioxidant enzyme gene expression. *J Clin Invest*. 1993;91(2):499–508.
- 32 Jean JC, George E, Kaestner KH, Brown LA, Spira A, Joyce-Brady M. Transcription factor Klf4, induced in the lung by oxygen at birth, regulates perinatal fibroblast and myofibroblast differentiation. *PLoS One*. 2013;8(1):e54806.
- 33 Puente BN, Kimura W, Muralidhar SA, et al. The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell*. 2014;157(3):565–579.
- 34 Chess PR, D'Angio CT, Pryhuber GS, Maniscalco WM. Pathogenesis of bronchopulmonary dysplasia. *Semin Perinatol*. 2006;30(4):171–178.
- 35 Askie LM, Henderson-Smart DJ, Irwig L, Simpson JM. Oxygen-saturation targets and outcomes in extremely preterm infants. *New Engl J Med*. 2003;349:959–967.
- 36 Madan A, Brozanski BS, Cole CH, Oden NL, Cohen G, Phelps DL. A pulmonary score for assessing the severity of neonatal chronic lung disease. *Pediatrics*. 2005;115:e450–457.
- 37 Saugstad OD. Oxygen and oxidative stress in bronchopulmonary dysplasia. *J Perinat Med*. 2010;38(6):571–577.
- 38 Merritt TA, Deming DD, Boynton BR. The 'new' bronchopulmonary dysplasia: challenges and commentary. *Semin Fetal Neonatal Med*. 2009;14(6):345–357.
- 39 Wright CJ, Kirpalani H. Targeting inflammation to prevent bronchopulmonary dysplasia: can new insights be translated into therapies? *Pediatrics*. 2011;128(1):111–126.
- 40 Parad RB, Allred EN, Rosenfeld WN, Davis JM. Reduction of retinopathy of prematurity in extremely low gestational age newborns treated with recombinant human Cu/Zn superoxide dismutase. *Neonatology*. 2012;102(2):139–144.
- 41 Davis JM, Parad RB, Michele T, Allred E, Price A, Rosenfeld W. Pulmonary outcome at 1 year corrected age in premature infants treated at birth with recombinant human CuZn superoxide dismutase. *Pediatrics*. 2003;111(3):469–476.
- 42 Kinsella JP, Parker TA, Davis JM, Abman SH. Superoxide dismutase improves gas exchange and pulmonary hemodynamics in premature lambs. *Am J Respir Crit Care Med*. 2005;172(6):745–749.
- 43 Polglase GR, Dalton RG, Nitsos I, et al. Pulmonary vascular and alveolar development in preterm lambs chronically colonized with *Ureaplasma parvum*. *Am J Physiol Lung Cell Mol Physiol*. 2010;299(2):L232–241.
- 44 Velten M, Hutchinson KR, Gorr MW, Wold LE, Lucchesi PA, Rogers LK. Systemic maternal inflammation and neonatal hyperoxia induces remodeling and left ventricular dysfunction in mice. *PLoS One*. 2011;6(9):e24544.
- 45 Robin B, Kim YJ, Huth J, et al. Pulmonary function in bronchopulmonary dysplasia. *Pediatr Pulmonol*. 2004;37(3):236–242.
- 46 Doyle LW. Respiratory function at age 8–9 years in extremely low birthweight/very

- preterm children born in Victoria in 1991–1992. *Pediatr Pulmonol.* 2006;41(6):570–576.
- 47 Doyle LW, Faber B, Callanan C, Freezer N, Ford GW, Davis NM. Bronchopulmonary dysplasia in very low birth weight subjects and lung function in late adolescence. *Pediatrics.* 2006;118(1):108–113.
- 48 Smith VC, Zupancic JA, McCormick MC, et al. Rehospitalization in the first year of life among infants with bronchopulmonary dysplasia. *J Pediatr.* 2004;144(6):799–803.
- 49 Weisman LE. Populations at risk for developing respiratory syncytial virus and risk factors for respiratory syncytial virus severity: infants with predisposing conditions. *Pediatr Infect Dis J.* 2003;22(2 Suppl):S33–37; discussion S37–39.
- 50 Doyle LW, Faber B, Callanan C, Morley R. Blood pressure in late adolescence and very low birth weight. *Pediatrics.* 2003;111(2):252–257.
- 51 Roberts G, Anderson PJ, Doyle LW. Neurosensory disabilities at school age in geographic cohorts of extremely low birth weight children born between the 1970s and the 1990s. *J Pediatr.* 2009;154(6):829–834.e1.
- 52 Jobe AH, Kallapur SG. Long term consequences of oxygen therapy in the neonatal period. *Semin Fetal Neonatal Med.* 2010;15(4):230–235.
- 53 Patz A, Hoeck LE, De La Cruz E. Studies on the effect of high oxygen administration in retrolental fibroplasia. I. Nursery observations. *Am J Ophthalmol.* 1952;35(9):1248–1253.
- 54 Supplemental Therapeutic Oxygen for Prethreshold Retinopathy Of Prematurity (STOP-ROP), a randomized, controlled trial. I: primary outcomes. *Pediatrics.* 2000;105(2):295–310.
- 55 Askie LM, Henderson-Smart DJ, Irwig L, Simpson JM. Oxygen-saturation targets and outcomes in extremely preterm infants. *N Engl J Med.* 2003;349(10):959–967.
- 56 Carlo WA, Finer NN, Walsh MC, et al. Target ranges of oxygen saturation in extremely preterm infants. *N Engl J Med.* 2010;362(21):1959–1969.
- 57 Stenson BJ, Tarnow-Mordi WO, Darlow BA, et al. Oxygen saturation and outcomes in preterm infants. *N Engl J Med.* 2013;368(22):2094–2104.
- 58 Stevens TP, Dylag A, Panthagani I, Pryhuber G, Halterman J. Effect of cumulative oxygen exposure on respiratory symptoms during infancy among VLBW infants without bronchopulmonary dysplasia. *Pediatr Pulmonol.* 2010;45(4):371–379.
- 59 Warner BB, Stuart LA, Papes RA, Wispe JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. *Am J Physiol.* 1998;275(1 Pt 1):L110–117.
- 60 Bonikos DS, Bensch KG, Ludwin SK, Northway WH Jr. Oxygen toxicity in the newborn. The effect of prolonged 100 percent O₂ exposure on the lungs of newborn mice. *Lab Invest.* 1975;32(5):619–635.
- 61 Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H. Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. *Am J Physiol Lung Cell Mol Physiol.* 2002;282(4):L811–823.
- 62 Klekamp JG, Jarzecka K, Perket EA. Exposure to hyperoxia decreases the expression of vascular endothelial growth factor and its receptors in adult rat lungs. *Am J Pathol.* 1999;154(3):823–831.
- 63 Maniscalco WM, Watkins RH, Roper JM, Staversky R, O'Reilly MA. Hyperoxic ventilated premature baboons have increased p53, oxidant DNA damage and decreased VEGF expression. *Pediatr Res.* 2005;58(3):549–556.
- 64 Le Cras TD, Markham NE, Tudor RM, Voelkel NF, Abman SH. Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. *Am J Physiol Lung Cell Mol Physiol.* 2002;283(3):L555–562.
- 65 McGrath-Morrow SA, Cho C, Zhen L, Hicklin DJ, Tudor RM. Vascular endothelial growth factor receptor 2 blockade disrupts postnatal lung development. *Am J Respir Cell Mol Biol.* 2005;32(5):420–427.
- 66 Zhao L, Wang K, Ferrara N, Vu TH. Vascular endothelial growth factor co-ordinates proper development of lung epithelium and vasculature. *Mech Dev.* 2005;122(7–8):877–886.
- 67 Kunig AM, Balasubramaniam V, Markham NE, et al. Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. *Am J Physiol Lung Cell Mol Physiol.* 2005;289(4):L529–535.
- 68 Thebaud B, Ladha F, Michelakis ED, et al. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. *Circulation.* 2005;112(16):2477–2486.
- 69 Balasubramaniam V, Mervis CF, Maxey AM, Markham NE, Abman SH. Hyperoxia reduces bone marrow, circulating, and

- lung endothelial progenitor cells in the developing lung: implications for the pathogenesis of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol.* 2007;292(5):L1073–1084.
- 70 van Haaften T, Byrne R, Bonnet S, et al. Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. *Am J Respir Crit Care Med.* 2009;180(11):1131–1142.
- 71 Aslam M, Baveja R, Liang OD, et al. Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease. *Am J Respir Crit Care Med.* 2009;180(11):1122–1130.
- 72 Doyle LW. Cardiopulmonary outcomes of extreme prematurity. *Semin Perinatol.* 2008;32(1):28–34.
- 73 Yee M, White RJ, Awad HA, Bates WA, McGrath-Morrow SA, O'Reilly MA. Neonatal hyperoxia causes pulmonary vascular disease and shortens life span in aging mice. *Am J Pathol.* 2011;178(6):2601–2610.
- 74 Zyzorczyk C, Comte B, Cambonie G, et al. Neonatal oxygen exposure in rats leads to cardiovascular and renal alterations in adulthood. *Hypertension.* 2008;52(5):889–895.
- 75 Yee M, Vitiello PF, Roper JM, et al. Type II epithelial cells are critical target for hyperoxia-mediated impairment of postnatal lung development. *Am J Physiol Lung Cell Mol Physiol.* 2006;291(5):L1101–1111.
- 76 Auten RL, Mason SN, Auten KM, Brahmajothi M. Hyperoxia impairs postnatal alveolar epithelial development via NADPH oxidase in newborn mice. *Am J Physiol Lung Cell Mol Physiol.* 2009;297(1):L134–142.
- 77 O'Reilly MA. DNA damage and cell cycle checkpoints in hyperoxic lung injury: braking to facilitate repair. *Am J Physiol Lung Cell Mol Physiol.* 2001;281(2):L291–305.
- 78 Ahmed MN, Suliman HB, Folz RJ, et al. Extracellular superoxide dismutase protects lung development in hyperoxia-exposed newborn mice. *Am J Respir Crit Care Med.* 2003;167(3):400–405.
- 79 Auten RL, O'Reilly MA, Oury TD, Nozik-Grayck E, Whorton MH. Transgenic extracellular superoxide dismutase protects postnatal alveolar epithelial proliferation and development during hyperoxia. *Am J Physiol Lung Cell Mol Physiol.* 2006;290(1):L32–40.
- 80 Auten RL, Whorton MH, Nicholas Mason S. Blocking neutrophil influx reduces DNA damage in hyperoxia-exposed newborn rat lung. *Am J Respir Cell Mol Biol.* 2002;26(4):391–397.
- 81 McGrath-Morrow SA, Cho C, Soutiere S, Mitzner W, Tuder R. The effect of neonatal hyperoxia on the lung of p21Waf1/Cip1/Sdi1-deficient mice. *Am J Respir Cell Mol Biol.* 2004;30(5):635–640.
- 82 Yee M, Buczynski BW, O'Reilly MA. Neonatal hyperoxia stimulates the expansion of alveolar epithelial type II cells. *Am J Respir Cell Mol Biol.* 2014;50(4):757–766.
- 83 O'Reilly MA, Marr SH, Yee M, McGrath-Morrow SA, Lawrence BP. Neonatal hyperoxia enhances the inflammatory response in adult mice infected with influenza A virus. *Am J Respir Crit Care Med.* 2008;177(10):1103–1110.
- 84 O'Reilly MA, Yee M, Buczynski BW, et al. Neonatal oxygen increases sensitivity to influenza A virus infection in adult mice by suppressing epithelial expression of Ear1. *Am J Pathol.* 2012;181(2):441–451.
- 85 Johnston CJ, Stripp BR, Piedbeouf B, et al. Inflammatory and epithelial responses in mouse strains that differ in sensitivity to hyperoxic injury. *Exp Lung Res.* 1998;24(2):189–202.
- 86 Tryka AF, Witschi H, Gosslee DG, McArthur AH, Clapp NK. Patterns of cell proliferation during recovery from oxygen injury. Species differences. *The Am Rev Respir Dis.* 1986;133(6):1055–1059.
- 87 Buczynski BW, Yee M, Paige Lawrence B, O'Reilly MA. Lung development and the host response to influenza A virus are altered by different doses of neonatal oxygen in mice. *Am J Physiol Lung Cell Mol Physiol.* 2012;302(10):L1078–1087.
- 88 Maduekwe ET, Buczynski BW, Yee M, et al. Cumulative neonatal oxygen exposure predicts response of adult mice infected with influenza A virus. *Pediatr Pulmonol.* 2014.
- 89 Petousi N, Croft QP, Cavalleri GL, et al. Tibetans living at sea level have a hyporesponsive hypoxia-inducible factor system and blunted physiological responses to hypoxia. *J Appl Physiol (1985).* 2014;116(7):893–904.
- 90 Frisancho AR. Developmental functional adaptation to high altitude: review. *Am J Hum Biol.* 2013;25:151–168.
- 91 Beall CM, Brittenham GM, Strohl KP, et al. Hemoglobin concentration of high-altitude Tibetans and Bolivian Aymara. *Am J Phys Anthropol.* 1998;106(3):385–400.
- 92 Beall CM, Cavalleri GL, Deng L, et al. Natural selection on EPAS1 (HIF2alpha) associated with low hemoglobin concentration in Tibetan

- highlanders. *Proc Natl Acad Sci U S A*. 2010;107(25):11459–11464.
- 93 Yi X, Liang Y, Huerta-Sanchez E, et al. Sequencing of 50 human exomes reveals adaptation to high altitude. *Science*. 2010;329(5987):75–78.
- 94 Simonson TS, Yang Y, Huff CD, et al. Genetic evidence for high-altitude adaptation in Tibet. *Science*. 2010;329(5987):72–75.
- 95 Bigam A, Bauchet M, Pinto D, et al. Identifying signatures of natural selection in Tibetan and Andean populations using dense genome scan data. *PLoS Genet*. 2010;6(9):e1001116.
- 96 Song D, Li LS, Arsenault PR, et al. Defective Tibetan PHD2 binding to p23 links high altitude adaptation to altered oxygen sensing. *J Biol Chem*. 2014;289(21):14656–14665.
- 97 Erzurum SC, Ghosh S, Janocha AJ, et al. Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans. *Proc Natl Acad Sci U S A*. 2007;104(45):17593–17598.
- 98 Ambalavanan N, Nicola T, Hagoood J, et al. Transforming growth factor-beta signaling mediates hypoxia-induced pulmonary arterial remodeling and inhibition of alveolar development in newborn mouse lung. *Am J Physiol Lung Cell Mol Physiol*. 2008;295(1):L86–95.
- 99 Nicola T, Ambalavanan N, Zhang W, et al. Hypoxia-induced inhibition of lung development is attenuated by the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone. *Am J Physiol Lung Cell Mol Physiol*. 2011;301(1):L125–134.
- 100 Radom-Aizik S, Zaldivar FP, Nance DM, Haddad F, Cooper DM, Adams GR. Growth inhibition and compensation in response to neonatal hypoxia in rats. *Pediatr Res*. 2013;74(2):111–120.
- 101 Choudhuri JA, Ogden LG, Ruttenber AJ, Thomas DS, Todd JK, Simoes EA. Effect of altitude on hospitalizations for respiratory syncytial virus infection. *Pediatrics*. 2006;117(2):349–356.
- 102 Yan X. Pro: all dwellers at high altitude are persons of impaired physical and mental powers. *High Alt Med Biol*. 2013;14(3):208–211.
- 103 Voss JD, Allison DB, Webber BJ, Otto JL, Clark LL. Lower obesity rate during residence at high altitude among a military population with frequent migration: a quasi experimental model for investigating spatial causation. *PLoS One*. 2014;9(4):e93493.
- 104 Soria R, Julian CG, Vargas E, Moore LG, Giussani DA. Graduated effects of high-altitude hypoxia and highland ancestry on birth size. *Pediatr Res*. 2013;74(6):633–638.
- 105 Lumbroso D, Lemoine A, Gonzales M, Villalpando G, Seaborn T, Joseph V. Life-long consequences of postnatal normoxia exposure in rats raised at high altitude. *J Appl Physiol*. 2012;112(1):33–41.
- 106 Berner RA, Vandenbrooks JM, Ward PD. Evolution. Oxygen and evolution. *Science*. 2007;316(5824):557–558.
- 107 Fluck M, Webster KA, Graham J, Giomi F, Gerlach F, Schmitz A. Coping with cyclic oxygen availability: evolutionary aspects. *Integr Comp Biol*. 2007;47(4):524–531.
- 108 Stamati K, Mudera V, Cheema U. Evolution of oxygen utilization in multicellular organisms and implications for cell signalling in tissue engineering. *J Tissue Eng*. 2011;2(1):2041731411432365.
- 109 Thannickal VJ. Oxygen in the evolution of complex life and the price we pay. *Am J Respir Cell Mol Biol*. 2009;40(5):507–510.
- 110 Frazier MR, Woods HA, Harrison JF. Interactive effects of rearing temperature and oxygen on the development of *Drosophila melanogaster*. *Physiol Biochem Zool*. 2001;74(5):641–650.
- 111 Kho AT, Bhattacharya S, Mecham BH, Hong J, Kohane IS, Mariani TJ. Expression profiles of the mouse lung identify a molecular signature of time-to-birth. *Am J Respir Cell Mol Biol*. 2009;40(1):47–57.
- 112 Fanucchi MV, Plopper CG, Evans MJ, et al. Cyclic exposure to ozone alters distal airway development in infant rhesus monkeys. *Am J Physiol Lung Cell Mol Physiol*. 2006;291(4):L644–650.
- 113 Lee D, Wallis C, Van Winkle LS, Wexler AS. Disruption of tracheobronchial airway growth following postnatal exposure to ozone and ultrafine particles. *Inhal Toxicol*. 2011;23(9):520–531.
- 114 Gabehart K, Correll KA, Yang J, et al. Transcriptome profiling of the newborn mouse lung response to acute ozone exposure. *Toxicol Sci*. 2014;138(1):175–190.
- 115 Auten RL, Gilmour MI, Krantz QT, Potts EN, Mason SN, Foster WM. Maternal diesel inhalation increases airway hyperreactivity in ozone-exposed offspring. *Am J Respir Cell Mol Biol*. 2012;46(4):454–460.
- 116 Sekhon HS, Keller JA, Benowitz NL, Spindel ER. Prenatal nicotine exposure alters pulmonary function in newborn rhesus monkeys. *Am J Respir Crit Care Med*. 2001;164(6):989–994.
- 117 McGrath-Morrow S, Rangasamy T, Cho C, et al.

- Impaired lung homeostasis in neonatal mice exposed to cigarette smoke. *Am J Respir Cell Mol Biol.* 2008;38(4):393–400.
- 118 McGrath-Morrow SA, Lauer T, Collaco JM, et al. Neonatal hyperoxia contributes additively to cigarette smoke-induced chronic obstructive pulmonary disease changes in adult mice. *Am J Respir Cell Mol Biol.* 2011;45(3):610–616.
- 119 Van Winkle LS, Murphy SR, Boetticher MV, VandeVoort CA. Fetal exposure of rhesus macaques to bisphenol a alters cellular development of the conducting airway by changing epithelial secretory product expression. *Environ Health Perspect.* 2013;121(8):912–918.
- 120 Roy A, Bauer SM, Lawrence BP. Developmental exposure to bisphenol A modulates innate but not adaptive immune responses to influenza A virus infection. *PLoS One.* 2012;7(6):e38448.
- 121 Midoro-Horiuti T, Tiwari R, Watson CS, Goldblum RM. Maternal bisphenol a exposure promotes the development of experimental asthma in mouse pups. *Environ Health Perspect.* 2010;118(2):273–277.
- 122 Bauer SM, Roy A, Emo J, Chapman TJ, Georas SN, Lawrence BP. The effects of maternal exposure to bisphenol A on allergic lung inflammation into adulthood. *Toxicol Sci.* 2012;130(1):82–93.
- 123 Ledon-Rettig CC, Pfennig DW. Emerging model systems in eco-evo-devo: the environmentally responsive spadefoot toad. *Evol Dev.* 2011;13(4):391–400.
- 124 Cook LM, Saccheri IJ. The peppered moth and industrial melanism: evolution of a natural selection case study. *Heredity.* 2013;110(3):207–212.
- 125 Scheinfeldt LB, Tishkoff SA. Recent human adaptation: genomic approaches, interpretation and insights. *Nat Rev Genet.* 2013;14(10):692–702.
- 126 Barletta KE, Cagnina RE, Wallace KL, Ramos SI, Mehrad B, Linden J. Leukocyte compartments in the mouse lung: distinguishing between marginated, interstitial, and alveolar cells in response to injury. *J Immunol Methods.* 2012;375(1–2):100–110.
- 127 Streets AM, Zhang X, Cao C, et al. Microfluidic single-cell whole-transcriptome sequencing. *Proc Natl Acad Sci U S A.* 2014;111(19):7048–7053.
- 128 Treutlein B, Brownfield DG, Wu AR, et al. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature.* 2014;509(7500):371–375.
- 129 Rackley CR, Stripp BR. Building and maintaining the epithelium of the lung. *J Clin Invest.* 2012;122(8):2724–2730.
- 130 Carvalho TC, Peters JI, Williams RO III. Influence of particle size on regional lung deposition—what evidence is there? *Int J Pharm.* 2011;406(1–2):1–10.
- 131 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646–674.
- 132 Luch A. Nature and nurture – lessons from chemical carcinogenesis. *Nat Rev Cancer.* 2005;5(2):113–125.
- 133 Minamoto T, Mai M, Ronai Z. Environmental factors as regulators and effectors of multistep carcinogenesis. *Carcinogenesis.* 1999;20(4):519–527.

Lung Growth Through the “Life Course” and Predictors and Determinants of Chronic Respiratory Disorders

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Abstract

Strong observational and experimental evidence indicates that lung growth during fetal and early postnatal life is one of the strongest determinants of adult lung function. Genetic variation plays a critical role in determining maximal lung function reached in adult life. Factors that affect lung growth such as extreme prematurity with bronchopulmonary dysplasia, intrauterine growth retardation, exposure to tobacco smoke in utero and postnatally, and vitamin A and D deficiencies also play varying roles in determining lung function. However, catch-up growth seems to be able to reverse at least in part the negative effects of some of these conditions. Individuals who reach early adult life with lower levels of lung function are at increased risk of developing chronic obstructive pulmonary disease during the decline phase of lung function, after the third decade of life, and may also be more susceptible to the deleterious effects of active cigarette smoking.

Keywords:

Lung function, prematurity, intrauterine growth retardation, bronchopulmonary dysplasia, chronic obstructive pulmonary disease

Chronic respiratory diseases (CRD) are a major cause of morbidity and mortality worldwide. Asthma is the most frequent chronic illness in children, and chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States (1). In 2012, twelve million Americans had a diagnosis of COPD, but twenty-four million had some degree of chronic airway obstruction (2), suggesting that COPD is greatly underdiagnosed. Asthma and COPD are by far the most common chronic respiratory conditions, but chronic lung disease of prematurity, chronic infection, lung restrictive conditions, and cystic fibrosis are also important components of the CRD spectrum.

An important advantage that CRD researchers have with respect to their peers studying other chronic conditions is the availability of noninvasive pulmonary function tests that can be used across the life span. These tests provide an imperfect but extremely useful tool for the longitudinal and cross-sectional assessment of airway and parenchymal status in general population samples and in selected patient groups. The most widely

utilized among these tests is spirometry, which usually consists of a maximal forced exhalation from full inspiration to the point in which no more air can be blown out of the lungs (technically called total lung capacity and residual volume points, respectively). There are now in the market portable instruments using a pneumotachographic device linked to a computer, which transforms a flow signal into flow-volume and volume-time curves and produces easily readable outputs. The most frequently used indices derived from these curves are the forced expiratory volume in one second (FEV1), defined as the volume of air after one second of forced exhalation; the forced vital capacity (FVC), defined as the volume of air expired after a maximal forced exhalation of at least six seconds; and the ratio between FEV1 and FVC (FEV1/FVC). FEV1 (expressed as percentage of the expected value for the subject's age, sex, and stature) and FEV1/FVC are measures of airway patency, and when decreased, they indicate the degree of airway obstruction present, which is proportional to the measured value for the index.

Frequently, subjects are tested before and after use of a bronchodilator, to assess if the changes observed are reversed after airway smooth muscle relaxation.

Spirometry has now been measured in a standardized way in hundreds of thousands of individuals, from early school age up to senescence. Most important, groups of individuals have been tested repeatedly, providing invaluable data on the factors that determine different patterns of growth and decay of lung function across the life span. FEV1 and FEV1/FVC ratio are directly used to diagnose COPD and to determine the different levels of COPD severity (3). They are also used to determine variability of lung function and bronchial hyperresponsiveness to airway challenges, two key features of asthma.

In the last twenty years, similar curves have been acquired in newborns and young children, mainly in experimental centers, given the requirement for sedation and much longer testing sessions. In this age group, pressure is applied to the chest wall either at the end of tidal expiration or after expanding the lungs using the Hering-Breuer reflex, and flows are measured at the mouth using similar methods to those used for spirometry in older children and adults. Correlating indices of lung function assessed shortly after birth with those measured in the same individuals up to the adult years has thus become possible (see later). Analyses of the shape of passive tidal breathing curves in infants have also provided useful but less reliable information (4). A gap remains in the age group from one to five years, in which passive methods such as impulse oscillometry can also yield useful results, but they are not easy to correlate with the spirometry measurements.

Asthma, Wheezing, and Development of Lung Function

Asthma affects more than twenty million Americans, and there are almost 500,000 hospitalizations due to asthma in the United States alone each year (5). Asthma is a heterogeneous condition, and this is especially true during childhood. In the majority of cases, the disease is mild, acute episodes occur infrequently, and lung function is within normal ranges. In a substantial minority, however, symptoms and exacerbations occur frequently if not adequately controlled with inhaled

corticosteroids. Moreover, in this group of children, the disease is also more likely to persist into adult life. Two landmark longitudinal studies in Australasia (6,7) showed that children with moderate to severe disease had, as a group, lower levels of lung function, as assessed by either FEV1 or FEV1/FVC ratio during the early school years, and these lung function deficits remained relatively stable along the course of the disease and into adult life. These data thus suggested that the resting patency of the airways could be an important determinant of asthma severity, and that blocking the development of airflow limitation could be potential strategy for the prevention of the disease.

What remained undefined, however, was the timing of the inception of these lung function deficits. Specifically, it was unknown if the factors determining the deficits were operative in utero or mainly after birth. To elucidate this issue, birth cohort studies were needed that assessed lung function shortly after birth and followed those tested until a diagnosis of asthma could be ascertained. There are now several such cohort studies, and they have provided data addressing this issue specifically. Studies using shape indexes for the tidal flow volume curve (4) or maximal flows assessed with the chest compression technique (8) have shown that, as a group, newborns that will go on to develop persistent asthma symptoms have altered lung function at birth as compared with those who do not. In subjects with active asthma, further airflow limitation does emerge thereafter, more markedly during the preschool years (9,10) but also between the ages of six and eighteen years (11). As a result, approximately one-third of the deficits in lung function observed in adults with childhood onset asthma were already present at birth (Figure 16-1).

Newborn cohorts have also allowed for a detailed study of the natural history of asthma and asthma-related syndromes during childhood. The picture that emerges is one of marked heterogeneity. During the first years of life, many children have recurrent asthma-like symptoms (wheeze, cough, shortness of breath), especially during viral infections, but not all go on to develop the chronic, persistent form of the disease that is more often associated with the label of asthma. These children have been dubbed "transient early wheezers," and they are clinically undistinguishable in terms of severity and

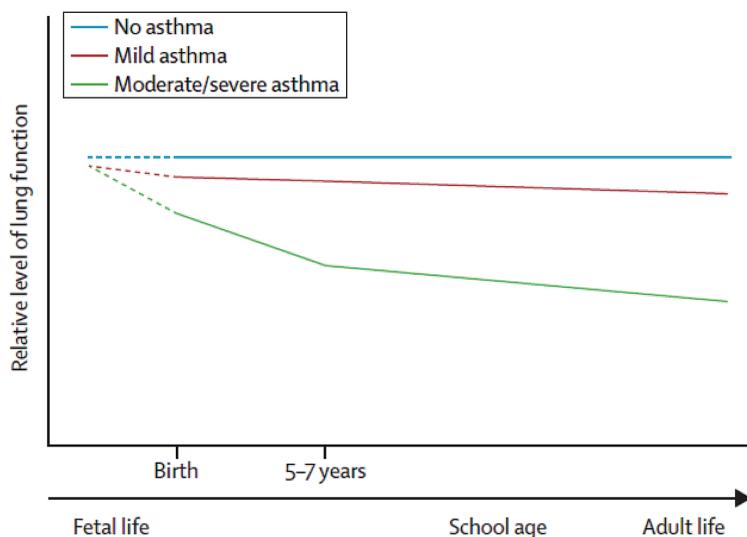


Figure 16-1. Schematic representation of the course of lung function for persons with asthma of different severities as compared with persons without asthma. Both lung function at birth and deficits occurring after birth play a role in determining the course of the disease for a lifetime. (Reprinted with permission from Stern, Morgan, Wright, Guerra, Martinez. *Lancet*. 2007;370(9589):758–764).

frequency of symptoms, from “persistent wheezers,” that is, those whose symptoms start before the age of three but are still present in the early school years. Interestingly, transient wheezers were found to have significantly lower levels of lung function measured shortly after birth than children who did not wheeze during the first years of life (12). These children’s lung function also improved concomitantly with the remission of their symptoms, but was still significantly diminished up to early adult life. It is thus plausible to surmise that the congenital airflow limitation present in these children may be causally involved in the pathogenesis of their disease. Because resistance to flow in a tubular structure is a function of the fourth power of the radius and normal infants have narrower airways, relative to the size of their lungs, than older children (13), any further narrowing of the airway would have significant functional consequences for the affected child. The improvement in lung function that these children show with aging could also explain why their symptoms are transient and do not persist beyond the early years.

In summary, the factors that control the level of lung function with which a child is born may play a critical role in determining that child’s susceptibility for the development of both transient and persistent airway obstruction, starting in early life.

Tracking of Lung Function with Age

The availability of cohorts in which lung function has been followed from birth and up to the adult

years has provided further support to the concept that factors active in utero may play a critical role in determining the potential level of lung function that a subject may attain into adult life. It has been known for years that lung function tracks markedly during the school years (14), but the role of prenatal versus postnatal influences had not been elucidated. Using data from the Tucson Children’s Respiratory Study, Stern et al. (15) showed that up to 14% of the variance in measurements of airway function in young adults, including FEV1 and FEV1/FVC ratio, was explained by the maximal flows at functional residual capacity, an index of airway function from partial expiratory flow-volume curves, measured in the same subjects at a mean age of two months. The study excluded premature children who required medical attention as newborns. A more detailed analysis of the data suggested that most of the association between infant airway function and adult lung function was attributable to subjects who had already diminished airway function shortly after birth. Individuals who were in the lowest quartile for lung function shortly after birth had mean predicted FEV1/FVC ratios of 75.1% at age twenty-two years, a level that was 5.2% lower than mean values for subjects in the other three quartiles. The authors suggested that this could indicate that subjects with airflow obstruction at birth are those more likely to remain in the lowest end of the distribution until early adult life, whereas tracking of airway function may be less evident in children with normal airways. Thus, combinations of genetic and

environmental factors that strongly affect lung growth in utero may have deleterious effects that last into adult life.

COPD and Development of Lung Function

COPD is a disease mostly affecting older adults that is both defined and classified into different stages of severity predominantly based the level of lung function after the administration of a bronchodilator (3). Specifically, for example, subjects who have an FEV₁/FVC ratio <70% and an FEV₁ that is less than 80% of the value predicted for their sex, size, and age are classified as having stage 2 COPD, stage 1 being mostly a preclinical phase in which only FEV₁/FVC is abnormal. Until recently, COPD was considered mostly a disease due to smoking, based on the fact that the great majority of affected individuals in the developed world are indeed smokers. Because only 15–50% of smokers develop COPD, the most accepted hypothesis about the pathogenesis of the disease was that certain subjects were more sensitive than others to the direct toxic effects of cigarettes. Thus, the assumption was that patients with COPD reached early adult life with essentially normal lung function, and that most of their airflow limitation was the result of subsequent accelerated decline in lung function (curves (c) and (d), Figure 16-2). However, recent longitudinal studies of COPD have challenged the idea that this is the only course that the disease can take. In the ECLIPSE study, for example, more than half of patients with COPD followed for a

period of three years showed no more decline in FEV₁ than that which is observed in subjects without lung disease (16). If COPD is not invariably a progressive disease, the origins of the airflow limitation present in those who do not show excessive lung function decline remained undefined.

One potential explanation could be that genetic and environmental factors that control the development of lung function in utero and during childhood and, therefore, determine the maximum level of lung function attained in adulthood, play an important role in the pathogenesis of COPD. This "early origins" hypothesis (represented in curve (b), Figure 16-2) postulates that many smokers with COPD develop the disease not because of excessive decline in lung function as adults, but because they reach early adult life with a certain degree of irreversible airflow limitation, which in most cases is still asymptomatic. When the normal process of lung function decline takes place, these subjects are at increased risk of reaching the critical level of airway obstruction that defines the different stages of COPD and are also more likely to develop respiratory symptoms associated with smoking, thus fitting the typical clinical presentation of the disease. All this is without the need to show any additional decline of lung function with aging beyond what is characteristic of this age group.

The data from ECLIPSE and other recent longitudinal studies suggest that up to half of all patients with COPD may follow this "early origins" trajectory to the inception of the disease. It is thus critical to understand what

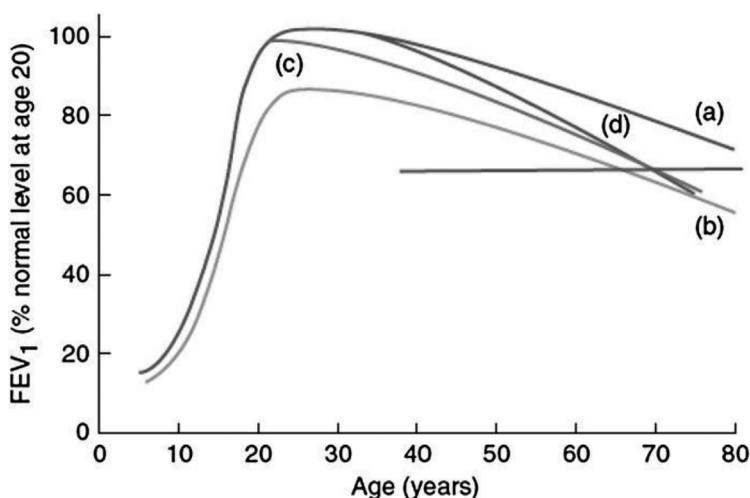


Figure 16-2. Different pathways to chronic obstructive pulmonary disease (COPD). (a) Normal course of lung function growth and decline in subjects without COPD. (c, d) Premature and late accelerated decline in lung function, mainly associated with active cigarette smoking in subjects who reach early adult life with normal levels of lung function. (b) The early origins of COPD hypothesis: a critically low level of lung function (horizontal line) is achieved by subjects who show normal decline in lung function with aging, but who reached only a fraction of the peak of lung function at age twenty years (Reprinted with permission from Proceedings of the American Thoracic Society, 6, 2009).

determines the level of lung function reached in early adult life.

Genetics of Lung Function

There is now solid evidence indicating that levels of lung function, adjusted for age, sex, and height, are genetically controlled. Heritability of lung function, that is, the fraction of phenotype variability that can be attributed to genetic variation, can be calculated both from hundreds of thousands of single-nucleotide polymorphisms (SNPs) that span the whole genome and that have been widely used to study genetic association with a large number of human phenotypes, and from segregation of lung function within pedigrees. Similar heritability coefficients (0.50 for FEV₁ to 0.66 for FEV₁/FVC) were reported for genome-wide association studies (GWAS)- and pedigree-based studies when both methods have been assessed in the same population (17). A meta-analysis of the results for GWAS for spirometry performed in four cohorts for a total of 20,890 individuals of European ancestry (18) identified eight loci associated with FEV₁/FVC: the gene for hedgehog interacting protein (*HHIP*) on chromosome 4q31, which is known to participate in developmental processes; the gene for a G protein-coupled receptor, *GPR126* on chromosome at 6q24.1, known to be involved in the genetics of stature (19), and thus possibly resulting from insufficient adjustment of the results for height; a gene for a member of the “a disintegrin and metalloprotease” (ADAM) family of membrane-anchored glycoproteins that control cell-matrix interactions and help regulate growth and morphogenesis, *ADAM19 at 5q33.3*; a locus between the genes for the advanced glycosylation end product (*AGER*) and Palmitoyl-Protein Thioesterase 2 (*PPT2*) at 6p21; a gene of unknown function, *FAM13A* at 4q22.1; a gene for Protein patched homolog 1, *PTCH1*, which is also a hedgehog interacting protein, at 9q22.32; and genes for phosphotyrosine interaction domain containing 1 (*PIDI*) at 2q36.3 and 5-hydroxytryptamine (serotonin) receptor 4 (*HTR4*) at 5q31-33. One locus associated with FEV₁ contained several genes and was located at 4q24. A second meta-analysis of GWAS results from the SpiroMeta consortium (20), which included 20,288 individuals of European ancestry, replicated many of the loci described earlier, and

described one additional locus associated with FEV₁/FVC ratio, thrombospondin type I domain containing 4 (*THSD4*) at 15q23, which interestingly is also related to the ADAM family.

Subsequent to the meta-analysis described earlier, an even larger GWAS assessed genetic determinants of FEV₁/FVC ratio and FEV₁ in 48,201 individuals of European ancestry with follow-up of the top hits in up to an additional 46,411 individuals (21). This study identified an additional sixteen loci associated with lung function and replicated many of the loci reported earlier.

Both children and adults were included in these two very large studies, and results were not confounded by smoking, which suggests that the genes identified most likely regulate lung functional growth during fetal life and the growing postnatal years. However, as is true for almost all complex human phenotypes, the loci identified explain only a small fraction of the variability of lung function in the population. In the Soler–Artigas study, which involved almost 100,000 persons, the authors calculated that the twenty-six newly identified and replicated loci explained approximately 3.2% of the additive polygenic variance for FEV₁/FVC and 1.5% of the variance for FEV₁ (21). Using statistical tools that estimate the number of susceptibility loci and the distribution of their effect sizes for a trait on the basis of discoveries from existing GWAS, the authors calculated that there were an additional seventy-six putative undiscovered variants, which, when added to the other twenty-six loci, collectively explain around 7.5% of the additive polygenic variance for FEV₁/FVC and 3.4% of the variance for FEV₁. Clearly, even when extremely large numbers of subjects are studied, a large gap remains between the heritability calculations described earlier (i.e., 50–66% of variation in lung function explained by genetic variation) and the low fraction of that variation detected through GWAS.

Several explanations have been proposed for this gap, the so-called hidden heritability. It is quite likely that, much like is the case for stature, lung function is a highly polygenic condition, and, therefore thousands of common and rare genetic variants with very small effects may be at play. If effects of common variants included in GWAS are too small, there would be very little power to detect them with a few thousand cases

and controls. In the case of rare variants, which are usually not included in GWAS chips, the only way to thoroughly address their role is to sequence the whole genomes of large numbers of individuals, which until recently was not feasible due to the high costs of such an enterprise. Fortunately, costs of sequencing are rapidly decreasing, and it is thus to be expected that studies of the association between rare variants and lung function will become available in the near future.

As explained earlier, adult lung function is determined by a combination of the level of lung function present at birth and the deficits in lung function growth that emerge during the different phases of postnatal development and senescence. GWAS of lung function have included mixed populations of children and adults, and it is thus not possible to know if the genetic variants identified in these studies are determinants of the “basal” lung function that tracks with age from birth or of the deficits in lung function growth (or accelerated decline) occurring in postnatal life. This issue is a complex one to address because it requires the availability of a rather large population in which both newborn and postnatal lung function has been reliably tested. Kreiner-Møller et al. (22) measured lung function at birth and at seven years of age in 284 children of asthmatic mothers and assessed the association between indices from these measurements and a composite score of genetic risk, calculated based on some of the reported SNPs for adult FEV1/FVC ratio and FEV1 described earlier, as the number of risk alleles weighted on the effect size reported in the original adult studies. They reported that the genetic risk scores were not significantly associated with lung function measures at age one month, but the genetic risk score for adult FEV1/FVC was significantly associated with reduced forced expiratory volume at 50% of vital capacity (FEF50) at age seven years and similarly with reduced growth in FEF50 from birth to age seven years. These results thus suggested that a significant proportion of the variance explained by the GWAS studies may reflect deficits of lung functional growth that occur after birth. However, this result may be influenced by the fact that all children in this study had mothers with asthma, and it is thus plausible to surmise that these children may be more likely to have asthma, with associated deficits in lung functional growth associated

with the disease. Larger studies involving unselected general populations are needed to further elucidate these complex issues.

It is also still possible that there are common genetic variants with meaningful effects that are not detected by the current GWAS chips. This could be the case for common variants that are not included and are not correlated (in technical terms: not in “linkage disequilibrium”) with the large number of SNPs included in those chips. An interesting example is vascular endothelial growth factor A (VEGFA). VEGFA is known to play a major role in the regulation of airway and alveolar growth in utero. Reduction of VEGF expression during fetal life is known to impair lung microvascular, airway, and airspace maturation in mice (23,24). Simpson et al. reported that genetic variants not present in available GWAS chips, and not correlated with variants included in those chips, were associated with lung function measured shortly after birth, during childhood, and up to adult life (25). The most strongly associated variant, rs305028, appeared to alter splicing and affect the balance between the inhibitory (VEGF-A165b) and active (VEGF-A165a) isoforms of the molecule. In summary, genetics plays a major role in determining the distribution of lung function in the general population, and these effects can be detected even shortly after birth.

Prematurity and Lung Function

There is increasing evidence that premature birth is associated with significant respiratory sequelae, both in terms of respiratory morbidity and long-term alterations in lung function. Data from a small number of adolescents and young adults born before 1973 and who developed bronchopulmonary dysplasia (BPD) showed that 68% had airway obstruction, as demonstrated by an abnormally low FEV1, and 24% had fixed airflow limitation (26). A Dutch study followed forty-two children born in 1983 with a gestational age of less than thirty-two weeks and/or a birth weight under 1,500 g up to the age of nineteen years (27). As compared to controls, former premature subjects had significantly lower FEV1 and a decreased diffusing capacity. Although the authors claimed that there were no differences in lung function between preterms that had or did not have a history of BPD, the numbers of subjects were probably too small to justify

definitive conclusions (28). In a study of all singleton infants born in Sweden in 1973–1979 ($n = 622,616$) and followed up to early adult life, Crump et al. (29) reported that those born extremely premature (twenty-three to twenty-seven weeks gestation) were 2.4 times more likely (adjusted 95% CI: 1.41–4.06) to be prescribed asthma medications in 2005–2007 than those who were born at term. No association was found between later preterm birth (twenty-eight–thirty-two or thirty-three–thirty-six weeks gestation) and asthma medications in young adulthood.

Subjects included in these three studies were born before the availability of surfactant for therapeutic use. More recent surveys have assessed long-term sequelae of current graduates of neonatal intensive care, in whom BPD is most often observed in extremely premature infants who have been treated with surfactant and also with gentler ventilatory regimens than in the past. Anatomical studies indicate that this so-called “new BPD” is characterized by decreased incidence of airway fibrosis (30), suggesting the possibility that the long-term impairment in airway function in these children may be less severe than that observed in former preterms from the presurfactant era. Friedrich et al. (31) showed that healthy infants born prematurely had decreased forced expiratory flows and normal forced vital capacities during the first and second years of life. Based on their finding that increase in lung function with growth was similar to those observed in full-term children, they speculated that premature birth is associated with altered lung function development that may persist beyond the first years of life. Fawke et al. (32) reported the results of lung function measurements at age eleven in 182 participants enrolled in the EPICure Study, which included all children born at or less than twenty-five full weeks of gestation in the United Kingdom and Ireland between March and December 1995. They found that, as a group and compared with appropriate controls, these children had significantly lower mean \pm SE pre-bronchodilator FEV1 ($83 \pm 14\%$ predicted vs. $100 \pm 12\%$ predicted) and FEV1/FVC ratio ($86 \pm 0.1\%$ vs. $79 \pm 0.1\%$).

Interestingly, alterations were most severe among participants with prior BPD (71% of entire cohort): mean \pm SE values were $80 \pm 13\%$ predicted and $78 \pm 10\%$ for FEV1 and FEV1/FVC

ratio, respectively. Reports of asthma-like symptoms were also higher in all preterms, but especially among those with BPD. These results have been recently confirmed by a longitudinal study of children with BPD followed up to a mean age of 9.5 years (33), which also showed a mild restrictive component (i.e., a significant decrease in FVC) in these children.

A recent meta-analysis (34) assessed all available studies in which FEV1 was assessed in later life in preterm-born subjects, with or without BPD, compared with term-born controls. Fifty-nine studies were included. Former preterm-born subjects without BPD, those with BPD with supplemental oxygen requirement at twenty-eight days, and those with BPD with supplemental oxygen dependency at thirty-six weeks postmenstrual age had deficits in size-adjusted FEV1 of 7.2%, 16.2%, and 18.9%, respectively, when compared with term-born controls. Pooled mean % predicted FEV1 for all subjects included in the different studies was 91% (95% confidence interval 88.8% to 93.1%) for preterm born subjects without BPD, 83.7% (80.2% to 87.2%) for BPD with supplemental oxygen requirement at twenty-eight days, and 79.1% (76.9% to 81.3%) for BPD with supplemental oxygen dependency at thirty-six weeks postmenstrual age.

Few studies have studied lung function longitudinally to determine if there is any improvement during childhood in children with different degrees of prematurity. Filbrun et al. (35) assessed lung function on two occasions in preschool children with a history of BPD. They confirmed that these children had a marked obstructive spirometric pattern with, in addition, a modest restrictive component. In the group as a whole, maximal flows and volumes tracked with age, thus suggesting no apparent catch-up growth in lung function. However, children with above average somatic growth during the interval between the two tests showed improvement in both restrictive and obstructive pattern as compared with their peers with less somatic growth. This finding strongly supports the contention that aggressive postnatal nutritional care may have a favorable effect on the respiratory outcome of children with a history of BPD (36). Using data from the Avon Longitudinal Study of Parents and Children (ALSPAC), spirometric indices were compared at eight to nine years of age ($n = 6705$) and

fourteen to seventeen years of age ($n = 4508$) between term-born children and those born after twenty-five to thirty-two, thirty-three to thirty-four, and thirty-five to thirty-six weeks gestation, respectively (37). No significant deficits in any lung function measurements were found among children born after thirty-five to thirty-six weeks at either age eight to nine or fourteen to seventeen years. At eight to nine years of age, FEV1, but not FVC, was significantly lower, in the twenty-five- to thirty-two-week gestation group compared with the term group. At this same age, FEV1 and FVC were significantly lower in the children born after thirty-three to thirty-four weeks gestation as compared with the term group. Mean deficits compared with term infants after adjustment for age, gender, and height were 143 mL for FEV1 and 98 mL for FVC in the thirty-three- to thirty-four-week gestation group and 121 mL for FEV1 and 65 mL for FVC in the twenty-five- to thirty-two-week gestation group. At fourteen to seventeen years, FEV1 and FVC were no longer significantly different in the thirty-three- to thirty-four-week gestation group as compared with term infants. Implied in this finding is the possibility that the lung function deficits observed early in life in these late preterm children could improve with time. In addition, the finding that both FEV1 and FVC were affected suggested that prematurity may be not only be associated with an obstructive respiratory defect but also with a restrictive one.

It is plausible to surmise that, as is the case for other young adults who do not reach their highest possible level of lung function in early adult life, adults born extremely premature should also be more susceptible to the effects of active cigarette smoking and other harmful exposures. Surprisingly, there are no studies assessing the specific effects of either smoking (passive or active) or air pollution on rates of decline in lung function among extremely premature children.

In summary, and given that lung function tracks markedly with age, it is likely that a large proportion of extremely premature infants, and especially those with BPD, will reach adult life with 10–20% lower levels of FEV1 as compared to their age peers. With the normal decline of lung function that occurs with aging, these children are thus at high risk of reaching the critical level of airflow limitation that would classify them as having COPD. There is some evidence

that extreme prematurity and BPD may also be associated with a restrictive spirometric profile (33,35); such a profile, when present in adults, has been shown to increase subsequent risk for cardiovascular death (38). Mild to moderate prematurity is associated with modest changes in lung function, which may not have major impact on the risk for chronic respiratory impairment later in life.

Genetics of BPD

Although the association of extremely premature birth, and especially that associated with BPD, to subsequent impairment in lung function is clearly established, not all extremely premature infants go on to develop BPD. This raises the possibility that genetic factors may predispose to the development of BPD and, therefore, to the deleterious long-term respiratory outcomes associated with extreme prematurity. Comparisons of concordance for complex diseases such as BPD among monozygotic and dizygotic twins provide a strong tool to assess the role of genetic factors in the development of these diseases. Two studies (39, 40) that have addressed this issue yielded similar results: Concordance of BPD was significantly higher among monozygotic than among dizygotic twins, with a calculated heritability (i.e., the proportion of the variability of the trait that is explained by genetic factors) of between 53 and 80%. To determine which genetic variants may explain this striking role of hereditary factors on BPD, GWAS have been recently attempted. In these studies, the frequencies of hundreds of thousands of SNPs distributed across the whole genome are compared between affected subjects and appropriate controls. Hadchouel et al. (41) assessed a relatively small number of infants ($n = 418$) born after < 28 weeks gestation of European and African ancestry, of whom 22% developed BPD. Genetic variants in only one gene, *SPOCK2* on chromosome 10q22.1, were found to be significantly associated with BPD in both ancestries, and results were replicated in a third population in Finland. Although the observed odds ratios for the most strongly associated SNP (rs1245560: 3.0 among Whites and 4.9 among Blacks, respectively) are high for these types of studies, the proportion of the total variance explained by this SNP was very low. *SPOCK2*, also called Testican-2, is a member of the testican group of

extracellular chondroitin and heparan sulfate proteoglycans. Studies in rats showed that *SPOCK2* is expressed in the developing rat lung and that mRNA levels changed with stages of lung development. *SPOCK2* expression was low during the canalicular and saccular stages of rat lung development and increased significantly at the beginning of alveolarization, remaining high until the alveolarization was completed. It has been suggested that this pattern of expression may be consistent with the involvement of *SPOCK2* in the control of septation (42). Interestingly, *SPOCK2* was recently found to play an active role in the transdifferentiation of surfactant-producing pulmonary alveolar epithelial type II (AT2) cells into type I (AT1) cells (42). These results thus seemed to provide support to the contention that genetic studies could provide clues to the pathogenesis of BPD and also potential therapeutic targets for the prevention of BPD and of the deficits in lung function that are often a long-term sequelae of the disease.

A more recent GWAS, however, yielded disappointing results. The new study (43) included 1,726 very low-birth-weight infants with a gestational age of twenty-five to less than twenty-nine weeks who had a minimum of three days of intermittent positive pressure ventilation and were in the hospital at thirty-six weeks postmenstrual age. At that age, BPD cases (52%) required continuous supplemental oxygen and were defined as having BPD, whereas controls (48%) did not. Almost 1.8 million SNPs were successfully genotyped, and none showed significant genome-wide association with BPD status (preestablished at $p \leq 5 \times 10^{-8}$). Moreover, SNPs identified in both candidate gene studies and GWAS, including those in *SPOCK2*, could not be replicated. There are many possible explanations for these discrepancies between studies, including (among others) differences in the definition of the phenotypes, insufficient power, and inclusion of subjects from different ethnic backgrounds. The negative study by Wang et al., for example, included mostly children born in the United States of Hispanic/Mexican origins. There is strong evidence that different sets of genetic variants and genes may determine susceptibility of the same phenotypes in Mexican-Americans, who as a group show evidence of European–Native American admixture, as compared with subjects of European origins (44,45). As has been

the case for most other complex phenotypes, the genetic variants that explain most of the “hidden heritability” of BPD remain to be ascertained, and their identification may require large, whole genome sequencing studies.

Lung Structure and Function in Animal Models of Premature Birth and BPD

Preterm sheep and baboons have been used to develop experimental models of both BPD, associated with the requirement for neonatal mechanical ventilation, and preterm status per se, in the absence of respiratory support. In models of BPD, observed pathological changes are similar in preterm infants with BPD: Chronically ventilated preterm baboons and preterm lambs show alveolar simplification, persistent smooth muscle hypertrophy of both pulmonary arterioles and small airways (46). The main factor determining alveolar simplification is insufficient growth of secondary septa from the walls of the saccules, which in turn decreases the number of alveoli and the airspace surface area for gas exchange. Elastin synthesis plays a major role in septa growth, and it has been shown that elastin synthesis is continuously upregulated in chronically ventilated preterm baboons and preterm lambs (47). Decreased alveolarization in baboon models of BPD has been shown to persist up to thirty-three weeks, which corresponds to approximately two years of age in humans (48). In addition, these animals show stunted development of septal capillaries and thickened airspace walls, which could explain the restrictive spirometric pattern observed in some studies of adult with a history of BPD.

Baboon and lamb models of preterm birth followed by mechanical ventilation also shed light on the development of chronic airflow limitation that is observed in many survivors of BPD. Indeed, these animals have increased airway expiratory resistance and lower lung compliance. The main histopathological change associated with these functional impairments is increased accumulation of airway smooth muscle and a thickened airway wall (46).

Fewer studies have used animal models to study the association of prematurity per se in the absence of any ventilatory requirement, with the subsequent development of respiratory

impairment. In sheep, these studies are limited to animals delivered two weeks premature (at 133 days gestation), the earliest gestational age at which mechanical ventilation is not required (49). Respiratory system compliance was not different at term equivalent age in preterm lambs as compared with term lambs, but was higher in preterm lambs at six weeks post-term-equivalent age. Pulmonary resistance was slightly, but not significantly, higher in preterm lambs than in controls at term-equivalent age, but this difference was no longer present at age six to eight weeks post-term age. Alveolar septa were 33% thicker and the blood–air barrier was 26% thicker in preterm lambs than in controls at term-equivalent age and remained thicker at six weeks post-term-equivalent age. In preterm lambs, the airway epithelium was also thicker at term-equivalent age and at six weeks post-term-equivalent age (50). These studies thus confirm the conclusion reached from studies in humans that mild to moderate preterm birth by itself is associated with only mild disruption of lung and airway structure (51), which does not seem to have clinically important long-term functional consequences during childhood and adult life.

Intrauterine Growth Retardation and Lung Function

The role of an adverse intrauterine environment on subsequent lung function and COPD risk has been an essential component of the “fetal origins” hypothesis first proposed by Barker (52). It is reasonable to assume that the mechanisms responsible for potential harmful effects on lung growth would be different between premature birth and intrauterine growth retardation (IUGR) associated with malnutrition or other noxious exposures occurring during pregnancy such as maternal smoking. In many studies addressing this issue, however, either gestational age was not available or birth weight was directly related to subsequent lung function, without attempting to specifically determine the separate roles of prematurity and IUGR. A meta-analysis (53) of nine studies among adults showed a positive association between birth weight and FEV1, with FEV1 increasing by 0.048 l (95% CI 0.026 to 0.070) for each increase of 1 kg birth weight. More recently, Hancox et al. (54) related birth weight to lung function at age thirty-two years in a birth

cohort in Dunedin, New Zealand. Birth weight was not significantly associated with either FEV1 or FVC when the latter were adjusted for height and sex, although the coefficient for FEV1 was very similar to that reported in the previous meta-analysis (50 ml, 95% CI –8 to 120).

A few studies have specifically assessed lung function across the life span in small-for-gestational-age (SGA) children and compared results with those for their appropriate-for-gestational-age (AGA) peers. Results of a study of SGA (<10th percentile birth weight for gestational age) infants suggested that both flows and volumes derived from flow-volume loops measured with the raised volume technique were significantly lower than those of AGA infants after adjusting for relevant maternal and infant characteristics (55). In a study limited to premature children, mean airway resistance was found to be significantly higher at ages six to twenty-four months in thirty-one SGA infants with a mean gestational age of thirty-one weeks as compared to their AGA peers with a mean gestational age of twenty-eight weeks (38 vs. 34 cmH₂O/L*s, respectively, $p = 0.004$) (56).

Deficits in lung function in SGA individuals have also been reported in two large studies in older children. Rona et al. (57) first suggested that children ages five to eleven who were SGA at birth had significantly lower lung function than AGA children. They estimated that the difference in expected FEV1 between two children with thirty-eight weeks of gestation (expected birth weight of 3,300 g), but one having a birth weight of 2,500 g and the other 3,500 g, would be 26 mL, or 1.7% of the expected FEV1. In the ALSPAC study quoted earlier (34), term children ages seven to eight years who had showed evidence of intrauterine growth retardation at birth had mean deficits of 50 mL for FEV1 and 40 mL for FVC, adjusted for height, age, and sex, when compared with AGA children. Observed deficits were statistically significant, consistent, but relatively modest in both studies. There are no reports that have specifically assessed the association between SGA status and adult lung function.

Of interest is the possibility that, as was suggested for prematurity, deficits in lung function associated with IUGR could improve with time. Catch-up growth does occur between birth and puberty in many SGA children, and it is possible that lung function may also improve in these

children more than in those with no catch-up growth. In support of this contention, Suresh and coworkers compared lung function at age twenty-one years in subjects with IUGR at birth, that is, those in the lowest quintile for birth weight (58); no apparent adjustment was made for gestational age. Within the IUGR group, both FVC and FEV1 were significantly higher in children who showed catch-up growth than in those who did not, and this was true for catch-up growth assessed between birth and five years and between birth and fourteen years. This raises the possibility that aggressive nutritional care during childhood may reverse the significant effects of IUGR on long term-lung function up to adult life.

Role of Malnutrition and Exposure to Tobacco Smoke

Two known causes of IUGR in humans and their potential role in susceptibility to chronic respiratory impairment have been thoroughly studied: maternal malnutrition, which still affects a significant proportion of the population in the developing world; and maternal smoking during pregnancy. In the case of maternal malnutrition, the Dutch famine of 1944–45 has provided the most important clues. The Dutch famine was a circumscribed episode of severe human starvation that occurred during a six-month period lasting from October 1944 to May 1945 (59). At the height of the famine, from December 1944 to April 1945, the official daily rations varied between 400 and 800 kcal. Studies using records from that period show that subjects who were exposed to the famine during late and mid-gestation had a lower birth weight, body length, and ponderal index (weight divided by the cube of length) and smaller heads. However, paradoxically, those exposed to famine in early gestation had a higher weight and body length at birth than nonexposed subjects (60). That there may be catch-up lung growth during malnourished pregnancies seems to be corroborated by experimental studies. Protein restriction during pregnancy in mice resulted in fetuses with impaired branch morphogenesis (day 12 of gestation) but by day 13, rapid recovery had already occurred (61).

In a study of survivors of the Dutch famine, lung function was successfully measured in 733 individuals age 50 whose mothers had been

subjected to severe food restrictions during pregnancy. There was no difference in any spirometric parameter between subjects exposed to famine in utero and those not exposed (60). A more recent study assessed the impact on COPD of exposure to the Dutch famine during postnatal life (62). Women who reported to be exposed to moderate or severe famine were significantly more likely to have been hospitalized for COPD by a mean age of 60 years than their age peers not exposed to famine. These effects were particularly noticeable (and only significant) among women who were active smokers, suggesting that postnatal malnutrition may increase susceptibility to the deleterious effects of smoking.

The potential role of vitamin D deficiency during pregnancy on fetal lung growth and subsequent lung function has been the subject of intense scrutiny during the last decade. Vitamin D is known to play a role in alveolar growth in the embryo and fetus, and thus, vitamin D deficiency during pregnancy could plausibly affect lung function in the offspring. Older studies in rats (63) showed that offspring of mothers deprived of vitamin D during pregnancy had lower lung volumes and decreased lung compliance at fifty days postnatally when compared with offspring of mothers receiving usual doses of vitamin D. More recent studies showed increased bronchial responsiveness, decreased radial alveolar counts, and increased linear intercepts in offspring rats of vitamin D deficient pregnancies (64). In humans, no association was found between vitamin D levels in the mother at thirty-six weeks gestation and their offspring's mean FEV1 at age six to seven years (65). Likewise, no association was reported between cord blood levels of vitamin D and lung function assessed repeatedly during the first seven years of life in a newborn cohort of children at high risk for asthma in Denmark (66). In the only clinical trial currently available, 180 pregnant women were randomized at twenty-seven weeks gestation to either no vitamin D, 800 IU ergocalciferol daily until delivery, or a single oral bolus of 200,000 IU cholecalciferol. Supplementation improved but did not optimize vitamin D status. There was no difference between groups in lung function, as assessed by impulse oscillometry, or in any other respiratory outcome at age three (67). Larger, ongoing randomized trials evaluating the effects of vitamin D supplementation during pregnancy are likely

to provide more definitive answers in establishing the clinical significance of maternal vitamin D levels during pregnancy on lung function and asthma risk in their offspring(68).

Similar to vitamin D, vitamin A is known to influence alveolar and airway growth in fetal life. In a major study of vitamin A supplementation during pregnancy in Nepal, lung function was assessed at ages nine to thirteen years in children whose mothers had received weekly oral supplementation with either 7,000 µg retinol equivalents of vitamin A, 7000 µg retinol-equivalents of beta carotene, or placebo (69). Of note, this was a chronically undernourished population prior to initiation of the trial. Spirometry was performed in 1,371 offspring of the participating mothers. Children whose mothers had received vitamin A, but not those whose mothers had received beta carotene, had mean FEV1 and FVC levels that were significantly higher than those of children whose mothers had received placebo. The improvements in lung function (46 mL for both FEV1 and FVC) with vitamin A treatment amounted to a 2.6% and 2.9% improvement in FEV1 and FVC, respectively, which should be considered clinically meaningful.

The effects on lung function of exposure to tobacco smoke in utero and postnatally have been the matter of considerable research during the last thirty years. Experimental studies in animal models have convincingly shown that fetal exposure to nicotine negatively affects lung and airway development, reducing surface complexity of the lung parenchyma and increasing collagen deposition in the airways (70). In 1998, Cook and Strachan summarized the results of twenty-one studies comparing spirometric indices in children by exposure to parental smoking (71). They concluded that the reduction in FEV1 in children exposed to parental smoking compared with those not exposed was 1.4% (95% CI 1.0 to 1.9). These effects were considered "modest but statistically significant." Studies that distinguished between maternal and paternal exposure indicated that most of the effects were due to exposure in utero and during the neonatal period. Studies performed after 1998 have, in general, confirmed that children exposed to tobacco smoke in utero do have alteration in lung function, but the nature of these alterations differs from study to study. Hollams and coworkers (72), for example, assessed lung function at age

fourteen years in participants in a birth cohort in Perth, Australia. They found that offspring of mothers who smoked during pregnancy had similar FEV1 but larger FVC compared to those whose mothers did not smoke; as a consequence, diminished FEV1/FVC ratio was found in those exposed.

Whether effects of parental smoking persist into adult life has not been thoroughly explored. As part of the European Community Respiratory Health Survey, Svanes, et al. assessed lung function in 15,901 adults age twenty to forty-five who self-reported about the smoking habits of their parents (73). Adults who reported a history of maternal smoking during pregnancy had FEV1/FVC ratios that were 0.9% lower than those who did not ($p < 0.001$). Interestingly, they were also more likely to have chronic bronchitis and wheeze, but not asthma than their age peers not exposed to smoking during pregnancy. Guerra et al. (74) studied 519 adults enrolled in the Tucson Children's Respiratory Study. They found no association between parental smoking during childhood, assessed longitudinally, and adult lung function up to age twenty-six years, but the study was not powered to detect the small effects reported in the much larger study described earlier by Svanes et al. However, they did notice that adults who had been exposed to parental smoking and were themselves active smokers had levels of lung function that were significantly lower than those of control subjects exposed to neither, whereas those who were either exposed to parental smoking or smoked themselves, but not to both, had levels of lung function that were not different from those of controls. This suggests that an important sequela of exposure to tobacco smoke in utero and during the growing years may be increased susceptibility to noxious exposures in adult life.

Conclusions and Future Directions

Solid evidence suggests that lung growth during fetal and early postnatal life is one of the strongest determinants of adult lung function. Genetic variation plays a critical role in determining maximal lung function reached in adult life. Factors that affect lung growth such as extreme prematurity with BPD, IUGR, exposure to tobacco smoke in utero and postnatally, and vitamin A and D deficiencies also play varying

roles in determining lung function. However, catch-up growth seems to be able to reverse the negative effects of some of these conditions. Individuals who reach early adult life with lower levels of lung function are at increased risk of developing COPD during the decline phase of lung function after the third decade of life and may also be more susceptible to the deleterious

effects of active cigarette smoking. Future studies are needed to better identify children at risk for an early onset of chronic lung disease as well as susceptibility factors that contribute to disease progression. Such insights may lead to novel interventional strategies to reduce the burden of chronic respiratory disorders in adult life.

References

- Criner GJ, Bourbeau J, Diekemper RL, et al. Executive summary: prevention of acute exacerbation of Chronic Obstructive Pulmonary Disease: American College of Chest Physicians and Canadian Thoracic Society Guideline. *Chest*. 2014. Epub 2014/10/17. doi: 10.1378/chest.14-1677. PubMed PMID: 25320966.
- Mannino DM, Homa DM, Akinbami LJ, Ford ES, Redd SC. Chronic obstructive pulmonary disease surveillance—United States, 1971–2000. *MMWR Surveill Summ*. 2002;51(6):1–16. Epub 2002/08/30. PubMed PMID: 12198919.
- Vestbo J, Hurd SS, Agusti AG, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med*. 2013;187(4):347–365. Epub 2012/08/11. doi: 10.1164/rccm.201204-0596PP. PubMed PMID: 22878278.
- Haland G, Carlsen KC, Sandvik L, et al. Reduced lung function at birth and the risk of asthma at 10 years of age. *N Engl J Med*. 2006;355(16):1682–1689. Epub 2006/10/20. doi: 10.1056/NEJMoa052885. PubMed PMID: 17050892.
- Moorman JE, Zahran H, Truman BI, Molla MT. Current asthma prevalence—United States, 2006–2008. *MMWR Surveill Summ*. 2011;60 Suppl:84–86. Epub 2011/03/25. PubMed PMID: 21430629.
- Sears MR, Greene JM, Willan AR, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med*. 2003;349(15):1414–1422. Epub 2003/10/10. doi: 10.1056/NEJMoa022363. PubMed PMID: 14534334.
- Tai A, Tran H, Roberts M, Clarke N, Wilson J, Robertson CF. The association between childhood asthma and adult chronic obstructive pulmonary disease. *Thorax*. 2014;69(9):805–810. Epub 2014/03/22. doi: 10.1136/thoraxjnl-2013-204815. PubMed PMID: 24646659.
- Turner SW, Palmer LJ, Rye PJ, et al. The relationship between infant airway function, childhood airway responsiveness, and asthma. *Am J Respir Crit Care Med*. 2004;169(8):921–927. Epub 2004/02/07. doi: 10.1164/rccm.200307-891OC. PubMed PMID: 14764431.
- Morgan WJ, Stern DA, Sherrill DL, et al. Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence. *Am J Respir Crit Care Med*. 2005;172(10):1253–1258. Epub 2005/08/20. doi: 10.1164/rccm.200504-525OC. PubMed PMID: 16109980; PubMed Central PMCID: PMC2718414.
- Bisgaard H, Jensen SM, Bonnelykke K. Interaction between asthma and lung function growth in early life. *Am J Respir Crit Care Med*. 2012;185(11):1183–1189. Epub 2012/03/31. doi: 10.1164/rccm.201110-1922OC. PubMed PMID: 22461370.
- Strunk RC, Weiss ST, Yates KP, Tonascia J, Zeiger RS, Szefer SJ. Mild to moderate asthma affects lung growth in children and adolescents. *J Allergy Clin Immunol*. 2006;118(5):1040–1047. Epub 2006/11/08. doi: 10.1016/j.jaci.2006.07.053. PubMed PMID: 17088127.
- Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N Engl J Med*. 1995;332(3):133–138. Epub 1995/01/19. doi: 10.1056/NEJM199501193320301. PubMed PMID: 7800004.
- McKay KO, Hogg JC. The contribution of airway structure to early childhood asthma. *Med J Aust*. 2002;177 Suppl:S45–47. Epub 2002/09/13. PubMed PMID: 12225256.
- Wang X, Dockery DW, Wypij D, et al. Pulmonary function growth velocity in children 6 to 18 years of age. *Am Rev Respir Dis*. 1993;148(6 Pt 1):1502–1508. Epub 1993/12/01. doi: 10.1164/ajrccm/148.6_Pt_1.1502. PubMed PMID: 8256891.
- Stern DA, Morgan WJ, Wright AL, Guerra S, Martinez FD. Poor airway function in early infancy and lung function by

- age 22 years: a non-selective longitudinal cohort study. *Lancet*. 2007;370(9589):758–764. Epub 2007/09/04. doi: 10.1016/S0140-6736(07)61379-8. PubMed PMID: 17765525; PubMed Central PMCID: PMC2831283.
- 16 Vestbo J, Edwards LD, Scanlon PD, et al. Changes in forced expiratory volume in 1 second over time in COPD. *New Engl J Med*. 2011;365(13):1184–1192. Epub 2011/10/14. doi: 10.1056/NEJMoa1105482. PubMed PMID: 21991892.
- 17 Klimentidis YC, Vazquez AI, de Los Campos G, Allison DB, Dransfield MT, Thannickal VJ. Heritability of pulmonary function estimated from pedigree and whole-genome markers. *Front Genet*. 2013;4:174. Epub 2013/09/24. doi: 10.3389/fgene.2013.00174. PubMed PMID: 24058366; PubMed Central PMCID: PMC3766834.
- 18 Hancock DB, Eijgelsheim M, Wilk JB, et al. Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. *Nat Genet*. 2010;42(1):45–52. Epub 2009/12/17. doi: 10.1038/ng.500. PubMed PMID: 20010835; PubMed Central PMCID: PMC2832852.
- 19 Zhao J, Li M, Bradfield JP, et al. The role of height-associated loci identified in genome wide association studies in the determination of pediatric stature. *BMC Med Genet*. 2010;11:96. Epub 2010/06/16. doi: 10.1186/1471-2350-11-96. PubMed PMID: 20546612; PubMed Central PMCID: PMC2894790.
- 20 Repapi E, Sayers I, Wain LV, et al. Genome-wide association study identifies five loci associated with lung function. *Nat Genet*. 2010;42(1):36–44. Epub 2009/12/17. doi: 10.1038/ng.501. PubMed PMID: 20010834; PubMed Central PMCID: PMC2862965.
- 21 Soler Artigas M, Loth DW, Wain LV, et al. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. *Nat Genet*. 2011;43(11):1082–1090. Epub 2011/09/29. doi: 10.1038/ng.941. PubMed PMID: 21946350; PubMed Central PMCID: PMC3267376.
- 22 Kreiner-Moller E, Bisgaard H, Bonnelykke K. Prenatal and postnatal genetic influence on lung function development. *J Allergy Clin Immunol*. 2014. Epub 2014/05/27. doi: 10.1016/j.jaci.2014.04.003. PubMed PMID: 24857373.
- 23 Galambos C, Ng YS, Ali A, et al. Defective pulmonary development in the absence of heparin-binding vascular endothelial growth factor isoforms. *Am J Respir Cell Mol Biol*. 2002;27(2):194–203. Epub 2002/08/02. doi: 10.1165/ajrcmb.27.2.4703. PubMed PMID: 12151311.
- 24 Akeson AL, Cameron JE, Le Cras TD, Whitsett JA, Greenberg JM. Vascular endothelial growth factor-A induces prenatal neovascularization and alters bronchial development in mice. *Pediatr Res*. 2005;57(1):82–88. Epub 2004/11/24. doi: 10.1203/01.PDR.0000148070.89006.3F. PubMed PMID: 15557114.
- 25 Simpson A, Custovic A, Tepper R, et al. Genetic variation in vascular endothelial growth factor-a and lung function. *Am J Respir Crit Care Med*. 2012;185(11):1197–1204. Epub 2012/03/31. doi: 10.1164/rccm.201112-2191OC. PubMed PMID: 22461367; PubMed Central PMCID: PMC3373065.
- 26 Northway WH Jr, Moss RB, Carlisle KB, et al. Late pulmonary sequelae of bronchopulmonary dysplasia. *New Engl J Med*. 1990;323(26):1793–1799. Epub 1990/12/27. doi: 10.1056/NEJM199012273232603. PubMed PMID: 2247118.
- 27 Vrijlandt EJ, Gerritsen J, Boezen HM, Grevink RG, Duiverman EJ. Lung function and exercise capacity in young adults born prematurely. *Am J Respir Crit Care Med*. 2006;173(8):890–896. Epub 2006/02/04. doi: 10.1164/rccm.200507-1140OC. PubMed PMID: 16456146.
- 28 Kirkby J, Stanojevic S, Stocks J. Appropriate interpretation of lung function and exercise capacity in a longitudinal follow-up of preterm children. *Am J Respir Crit Care Med*. 2007;175(1):96–97; author reply 97. Epub 2006/12/21. doi: 10.1164/ajrccm.175.1.96. PubMed PMID: 17179500.
- 29 Crump C, Winkleby MA, Sundquist J, Sundquist K. Risk of asthma in young adults who were born preterm: a Swedish national cohort study. *Pediatrics*. 2011;127(4):e913–920. Epub 2011/03/23. doi: 10.1542/peds.2010-2603. PubMed PMID: 21422091; PubMed Central PMCID: PMC3387891.
- 30 Coalson JJ. Pathology of bronchopulmonary dysplasia. *Semin Perinatol*. 2006;30(4):179–184. Epub 2006/07/25. doi: 10.1053/j.semperi.2006.05.004. PubMed PMID: 16860157.
- 31 Friedrich L, Pitrez PM, Stein RT, Goldani M, Tepper R, Jones MH. Growth rate of lung function in healthy preterm infants. *Am J Respir Crit Care Med*. 2007;176(12):1269–1273. Epub 2007/09/22. doi: 10.1164/rccm.200703-476OC. PubMed PMID: 17885265; PubMed Central PMCID: PMC2176107.
- 32 Fawke J, Lum S, Kirkby J, et al. Lung function and respiratory symptoms at 11 years in children born extremely

- preterm: the EPICure study. *Am J Respir Crit Care Med*. 2010;182(2):237–245. Epub 2010/04/10. doi: 10.1164/rccm.200912-1806OC. PubMed PMID: 20378729; PubMed Central PMCID: PMC2913237.
- 33 Vom Hove M, Prenzel F, Uhlig HH, Robel-Tillig E. Pulmonary outcome in former preterm, very low birth weight children with bronchopulmonary dysplasia: a case-control follow-up at school age. *J Pediatr*. 2014;164(1):40–45.e4. Epub 2013/09/24. doi: 10.1016/j.jpeds.2013.07.045. PubMed PMID: 24055328.
- 34 Kotecha SJ, Edwards MO, Watkins WJ, et al. Effect of preterm birth on later FEV1: a systematic review and meta-analysis. *Thorax*. 2013;68(8):760–766. Epub 2013/04/23. doi: 10.1136/thoraxjnl-2012-203079. PubMed PMID: 23604458.
- 35 Filbrun AG, Popova AP, Linn MJ, McIntosh NA, Hershenson MB. Longitudinal measures of lung function in infants with bronchopulmonary dysplasia. *Pediatr Pulmonol*. 2011;46(4):369–375. Epub 2011/03/26. doi: 10.1002/ppul.21378. PubMed PMID: 21438170; PubMed Central PMCID: PMC3801101.
- 36 Jobe AH. Let's feed the preterm lung. *J Pediatr (Rio J)*. 2006;82(3):165–166. Epub 2006/06/15. doi: 10.2223/JPED.1481. PubMed PMID: 16773171.
- 37 Kotecha SJ, Watkins WJ, Paranjothy S, Dunstan FD, Henderson AJ, Kotecha S. Effect of late preterm birth on longitudinal lung spirometry in school age children and adolescents. *Thorax*. 2012;67(1):54–61. Epub 2011/09/29. doi: 10.1136/thoraxjnl-2011-200329. PubMed PMID: 21953066.
- 38 Guerra S, Sherrill DL, Venker C, Ceccato CM, Halonen M, Martinez FD. Morbidity and mortality associated with the restrictive spirometric pattern: a longitudinal study. *Thorax*. 2010;65(6):499–504. Epub 2010/06/05. doi: 10.1136/thx.2009.126052. PubMed PMID: 20522846; PubMed Central PMCID: PMC3036842.
- 39 Parker RA, Lindstrom DP, Cotton RB. Evidence from twin study implies possible genetic susceptibility to bronchopulmonary dysplasia. *Semin Perinatol*. 1996;20(3):206–209. Epub 1996/06/01. PubMed PMID: 8870123.
- 40 Lavoie PM, Pham C, Jang KL. Heritability of bronchopulmonary dysplasia, defined according to the consensus statement of the national institutes of health. *Pediatrics*. 2008;122(3):479–485. Epub 2008/09/03. doi: 10.1542/peds.2007-2313. PubMed PMID: 18762515.
- 41 Hadchouel A, Durrmeyer X, Bouzigon E, et al. Identification of SPOCK2 as a susceptibility gene for bronchopulmonary dysplasia. *Am J Respir Crit Care Med*. 2011;184(10):1164–1170. Epub 2011/08/13. doi: 10.1164/rccm.201103-0548OC. PubMed PMID: 21836138.
- 42 Morales Johansson H, Newman DR, Sannes PL. Whole-genome analysis of temporal gene expression during early transdifferentiation of human lung alveolar epithelial type 2 cells in vitro. *PLoS One*. 2014;9(4):e93413. Epub 2014/04/03. doi: 10.1371/journal.pone.0093413. PubMed PMID: 24690998; PubMed Central PMCID: PMC3972118.
- 43 Wang H, St Julien KR, Stevenson DK, et al. A genome-wide association study (GWAS) for bronchopulmonary dysplasia. *Pediatrics*. 2013;132(2):290–297. Epub 2013/07/31. doi: 10.1542/peds.2013-0533. PubMed PMID: 23897914; PubMed Central PMCID: PMC3727675.
- 44 Burchard EG. Medical research: missing patients. *Nature*. 2014;513(7518):301–302. Epub 2014/09/19. doi: 10.1038/513301a. PubMed PMID: 25230631.
- 45 Moreno-Estrada A, Gignoux CR, Fernandez-Lopez JC, et al. Human genetics. The genetics of Mexico recapitulates Native American substructure and affects biomedical traits. *Science*. 2014;344(6189):1280–1285. Epub 2014/06/14. doi: 10.1126/science.1251688. PubMed PMID: 24926019; PubMed Central PMCID: PMC4156478.
- 46 Albertine KH. Progress in understanding the pathogenesis of BPD using the baboon and sheep models. *Semin Perinatol*. 2013;37(2):60–68. Epub 2013/04/16. doi: 10.1053/j.semperi.2013.01.001. PubMed PMID: 23582959; PubMed Central PMCID: PMC3664547.
- 47 Pierce RA, Albertine KH, Starcher BC, Bohnsack JF, Carlton DP, Bland RD. Chronic lung injury in preterm lambs: disordered pulmonary elastin deposition. *Am J Physiol*. 1997;272(3 Pt 1):L452–460. Epub 1997/03/01. PubMed PMID: 9124602.
- 48 Coalson JJ, Winter V, deLemos RA. Decreased alveolarization in baboon survivors with bronchopulmonary dysplasia. *Am J Respir Crit Care Med*. 1995;152(2):640–646. Epub 1995/08/01. doi: 10.1164/ajrccm.152.2.7633720. PubMed PMID: 7633720.
- 49 De Matteo R, Blasch N, Stokes V, Davis P, Harding R. Induced preterm birth in sheep: a suitable model for studying the developmental effects of moderately preterm birth. *Reprod Sci*. 2010;17(8):724–733. Epub 2010/05/07. doi:

- 10.1177/19337191110369182. PubMed PMID: 20445008.
- 50 De Matteo R, Stacy V, Probyn ME, Brew N, Blasch N, Harding R. Does moderate preterm birth lead to altered arterial pressure? Studies in sheep. *Clin Exp Pharmacol Physiol.* 2008;35(12):1426–1432. Epub 2008/08/02. doi: 10.1111/j.1440–1681.2008.05014.x. PubMed PMID: 18671717.
- 51 Maritz G, Probyn M, De Matteo R, Snibson K, Harding R. Lung parenchyma at maturity is influenced by postnatal growth but not by moderate preterm birth in sheep. *Neonatology.* 2008;93(1):28–35. Epub 2007/07/17. doi: 10.1159/000105522. PubMed PMID: 17630495.
- 52 Barker DJ. The intrauterine origins of cardiovascular and obstructive lung disease in adult life. The Marc Daniels Lecture 1990. *J R Coll Physicians Lond.* 1991;25(2):129–133. Epub 1991/04/01. PubMed PMID: 2066923.
- 53 Lawlor DA, Ebrahim S, Davey Smith G. Association of birth weight with adult lung function: findings from the British Women's Heart and Health Study and a meta-analysis. *Thorax.* 2005;60(10):851–858. Epub 2005/08/02. doi: 10.1136/thx.2005.042408. PubMed PMID: 16055617; PubMed Central PMCID: PMC1747204.
- 54 Hancox RJ, Poulton R, Greene JM, McLachlan CR, Pearce MS, Sears MR. Associations between birth weight, early childhood weight gain and adult lung function. *Thorax.* 2009;64(3):228–232. Epub 2008/12/05. doi: 10.1136/thx.2008.103978. PubMed PMID: 19052051.
- 55 Dezateux C, Lum S, Hoo AF, Hawdon J, Costeloe K, Stocks J. Low birth weight for gestation and airway function in infancy: exploring the fetal origins hypothesis. *Thorax.* 2004;59(1):60–66. Epub 2003/12/25. PubMed PMID: 14694251; PubMed Central PMCID: PMC1758850.
- 56 Greenough A, Yuksel B, Cheeseman P. Effect of in utero growth retardation on lung function at follow-up of prematurely born infants. *Eur Respir J.* 2004;24(5):731–733. Epub 2004/11/02. doi: 10.1183/09031936.04.00060304. PubMed PMID: 15516664.
- 57 Rona RJ, Gulliford MC, Chinn S. Effects of prematurity and intrauterine growth on respiratory health and lung function in childhood. *BMJ.* 1993;306(6881):817–820. Epub 1993/03/27. PubMed PMID: 8490372; PubMed Central PMCID: PMC1677317.
- 58 Suresh S, O'Callaghan M, Sly PD, Mamun AA. Impact of childhood anthropometry trends on adult lung function. *Chest.* 2014. Epub 2014/10/24. doi: 10.1378/chest.14-0698. PubMed PMID: 25340561.
- 59 Roseboom TJ, Painter RC, van Abeelen AF, Veenendaal MV, de Rooij SR. Hungry in the womb: what are the consequences? Lessons from the Dutch famine. *Maturitas.* 2011;70(2):141–145. Epub 2011/08/02. doi: 10.1016/j.maturitas.2011.06.017. PubMed PMID: 21802226.
- 60 Lopuhaa CE, Roseboom TJ, Osmond C, et al. Atopy, lung function, and obstructive airways disease after prenatal exposure to famine. *Thorax.* 2000;55(7):555–561. Epub 2000/06/17. PubMed PMID: 10856314; PubMed Central PMCID: PMC1745806.
- 61 Langley-Evans SC, Daniel ZC, Wells CA, Ryan KJ, Plant R, Welham SJ. Protein restriction in the pregnant mouse modifies fetal growth and pulmonary development: role of fetal exposure to {beta}-hydroxybutyrate. *Exp Physiol.* 2011;96(2):203–215. Epub 2010/09/21. doi: 10.1113/expphysiol.2010.054460. PubMed PMID: 20851857.
- 62 van Abeelen AF, Elias SG, de Jong PA, et al. Famine in the young and risk of later hospitalization for COPD and asthma. *PLoS One.* 2013;8(12):e82636. Epub 2014/01/01. doi: 10.1371/journal.pone.0082636. PubMed PMID: 24376558; PubMed Central PMCID: PMC3871614.
- 63 Gaultier C, Harf A, Balmain N, Cuisinier-Gleizes P, Mathieu H. Lung mechanics in rachitic rats. *Am Rev Respir Dis.* 1984;130(6):1108–1110. Epub 1984/12/01. PubMed PMID: 6508008.
- 64 Yurt M, Liu J, Sakurai R, et al. Vitamin D supplementation blocks pulmonary structural and functional changes in a rat model of perinatal vitamin D deficiency. *Am J Physiol Lung Cell Mol Physiol.* 2014;307(11):L859–867. Epub 2014/10/12. doi: 10.1152/ajplung.00032.2014. PubMed PMID: 25305247.
- 65 Cremers E, Thijs C, Penders J, Jansen E, Mommers M. Maternal and child's vitamin D supplement use and vitamin D level in relation to childhood lung function: the KOALA Birth Cohort Study. *Thorax.* 2011;66(6):474–480. Epub 2011/03/23. doi: 10.1136/thx.2010.151985. PubMed PMID: 21422038.
- 66 Chawes BL, Bonnelykke K, Jensen PF, Schoos AM, Heickendorff L, Bisgaard H. Cord blood 25(OH) – vitamin D deficiency and childhood asthma, allergy and eczema: the COPSAC2000 birth cohort study. *PLoS One.* 2014;9(6):e99856. Epub 2014/06/14. doi: 10.1371/journal.pone.0099856. PubMed PMID: 24925304; PubMed Central PMCID: PMC4055727.

- 67 Goldring ST, Griffiths CJ, Martineau AR, et al. Prenatal vitamin d supplementation and child respiratory health: a randomised controlled trial. *PLoS One*. 2013;8(6):e66627. Epub 2013/07/05. doi: 10.1371/journal.pone.0066627. PubMed PMID: 23826104; PubMed Central PMCID: PMC3691177.
- 68 Litonjua AA, Lange NE, Carey VJ, et al. The Vitamin D Antenatal Asthma Reduction Trial (VDAART): rationale, design, and methods of a randomized, controlled trial of vitamin D supplementation in pregnancy for the primary prevention of asthma and allergies in children. *Contemp Clin Trials*. 2014;38(1):37–50. Epub 2014/03/13. doi: 10.1016/j.cct.2014.02.006. PubMed PMID: 24614387; PubMed Central PMCID: PMC4086903.
- 69 Checkley W, West KP Jr, Wise RA, et al. Maternal vitamin A supplementation and lung function in offspring. *New Engl J Med*. 2010;362(19):1784–1794. Epub 2010/05/14. doi: 10.1056/NEJMoa0907441. PubMed PMID: 20463338.
- 70 Maritz GS, Harding R. Life-long programming implications of exposure to tobacco smoking and nicotine before and soon after birth: evidence for altered lung development. *Int J Environ Res Public Health*. 2011;8(3):875–898. Epub 2011/05/11. doi: 10.3390/ijerph8030875. PubMed PMID: 21556184; PubMed Central PMCID: PMC3083675.
- 71 Cook DG, Strachan DP, Carey IM. Health effects of passive smoking. 9. Parental smoking and spirometric indices in children. *Thorax*. 1998;53(10):884–893. Epub 1999/04/08. PubMed PMID: 10193379; PubMed Central PMCID: PMC1745082.
- 72 Hollams EM, de Klerk NH, Holt PG, Sly PD. Persistent effects of maternal smoking during pregnancy on lung function and asthma in adolescents. *Am J Respir Crit Care Med*. 2014;189(4):401–407. Epub 2013/11/21. doi: 10.1164/rccm.201302-0323OC. PubMed PMID: 24251622.
- 73 Svanes C, Omenaas E, Jarvis D, Chinn S, Gulsvik A, Burney P. Parental smoking in childhood and adult obstructive lung disease: results from the European Community Respiratory Health Survey. *Thorax*. 2004;59(4):295–302. Epub 2004/03/30. PubMed PMID: 15047948; PubMed Central PMCID: PMC1763798.
- 74 Guerra S, Stern DA, Zhou M, et al. Combined effects of parental and active smoking on early lung function deficits: a prospective study from birth to age 26 years. *Thorax*. 2013;68(11):1021–1028. Epub 2013/07/13. doi: 10.1136/thoraxjnl-2013-203538. PubMed PMID: 23847259.
- 75 Martinez FD, Vercelli D. Asthma. *Lancet*. 2013;382(9901):1360–1372. Epub 2013/09/18. doi: 10.1016/S0140-6736(13)61536-6. PubMed PMID: 24041942.
- 76 Guerra S, Martinez FD. Epidemiology of the origins of airflow limitation in asthma. *Proc Am Thor Soc*. 2009;6(8):707–711. Epub 2009/12/17. doi: 10.1513/pats.200908-085DP. PubMed PMID: 20008881; PubMed Central PMCID: PMC2797072.

The Lung Structure Maintenance Program: Sustaining Lung Structure during Adulthood and Implications for COPD Risk

Norbert F. Voelkel and Masahiro Sakagami

Abstract

During lung development, alveolarization and the formation of the vast capillary network require the coordinate interactions of several growth factors during different stages of lung organ building; prominent among those are FGF10, PDGF, and epithelially secreted VEGF. Effective VEGF signaling is also required for the structure maintenance of the adult lung, as genetic modifications in mice and VEGF receptor blockade in adult rats lead to emphysematous airspace enlargement. The remarkable dependence of lung microvascular endothelial cells for their survival on autocrine VEGF signaling is illustrated in the lungs from patients with endstage COPD/emphysema; examination of such lungs shows severely decreased expression of VEGF and of VEGF receptor 1 and 2 proteins and of the VEGF gene upstream transcription factor HIF1 alpha. Copper is required for angiogenesis, and copper depletion causes emphysema in rodents. Thus copper is participating in the maintenance of the adult lung structure also because of its requirement for the activity of the collagen-crosslinking enzyme lysyl oxidase and the oxidant defense enzyme CuZn SOD. In addition, the “sphingosine-1-phosphate/ceramide rheostat” plays a role in the homeostasis of the lung structure. Taken together, this chapter provides the background and the rationale for concepts that can explain how genetic defects and environmental challenges can jeopardize the homeostatic lung structure maintenance program and cause destructive lung diseases in the adult.

Keywords:

Lung structure maintenance, emphysema, VEGF, HIF1 alpha, HDAC, copper, lysyl oxidase, ceramides

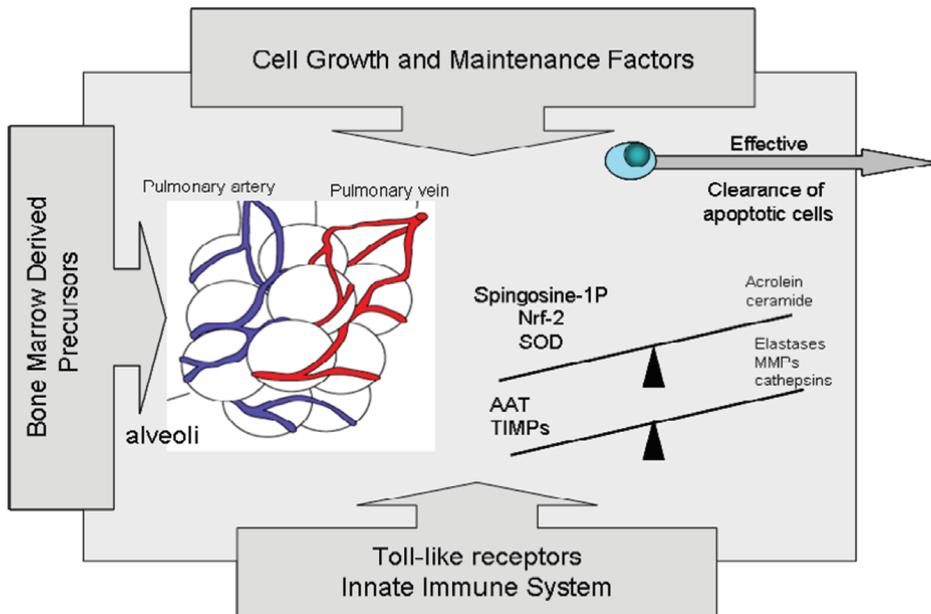
Introduction

Claude Bernard’s concept of homeostatic balances that are innate to biological systems, “the milieu interne,” provides an historical background for the postulate supporting the existence of a maintenance program that sustains and supports structure of the normal adult lung. In addition, such a program is likely built into the design of the adult lung and utilizes mechanisms and “blueprints” that have their origin during lung development. This maintenance program (1, 2) is normally “on” or *active* in the adult lung, but can be jeopardized by environmental challenges, such as inhaled dust and metal particles and endotoxins and nutritional deficiencies due to insufficient dietary supply of vitamins and trace metals. In addition,

there are likely genetic mechanisms that weaken or modulate the lung maintenance program.

Formulation of the postulate that such a lung structure maintenance program exists (Figure 17-1) – although somewhat intuitive – was initially prompted by the results of laboratory experiments designed to selectively block vascular endothelial growth factor (VEGF) signaling in adult rats, which demonstrated loss of alveolar surface area along with reduced vascular density (1–3). Previous studies had suggested that VEGF regulates endothelial cell *survival* (4,5), not only endothelial cell growth. In addition, even brief disruption of VEGF signaling after birth induced a developmental growth arrest of the distal airspace and vasculature and caused sustained pulmonary hypertension (3). Following our discovery that intact

Adult Lung Structure Maintenance Program



Taraseviciene-Stewart L, Voelkel NF. Molecular Pathogenesis of Emphysema. JCI, 2008

Figure 17-1. An illustration of the concept of the lung structure maintenance program. There are several homeostatic balances that contribute to lung cell growth and repair in response to environmental challenges. [Reproduced with permission from Taraseviciene-Stewart, Voelkel *J Clin Invest.* 2008;118:394–402.

VEGF signaling was an essential element of normal lung biology and required for the maintenance of lung structure, further studies revealed an important role for several other maintenance factors, which astonishingly appear to be linked in some fashion to VEGF, as will be illustrated next.

Although gene deletion strategies designed to knock out VEGF or its receptors confirmed the central role of VEGF in vascular biology (6), it was not obvious that the lung depends critically on intact signal transduction mechanisms, which follow binding of VEGF to its cognate receptors and phosphorylation of its intracellular tyrosine kinase (7). The first clue was the marked abundance of the VEGF gene expression in the adult lung (7), which led to questions regarding the potential functional roles of VEGF signaling in the adult lung, which in contrast with rapid lung growth during development does not grow during adulthood. One potential answer to this question was that VEGF was necessary for “organ maintenance.” This straightforward paradigm has recently been both enriched and complicated by the discovery of multiple VEGF isoforms and soluble receptors, which can influence VEGF signal transmission (8,9). In

Role of VEGF in the Autocrine Maintenance of Lung EC

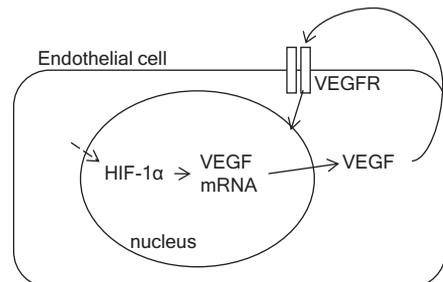


Figure 17-2. VEGF is of central importance for the survival of lung vascular endothelial cells. Microvascular lung endothelial cells generate and secrete large amounts of VEGF protein, which signals in an autocrine fashion.

addition, many cell types can generate and secrete VEGF in the lung, including epithelial cells, vascular smooth muscle cells, lymphocytes, mast cells and macrophages, and important, lung microvascular endothelial cells from which VEGF acts on these endothelial cells in an autocrine fashion (10). Thus, the lung microvascular endothelium generates its own survival/maintenance factor and may further modulate airspace structure as well (Figure 17-2). In the following sections we will relate the

failure of the adult lung structure maintenance program to the loss of lung capillaries and airspace enlargement in COPD/emphysema.

Development of the Lung Vessels

Lung development follows the highly coordinated, transcriptionally controlled complex interactions between cells and cells and matrix. A “blueprint” for pattern formation and branching morphogenesis exists in the primitive lung bud. Alveolarization and formation of the capillary network during late fetal and postnatal life require diverse factors such as platelet-derived growth factor (PDGF), VEGF, and retinoic acid (11). Epithelial-endothelial signal exchange is critical for the building of a functional blood/gas interface.

In fact, vascular ablation results in a perturbed branching stereotypy and a flat branching morphology (12). Interestingly, the design-controlling role of the vasculature is independent of perfusion, and apparently loss of vasculature perturbs the spatial expression pattern of the key lung branching mediator, Fibroblast Growth Factor 10 (FGF 10) (12). VEGF 164 stimulates mouse branching morphogenesis and increases the number of Flk-1 + mesenchymal cells as well as the expression of bone morphogenetic protein 4 (BMP-4) and surfactant protein C (13).

Remarkably, the pulmonary arterial wall is constructed radially, from the inside out, and there is evidence for the formation of a capillary plexus via distal angiogenesis (14,15) (Figure 17-3). The intricate and simultaneous development of airways and vessels during fetal life and, most important, their interdependence is illustrated by the fact that

epithelial branches are invested with an endothelial network that develops under the control of *epithelially* secreted VEGF. Reduction of available VEGF using a soluble VEGF receptor disrupts both capillary network and epithelial branching, whereas increased VEGF signaling stimulates vessel formation *and* airway branching (16).

At low fetal lung oxygen tension (23 torr), mammalian target of rapamycin complex 1 (mTORC1) amplifies epithelial hypoxia-inducible factor 1 alpha (HIF-1alpha)-induced epithelial VEGF production and vasculogenic activity (17). Another transcription factor upstream from VEGF is peroxisome proliferator-activated receptor gamma coactivator (PGC-1alpha) (18). Whether PGC-1alpha is involved in hypoxic stimulation of VEGF transcription in the fetal lung is unknown; however, it has been reported that FOXF1 is required for the formation of the embryonic vasculature through regulation of VEGF signaling in endothelial cells (19). Regardless, it is clear that HIF-1alpha, HIF-2alpha, and VEGF play central and critical roles during lung development. As discussed later, these same actors play active roles in tissue destruction and the pathobiology of emphysema in the adult lung.

Recently, Dr. Mark Krasnow’s laboratory discovered alveolar progenitor/stem cells that coexpress alveolar type 1 (AT1) and alveolar type 2 (AT2) cell markers, and that these bipotent progenitors are the source of most, if not all, AT1 and AT2 cells during lung development. Thereafter, mature AT2 cells function as stem cells that are intermittently activated for alveolar renewal and repair (20). There are functional endothelial colony-forming cells (ECFC) in human fetal and

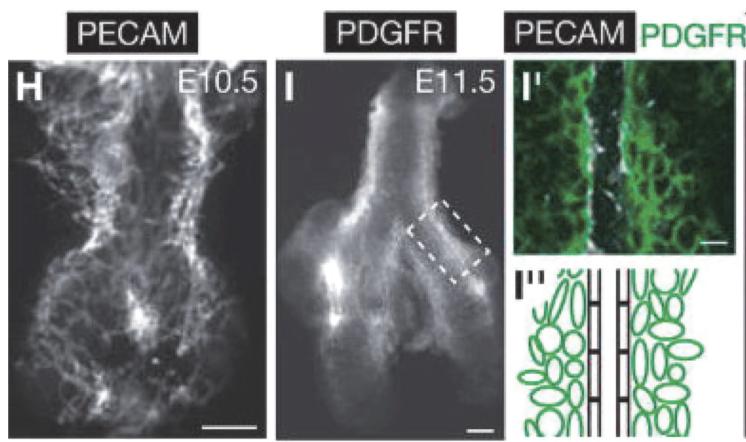


Figure 17-3. Development of pulmonary arteries in the mouse. Embryonic day 10.5 shows a vascular plexus that gives rise to the pulmonary arterial tube. (Reproduced from Greif, Kumar, Lighthouse, et al. *Dev Cell*. 2012;23:482–493 with permission).

rat neonatal lungs that have repair potential (21). c-Kit+/CD34+/VEGFR2+/Tie-2+ cells have also been described as a putative endothelial progenitor cell population in developing human lungs (22).

Can the Adult Lung Structure Maintenance Program Fail?

To further advance the concept of the lung structure maintenance program, we need to provide an experimental underpinning and search for “experiments of nature,” such as genetic and epigenetic conditions, that support this concept. The search for failure mechanisms began with the remark of a pathologist who, while examining lung tissue from a patient with severe COPD/emphysema, stated: “Emphysema is boring, there is just nothing there!” This “nothing there” or loss of alveolar structures and hole formation in the lung led to the hypothesis that alveolar cells undergo apoptosis and disappear in emphysema (23). We had been guided by a statement by the late Gordon Snider that “elastin degradation gives rise to emphysema, but not all airspace enlargements occur on such a background. Airspace enlargement may result from a connective tissue repair or growth process that is unable to preserve the normal alveolar structure of the lungs” (23). But the most important fact that argued for the existence of an adult lung structure maintenance program is the occurrence of emphysema in patients that never smoked (24). A recent study from Stockholm showed that low birth weight and preterm birth are risk factors for adult women to develop COPD (25). In this study, women born before thirty-two weeks of gestation had a hazard ration for obstructive lung disease of 2.7 (25). Although cigarette smoke is toxic to lung cells, airspace enlargement occurs in nonsmokers because the usually very robust response to endogenous oxidant stress factors or/and the response to low-grade environment stress such as air pollution is weakened.

The Histone Deacetylase (HDAC)/HIF-1alpha/VEGF Axis

The availability of a novel small molecule inhibitor of the intracellular tyrosine kinase section of the VEGF receptors 1 and 2 provided the opportunity to directly test the hypothesis that VEGF was required for the maintenance of the lung structure.

This hypothesis was first tested in adult (1) and neonatal (3) rats. Animals were given the highly lipophilic drug, Sugen 5416 (semaxinib), as a single subcutaneous injection of a slurry. Four weeks later, treated animals showed impressive airspace enlargement and a dramatic loss of lung vessels when assessed by angiography (1). Semaxinib is presently in clinical use for the treatment of renal cell carcinoma. The important findings were that SU5416 decreased lung expression of VEGFR2 and Akt-1 and that cotreatment of the rats with a pan-caspase inhibitor prevented the development of SU5416-induced emphysema (1). In neonatal rat studies, animals were treated on the first day after birth with this VEGFR inhibitor, which markedly reduced alveolarization as well as pulmonary arterial density (3). This study was the first to demonstrate that angiogenesis is required for normal postnatal alveolarization during development, suggesting that impaired endothelial survival or function may contribute to developmental disorders of lung hypoplasia, such as bronchopulmonary dysplasia (3).

VEGF also commands antioxidant activity and is antiapoptotic, as VEGF treatment upregulates MnSOD and BCL-2 expression in endothelial cells (26, 27). Kasahara et al. translated the experimental findings to patient lung tissue samples and found decreased VEGF and VEGF receptor expression in lung tissue from patients with end-stage COPD/emphysema (28). These findings suggested that a first component or factor that was both necessary and required for the maintenance of the lung structure (e.g., VEGF) had been identified. This finding was surprising in view of the fact that the major transcription factor controlling VEGF gene expression is HIF-1alpha, and that HIF-1 alpha protein is stabilized by hypoxia. As many end-stage COPD patients are hypoxic, we speculated that there was either a transcriptional block, assuming that the expression of HIF-1alpha protein was elevated in the emphysematous lungs, or alternatively, that HIF-1alpha protein expression was paradoxically decreased in the hypoxic lung. Subsequent studies of tissue samples from banked human COPD lungs demonstrated marked reductions in VEGF and HIF-1 alpha gene and protein expression in the emphysematous lung tissue samples (29; Figure 17-4). Figure 17-5 shows the decreased expression of VEGF and phospho-Akt, reflecting decreased signaling.

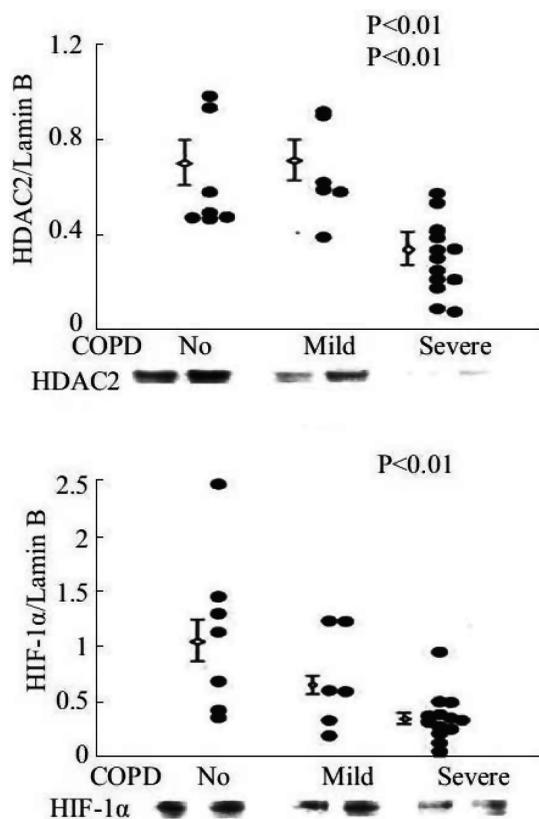


Figure 17-4. Expression of HDAC2 and HIF-1 alpha proteins in lung tissue samples of patients with severe COPD/emphysema. (Reproduced from Yasuo, Mizuno, Kraskauskas, et al. *Eur Respir J.* 2011; 37(4):775–78 with permission).

Thus, the diminished expression of lung tissue HIF-1 alpha, despite marked tissue hypoxia, was paradoxical and begged for a mechanistic explanation. Based on past studies suggesting key interactions between p53 (the “guardian of the genome”), HDAC and HIF-1alpha, HDAC 2 (Figure 17-4), and p53 protein expression were measured in COPD lung tissue samples (29). Ito and coworkers had examined lung samples from human COPD/emphysema patients and found a decreased expression and activity of HDAC proteins (30). This in turn led to the question whether experimental chronic inhibition of HDAC activity would lead to experimental emphysema. This indeed was shown in adult rats by Mizuno et al. (31). As histone modifications are part of the epigenetic repertoire, cigarette smoke exposure of lung cells, including endothelial cells, causes epigenetic changes that are partly dependent on HDAC expression and activity. One consequence

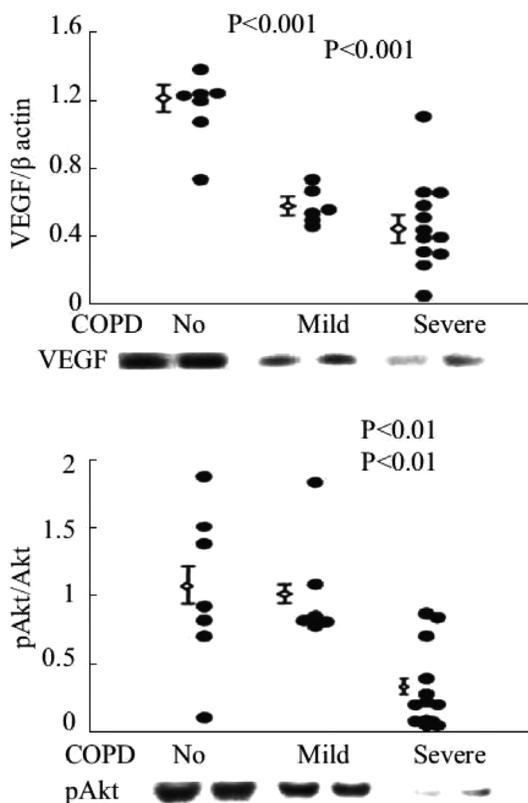


Figure 17-5. Expression of VEGF protein and the ratio of phosphorylated/non-phosphorylated Akt in lung tissue samples from patients with severe COPD/emphysema. (Reproduced with permission from Yasuo, Mizuno, Kraskauskas, et al. *Eur Respir J.* 2011; 37(4):775–783).

of decreased HDAC expression in emphysematous lungs may be the decreased HIF-1alpha gene expression from end-stage COPD patients. If so, HDAC is situated upstream in the hierarchy of components that make up the adult lung structure maintenance program (Figure 17-6). Indeed, when the expression of the HDAC2 gene was experimentally reduced in cultured lung cells, the expression of HIF-1alpha was downregulated, further supporting the role for a HDAC → HIF-1alpha → VEGF axis (29).

Copper and Sphingolipids

An experiment of nature, Menkes’ disease, which is based on a mutation of the gene encoding a copper transporter and impairs lung growth in infants, inspired our experimental work on the potential role for copper in the lung structure maintenance program. A further inspiration was the finding that the “blotchy mouse” carries the identical gene mutation as found in children with

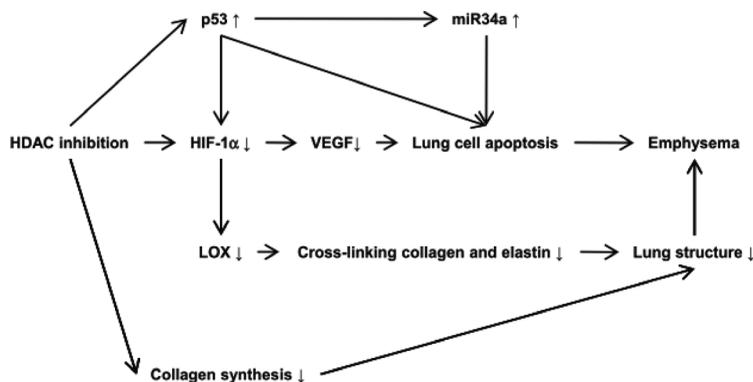


Figure 17-6. Proposed roles of HDAC 2, Hif-1alpha and VEGF in the lung structure maintenance program. Reproduced with permission from Mizuno, Yasuo, Bogaard, et al. *Am J Physiol Lung Cell Mol Physiol.* 2011;300:402–413.

Menkes' disease and that these mice develop emphysema without additional challenges (32). Copper is required for multiple metabolic activities, such as angiogenesis, collagen cross-linking (the activity of lysyl oxidase), antioxidant defenses (e.g., the activity of CuZn SOD), and transcriptional activity of HIF-1alpha. A copper-depleted diet or treatment with a copper chelator induced severe emphysema and decreased transcriptional activity of HIF-1 alpha in adult rats (32). Thus, copper is likely an essential component of the adult lung structure maintenance program. A study of dietary habits suggests that a section of the population is copper deficient (33), and there is also a study that shows smokers are copper deficient (34), as are patients with alpha-1 antitrypsin deficiency (35).

Spiegel and her group have provided evidence supporting the importance of sphingolipids in several diseases and introduced the concept of a sphingosine 1-phosphate /ceramide "rheostat." (36) In addition, Petrache and coworkers established that lung tissue from COPD/emphysema patients are enriched with ceramides, that smoking induces human lung endothelial cell adaptation to apoptotic stress, and that ceramide reduces cell growth and induces apoptosis of lung endothelial cells (37,38). Yasuo et al. (39) treated adult rats with fenretinide, a synthetic derivative of retinoic acid that enhances ceramide production, and found that fenretinide caused emphysema. Interestingly, these structural changes were associated with an increase in the number of caspase-expressing lung cells and a decrease in lung VEGF protein expression. Concomitant treatment with sphingosine-1 phosphate injections reduced emphysema and restored lung HIF-1alpha, HDAC2, and VEGF protein expression. Overall,

these experiments illustrate the importance of a balanced sphingosine metabolism for cell survival and growth in adult lung models of emphysema.

Conclusions and Future Directions

In this chapter, we develop the data to support the concept of a complex system of interactive factors that sustain homeostatic balance and provide an environment that supports lung structure stability. Genetic abnormalities or extrinsic challenges that result in epigenetic alterations can disrupt the regulation of cell growth and cell death. If we consider a "hub and spoke model," we can place the HDAC/HIF-1alpha/VEGF interactions in the position of a hub (Figure 17-6), which is connected with oxidant/antioxidant, protease/antiprotease, apoptosis/cell growth, and angiogenic/antiangiogenic mechanisms. Both deletion/depletion strategies and strategies that generate damaging metabolites demonstrate the importance of individual components of these balances and their connections with the hub. The concept of a lung structure maintenance program (40) not only connects with the design and building principles of developmental biology, but it also points the way toward identifying molecular targets for developing novel treatment strategies that could protect the adult lung structure, prevent the progression of COPD/emphysema and potentially could be applied to repair injured lungs (41–43). Examples of the experimental models with dexamethasone-, SU5416-, and fenretinide-induced emphysema should alert us to the possibility that there may be drug-induced forms of lung tissue destruction (44). Future studies are needed to further define mechanisms of a lung maintenance program and how disruption of these key pathways can lead to COPD/emphysema in human disease.

References

- 1 Kasahara Y, Tuder RM, Taraseviciene-Stewart L, et al. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest*. 2000;106:1311–1319.
- 2 Taraseviciene-Stewart L, Voelkel NF. Molecular pathogenesis of emphysema. *J Clin Invest*. 2008;118:394–402.
- 3 Jakkula M, Le Cras TD, Gebb S, et al. Inhibition of angiogenesis decreases alveolarization in the developing lung. *Am J Physiol Lung Cell Mol Physiol*. 2000;279:L600–607.
- 4 Gerber HP, McMurtrey A, Kowalski J, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway: requirement for Flt/Kdr activation. *J Biol Chem*. 1998;273:30336–30343.
- 5 Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med*. 1995;1:1024–1028.
- 6 Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. *Nature*. 1995;376:62–66.
- 7 Voelkel NF, Vandivier RW, Tuder RM. Vascular endothelial growth factor in the lung. *Am J Physiol Lung Cell Mol Physiol*. 2006;290:L209–221.
- 8 Esquibies AE, Karihaloo A, Quaggin SE, et al. Heparin binding VEGF isoforms attenuate hyperoxic embryonic lung growth retardation via a FLK1-neuropilin-1-PCK dependent pathway. *Respir Res*. 2014 March 19;15:32.
- 9 Voelkel NF, Gomez – Arroyo J. The role of vascular endothelial growth factor in pulmonary arterial hypertension: the angiogenesis paradox. *Am J Respir Cell Mol Biol*. 2014;51:474–484.
- 10 Stevens T, Kasper M, Cool CD, Voelkel NF. Pulmonary circulation and pulmonary hypertension. In: Aird WC, ed. *Endothelial Cells in Health and Disease*. Boca Raton, FL: Taylor & Francis; 2005:567–505.
- 11 Kumar VH, Lakshminrusimha S, El Abiad MT, et al. Growth factors in lung development. *Adv Clin Chem*. 2005;40:261–316.
- 12 Lazarus A, Del-Moral PM, Mishani E, Warburton D, Keshet E. A perfusion-independent role of blood vessels in determining branching stereotypy of lung airways. *Development*. 2011;138: 2359–2568.
- 13 Del Moral PM, Sala FG, Tefft D, et al. VEGF-A signaling through Flk-1 is a critical facilitator of early embryonic lung epithelial to endothelial crosstalk and branching morphogenesis. *Dev Biol*. 2006;290: 177–188.
- 14 Parera MC, van Dooren M, van Kempen M, et al. Distal angiogenesis: a new concept for lung vascular morphogenesis. *Am J Physiol Lung Cell Mol Physiol*. 2005;288:L141–149.
- 15 Greif DM, Kumar M, Lighthouse JK, et al. Radial construction of an arterial wall. *Dev Cell*. 2012;23:482–493.
- 16 Jesudason EC, Keshet E, Warburton D. Entrained pulmonary clocks: epithelium and vasculature keeping pace. *Am J Physiol Lung Cell Mol Physiol*. 2010;299:L453–454.
- 17 Scott CL, Walker DJ, Cwiklinski EL, et al. Control of HIF-1{alpha} and vascular signaling in fetal lung involves cross talk between mTORC1 and the FGF-10/FGFR2b/Spry2 airway branching periodicity clock. *Am J Physiol Lung Cell Mol Physiol*. 2010;299:455–471.
- 18 Thom R, Rowe GC, Jang C, et al. Hypoxic induction of vascular endothelial growth factor (VEGF) and angiogenesis in muscle by truncated peroxisome proliferator-activated receptor gamma coactivator (PGC-1alpha). *J Biol Chem*. 2014;280: 8810–8817.
- 19 Ren X, Ustijan V, Pradhan A, et al. FOXF1 transcription factor is required for the formation of embryonic vasculature by regulating VEGF signaling in endothelial cells. *Circ Res*. 2014;115:709–720.
- 20 Desai TJ, Brownfield DG, Krasnow MA. Alveolar progenitor and stem cells in lung development, renewal and cancer. *Nature*. 2014;507:190–194.
- 21 Alphonse RS, Vadivel A, Fung M, et al. Existence, functional impairment and lung repair potential of endothelial colony-forming cells in oxygen-arrested alveolar growth. *Circulation*. 2014;129:2144–2157.
- 22 Suzuki T, Suzuki S, Fujino N, et al. c-Kit immunoreexpression delineates a putative endothelial progenitor cell population in developing human lungs. *Am J Physiol Lung Cell Mol Physiol*. 2014;306:855–866.
- 23 Tuder RM, Voelkel NF. The pathobiology of chronic bronchitis and emphysema. In: Voelkel NF, MacNee W, eds. *Chronic Obstructive Lung Diseases*. Hamilton, London: BC Decker; 2002:90–113.
- 24 Celli BR, Halbert RJ, Nordyke RJ, Schau B. Airway obstruction in never smokers: results from the Third National Health and Nutrition Survey.

- Am J Med.* 2005;118: 1364–1372.
- 25 Broström EB, Akre O, Katz-Salamon M, Jaraj D, Kaijser M. Obstructive pulmonary disease in old age among individuals born preterm. *Eur J Epidemiol.* 2013;28(1): 79–85.
- 26 Abid MR, Tsai JC, Spokes KC, Deshpande SS, Irani K, Aird WC. Vascular endothelial growth factor induces manganese superoxide dismutase in endothelial cells by a Rac1-regulated NADPH oxidase-dependent mechanism. *FASEB J.* 2001;15(13):2548–2550.
- 27 Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins BCL-2 and A1 in vascular endothelial cells. *J Biol Chem.* 1998;273(21):13313–13316.
- 28 Kasahara Y, Tudor RM, Cool CD, Lynch DA, Flores SC, Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and its receptor KDR/flt1 in smoking-induced emphysema. *Am J Respir Crit Care Med.* 2001;163(3 Pt 1):737–744.
- 29 Yasuo M, Mizuno S, Kraskauskas D, et al. Hypoxia-inducible factor 1 alpha in human emphysema lung tissue. *Eur Respir J.* 2011; 37(4):775–783.
- 30 Ito K, Ito M, Elliott WM, et al. Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N Engl J Med.* 2005;352:1967–1976.
- 31 Mizuno S, Yasuo M, Bogaard HJ, et al. Inhibition of histone deacetylase causes emphysema. *Am J Physiol Lung Cell Mol Physiol.* 2011;300:402–413.
- 32 Mizuno S, Yasuo M, Bogaard HJ, et al. Copper deficiency induced emphysema is associated with focal adhesion kinase inactivation. *PLoS One.* 2012;7(1):e30678.
- 33 Roman Viñas B, Ribas Barba L, Ngo J, et al. Projected prevalence of inadequate nutrient intakes in Europe. *Ann Nutr Metab.* 2011;59:84–95.
- 34 Schwartz J, Weiss ST. Dietary factors and their relation to respiratory symptoms. The second national health and nutrition examination survey. *Am J Epidemiol.* 1990;132:67–76.
- 35 Ghio AJ, Soukup JM, Richards BM, et al. Deficiency of alpha-1-antitrypsin influences iron homeostasis. *Int J Chron Obstruct Pulmon Dis.* 2013;8:45–51.
- 36 Maceyka M, Spiegel S. Sphingolipid metabolites in inflammatory disease. *Nature.* 2014;510:58–67.
- 37 Petrache I, Petrusa DN. The involvement of sphingolipids in chronic obstructive disease. *Handb Exp Pharmacol.* 2013;216:247–264.
- 38 Medler TR, Petrusa DN, Lee PJ, et al. Apoptotic sphingolipid signaling by ceramides in lung endothelial cells. *Am J Respir Cell Mol Biol.* 2008;38:639–646.
- 39 Yasuo M, Mizuno S, Allegood J, et al. Fenretinide causes emphysema, which is prevented by sphingosine 1-phosphate. *PLoS One.* 2013;8(1): e53927.
- 40 Tudor RM, Yoshida T, Fijalowka I, Biswal S, Petrache I. Role of the lung maintenance program in the heterogeneity of lung destruction in emphysema. *Proc Am Thorac Soc.* 2006;3:673–679.
- 41 Diab KJ, Adamowicz JJ, Kamocki K, et al. Stimulation of sphingosine 1-phosphate signaling as an alveolar survival strategy in emphysema. *Am J Respir Crit Care Med.* 2010;18:344–352.
- 42 Tibboel J, Reiss E, de Jongste JC, Post M. Sphingolipids in lung growth and repair. *Chest.* 2014;145:120–128.
- 43 Saluja B, Li H, Desai UR, Voelkel NF, Sakagami M. Sulfated caffeic acid dehydropolymer attenuates elastase and cigarette smoke extract-induced emphysema in rats: sustained activity and a need of pulmonary delivery. *Lung.* 2014;192:481–492.
- 44 Voelkel NF, Mizuno S, Yasuo M. Does drug-induced emphysema exist? *Eur Respir J.* 2013;42:1464–1468.

Index

- a disintegrin and metalloprotease (ADAM) family, 290
- A549 (epithelial cell line), 15
- ABCA3 (adenosine triphosphate binding cassette A3), 65, 67, 71, 144–146, 154–155
- deficiency, 154
- ACD (alveolar capillary dysplasia), 24, 103–104, 132
- ACh (acetylcholine), 78–79, 225–226
- acidosis, 208–209, 216, 228
- acinar dysplasia (Type 0 CPAM; acinar agenesis), 103, 108–109
- acini, 127–129, 131, 198–199
- activins, 2–3, 13, 42–43
- Activin A, 12
- ADAM. *See* aerosol derived airway morphometry
- ADAM (a disintegrin and metalloprotease) family, 290
- adaptive immunity, 271
- ADC (apparent diffusion coefficient), 242–246
- adenocarcinomas, 108–109, 115, 246
- adenosine, 165, 233. *See also* ABCA3
- adenosine triphosphate (ATP), 154–155. *See also* ABCA3
- adenosine triphosphate binding cassette A3. *See* ABCA3
- adrenaline, 166–167, 172
- advanced glycosylation end product (AGER), 290
- AEG syndrome, 97
- aerosol derived airway morphometry (ADAM), 243
- early studies using, 244
- merits and demerits of, 243–244
- AGER (advanced glycosylation end product), 290
- air-liquid interface cultures, 14
- airway liquid clearance, 166–172
- at birth, 167
- after birth, 168–169
- changes in ductus arteriosus flow at birth, 172
- consequences of lung recoil, 169–170
- facilitating, 172–173
- hemodynamic consequences of lung aeration, 170–172
- during labor, 166–167
- before labor, 166
- Akt, 4–5, 306
- ALK (anaplastic lymphoma kinase), 109
- Alk1, 42–43, 49
- allergic sensitization, 80–81, 84
- α_1 -antitrypsin, 50, 213
- α v integrins, 46
- Altes, T.A., 244
- alveolar capillary dysplasia (ACD), 24, 103–104, 132
- alveolar hypoplasia, 46
- alveolar macrophages, 146
- alveolar stage of lung development, 23, 38, 77–78, 94–95, 134–137, 187, 269–271
- alveolar formation, 60–61, 94–95
- bulk alveolarization, 133–136
- continued alveolarization, 133, 136–137
- microvascular maturation, 134, 136
- alveolar type 1 (AT1) pneumocytes, 1, 7, 9, 13–14, 61–62, 130–132, 170, 275, 293–294, 305–306
- alveolar type 2 (AT2) pneumocytes, 1, 4–5, 7, 9, 13–14, 61–62, 130–132, 147–148, 305–306
- ABCA3 deficiency, 155
- bronchopulmonary dysplasia, 293–294
- hyperoxia, 274–275
- lung recoil, 170
- maturation of, 146
- nicotine, 79, 197
- pulmonary arterial development, 41
- surfactant proteins, 144
- surfactant treatment, 151
- alveolarization, 44–45, 60–63
- alveoli, 1–2
- AMPA, 225–226
- amphibian lung structure and air flow, 269
- amphiregulin, 41–42
- Ang (angiopoietins), 42
- Ang1, 42
- Ang2, 48–49
- angiogenesis, 36–37, 49–50, 60–61
- angiostatic factors, 44–45
- angiostatin, 44–45
- Angus, G.E., 239–241
- animal models, 2
- chorioamnionitis, 188–189
- continued alveolarization phase, 244–245
- calorie related changes, 245
- following pneumonectomy, 244–245
- possible mechanisms of, 245
- in rabbits and rhesus monkeys, 245
- development of respiratory system, 2–9
- lung function development, 294–295
- lung tissue from pluripotent stem cells, 9–15
- study of lung maturation, 70–71
- ANOVA F-test statistic, 65
- antidepressants, 197
- anti-oxidant defenses, 271
- API, 67–69
- Apert syndrome, 99–100
- apnea of prematurity, 223–234
- biologic basis for therapeutic interventions, 232–234
- continuous positive airway pressure, 233
- methylxanthine therapy, 233–234
- optimization of gas exchange and blood gas status, 232–233
- optimization of mechanosensory inputs, 229–232
- central respiratory control
- maturation of central CO₂ chemosensitivity, 224–225
- neurochemistry of, 225–226
- overview, 223–224
- diagnostic challenges in cardiorespiratory monitoring, 231–232

- apnea of prematurity (cont.)
 heart rate, 232
 oxygenation, 232
 respiration, 231–232
 fetal breathing movements,
 229–230
 future directions, 234
 inflammatory mechanisms and,
 230–231
 peripheral afferents and carotid
 body chemosensitivity,
 226–229
 C fiber receptors, 228
 maturation of peripheral CO₂
 and hypoxic carotid body
 chemosensitivity, 228–229
 rapidly adapting receptors,
 227–228
 slowly adapting receptors, 227
 role of vulnerable respiratory
 system, 230
 apneic threshold, 225
 apparent diffusion coefficient
 (ADC), 242–246
Aqa5, 70–71
Aqp5, 9, 65
 arginases, 216
 arginine vasopressin (AVP),
 166–167
 Armour, J., 247
 arteriogenesis. *See* pulmonary
 arterial development
Ascl1, 9
 asphyxia, 165–166, 172–173,
 178–179
 asthma, 22–23, 238, 286
 environmental air pollution,
 84–85, 87
 genetic susceptibility to, 87
 hyperoxia, 273
 indoor air pollution, 84–85
 lung function development,
 287–288
 lung function tests, 258–261
 ozone, 276–277
 tobacco product exposure, 82–83,
 277–278
 Vitamin D deficiency, 296–297
 AT1 pneumocytes. *See* alveolar type
 1 pneumocytes
 AT2 pneumocytes. *See* alveolar type
 2 pneumocytes
 atelectrauma, 177–178
 ATI, 9
 ATP (adenosine triphosphate),
 154–155. *See also* ABCA3
 Auten, R.L., 84–85
 Avery, M.E., 141–142, 147
 avian lung structure and air flow,
 269
 AVP (arginine vasopressin),
 166–167
 Ballard, H.O., 194–195
 Barker, D.J., 295
 basal cells, 9, 13–14, 25
 BCL-2, 306
 Bernard, Claude, 303
 β1 integrins, 46
 β3 integrins, 46
 β-catenin, 4–6, 8, 15–16
 branching morphogenesis, 27
 pulmonary arterial development,
 39, 43
 β-galactosidase, 48
 betamethasone, 189, 195–196
 B-H (Breuer-Hering) reflex, 227,
 287
 BH4 (tetrahydrobiopterin), 216
 bilateral pulmonary agenesis, 102
 biomass fuel exposure, 85–86,
 197–198, 276–277
 bisphenol A (BPA), 276–278
 bleomycin, 41–42, 156, 212–213
 Bloch-Salisbury, E., 229–232
 BMPs (bone morphogenetic
 protein receptors), 5–6
Bmpr1a, 5–7, 28
Bmpr1b, 5–7
Bmpr2, 43
 BMPs (bone morphogenetic
 proteins), 2, 4–8, 13, 42–43
 BMP2, 43
 BMP4, 6–8, 12–16, 28, 43, 305
 BMP5, 43
 BMP6, 43
 BMP7, 13–14, 43
 BMP9, 49
 Boyden, E.A., 131
 BPA (bisphenol A), 276–278
 BPD. *See* bronchopulmonary
 dysplasia
 BPD-associated PHT. *See*
 bronchopulmonary
 dysplasia-associated
 pulmonary hypertension
 brachyury (T), 3–4, 12
BRAF, 115
 brain-lung-thyroid syndrome, 105,
 156–157
 branching morphogenesis, 2, 4, 7–8,
 13–16, 22–29, 128–129, 270
 epigenetic influences on, 25–26
 essential nature of, 22–23
 influence of extracellular matrix
 on, 28–29
 modes of, 24
 nicotine and, 197
 overview of, 23–24
 signaling pathways in, 26–28
 transcription factors in, 24–25
 Breuer, Josef, 227
 Breuer-Hering (B-H) reflex, 227,
 287
 BRICHOS domain, 155–156
 bronchial atresia, 15, 101, 104, 115
 bronchial cartilaginous dysplasia,
 115
 bronchial stenosis, 101, 115
 bronchioles, 1
 bronchodilators, 212
 bronchogenic cysts, 97–99, 104–105,
 111–112
 bronchomalacia, 101–102, 130
 bronchopulmonary dysplasia
 (BPD), 126–127, 134, 137,
 173–174, 178–179, 194–196,
 198, 206–218, 230–231,
 233–234, 273–274
 in the current era, 206
 factors contributing to, 207–210
 genetics, 207
 hyperbilirubinemia, 209
 oxygen toxicity, 207–208
 packed red blood cell
 transfusion, 209–210
 patent ductus arteriosus, 210
 sepsis, 209
Ureaplasma spp., 209
 ventilation induced injury,
 208–209
 future directions, 216–217
 genetics of, 293–294
 interventions, 210–212
 bronchodilators, 212
 caffeine, 211
 inflammation, 212–213
 inhibition of neutrophil
 elastase, 213
 nitric oxide, 215
 non-steroidal antiinflammatory
 drugs, 211–212
 phosphodiesterase inhibitors,
 215
 Rho-kinase inhibition, 216
 steroids, 211–212
 targeting lung growth,
 210–211
 targeting peroxynitrite,
 214–215
 lung function in animal models,
 294–295
 lung function tests, 254–255, 262,
 264–265
 pathological features of, 206
 prematurity and lung function,
 291–293
 bronchopulmonary dysplasia-
 associated pulmonary
 hypertension (BPD-
 associated PHT), 205–207
 future directions, 217–218
 interventions, 213–214
 bulk alveolarization phase,
 133–136
 Burri, P.H., 240, 246
 Butler, J.P., 246

- C fiber receptors, 224, 228
 C10, 15
 caffeine, 211, 216–217, 233–234
 calreticulin, 143–144
 cAMP (cyclic adenosine monophosphate), 13–14, 166–167, 215
 canalicular stage of lung
 development, 2, 23–24, 77–78, 94–95, 130–133, 187, 269–271
 “canalization” of lung parenchyma, 130
 epithelial differentiation, 130–131
 pulmonary vascular development, 34–35
Candida spp., 188–189
 Candida albicans, 188
 capsaicin, 228
 carbohydrate recognition domain (CRD), 143–144
 carbon monoxide (CO), 84–86, 259
 carboxyhemoglobin, 85, 196–197
 Cardio-Facio-Cutaneous syndrome, 117
 cardiopulmonary progenitor (CPP)
 cells, 39–40, 47, 49–50
 carnitine, 50
 catecholamines, 226
 caveolins (Cavs), 193–194
 CAV1, 67–69, 193–194
 CC10, 13–14
 CCHS (congenital hypoventilation syndrome), 225
 CD14, 143–144
 CD25, 84–85
 CD31 (PECAM), 36–37
 CD34, 305–306
 CD56 (NCAM), 12–13
 CD91, 143–144
 CD271 (NGFR), 12–13
 CDH (congenital diaphragmatic hernia), 47, 130, 238
 CDH1, 67–69
 Cdx1-4, 4, 12–13, 15–16
 Cdx2, 12–13
 CEBPA, 65, 67–69
 central pattern generator (CPG), 223–226
 Cerberus, 4
 CF. *See* cystic fibrosis
 CFTR (cystic fibrosis transmembrane conductance regulator), 14, 100
 cGMP (cyclic guanosine monophosphate), 215–216
 CHARGE syndrome, 97
 CHD7, 97
 CHiP (chromatin immunoprecipitation), 63–64, 71
 cholecystokinin, 226
 Chordin, 4
 chorioamnionitis, 143–144, 152, 187–193, 212, 230–231
 animal models of, 188–189
 defined, 187–188
 effects on fetal lung development, 195–196
 human studies of, 194
 inflammation/injury of fetal lung, 190–191
 innate immune responses to, 191–193
 maturation of fetal lung and, 189
 chromatin immunoprecipitation (CHiP), 63–64, 71
 chromobox, 67–69
 chronic lung diseases (CLDs), 194, 246–248, 286. *See also* bronchopulmonary dysplasia
 chronic obstructive pulmonary disease (COPD), 22–23, 238, 242–243, 247–248, 286
 genetic susceptibility to, 86–87
 indoor air pollution, 85
 lung function development, 289–290
 malnutrition, 296
 tobacco product exposure, 82, 277
 chylothorax, 48
 ciliated cells, 9, 13–14
 CINC-1, 212–213
 c-Kit, 12, 305–306
 Clara cells, 144
 clarithromycin, 194–195
Cldn18, 67
 CLDs (chronic lung diseases), 194, 246–248, 286. *See also* bronchopulmonary dysplasia
 Clements, J.A., 141–142
 climate change, 87
 CLO (congenital lobar overinflation), 102–116
 club cells, 14
 clustering analysis, 65
 c-Myc, 11
 CNB1, 65
 CO (carbon monoxide), 84–86, 259
 Coalson, J.J., 246
 Coleman, T., 82–83
 collagens, 28
 collagen IV, 28
 nicotine exposure, 79, 81
 pulmonary arterial development, 46
 Collins, M.H., 78–79
 computer assisted sterology, 60–61
 congenital alveolar dysplasia, 103.
 See alveolar capillary dysplasia
 congenital bronchial cartilage deficiency, 115
 congenital bronchogenic cysts, 97–99
 congenital cystic adenomatoid malformations. *See* congenital pulmonary airway malformations
 congenital diaphragmatic hernia (CDH), 47, 130, 238
 congenital hypoventilation syndrome (CCHS), 225
 congenital lobar emphysema. *See* congenital lobar overinflation
 congenital lobar overinflation (CLO), 102–116
 congenital malformations, 94–118, 126–127
 associated with lung growth and structure, 104–118
 congenital lobar overinflation, 102–116
 congenital pulmonary airway malformations, 104–109
 pleuropulmonary blastoma, 109–111
 pulmonary hypoplasia, 105–107
 pulmonary lymphangiectasia, 116–118
 pulmonary sequestration, 104–115
 associated with lung initiation and formation, 95–104
 conducting airway abnormalities, 99–102
 lung/foregut abnormalities, 96–99
 pulmonary parenchymal malformations, 102–104
 future directions, 118
 overview of lung development, 94–95
 congenital pulmonary airway malformations (CPAM), 104–109, 111–114
 congenital pulmonary lymphangiectasia. *See* pulmonary lymphangiectasia
 connective tissue disorders, 100
 continued alveolarization phase, 133–137, 238–248
 in animals, 244–245
 calorie related changes, 245
 following pneumonectomy, 244–245
 possible mechanisms of, 245
 in rabbits and rhesus monkeys, 245

- continued alveolarization phase (cont.)
 difficulties and current concepts, 238–239
 future directions, 248
 histological techniques, 242
 in humans, 245–248
 alveolar plasticity, 247
 following pneumonectomy, 246
 metabolic demand, 247
 non-invasive measurements, 245–246
 physiologic rationale for, 246
 recovery of alveolar structure following preterm birth, 246
 survivors of preterm birth, 247–248
 indirect measures, 244
 morphometric studies, 239–242
 drawbacks of, 240–242
 timeline, 239–240
 non-histological measures, 242–244
 aerosol derived airway morphometry, 243–244
³Helium magnetic resonance, 242–244
- continuous positive airway pressure (CPAP), 141–142, 151–152, 169, 173, 177–178, 209, 216–217, 227, 233
- convection zone, 127–128
- conventional vessels, 36, 128
- Cook, D.G., 297
- cook stoves, 85–86
- COPD. *See* chronic obstructive pulmonary disease
- corticosteroids, 137, 141–144, 152–153, 195–196, 287
- corticotropin releasing hormone, 152
- cortisol, 152, 166–167
- Costello syndrome, 117
- COUP-TFII, 45–47
- COX-2 (cyclooxygenase-2)
 inhibitors, 212
- Coxson, H.O., 247
- CPAM (congenital pulmonary airway malformations), 104–109, 111–114
- CPAP (continuous positive airway pressure), 141–142, 151–152, 169, 173, 177–178, 209, 216–217, 227, 233
- CPG (central pattern generator), 223–226
- CpG-DNA, 192–193
- CPP (cardiopulmonary progenitor) cells, 39–40, 47, 49–50
- CRD (carbohydrate recognition domain), 143–144
- Crowley, P.A., 195–196
- Crump, C., 291–292
- CSFR2A, 158
- CSFR2B, 158
- CTGF, 193–194, 211
- Cunningham, J., 78
- curcumin, 211
- CuZn superoxide dismutase, 273, 307–308
- CXCL9, 193
- CXCL10, 193
- CXCR2, 212–213
- CXCR4, 12
- cyclic adenosine monophosphate (cAMP), 13–14, 166–167, 215
- cyclic guanosine monophosphate (cGMP), 215–216
- cyclooxygenase-2 (COX-2)
 inhibitors, 212
- cysteine thiols, 215
- cystic fibrosis (CF), 238, 286
 lung function tests, 254–255, 257–261
 tracheal stenosis, 100
- cystic fibrosis transmembrane conductance regulator (CFTR), 14, 100
- cytokines, 178, 209, 212–213, 230
- DA. *See* ductus arteriosus
- DAVID functional annotation tool, 66
- Davies, G., 241
- Dawson, J.A., 177
- definitive endoderm (DE), 3–4
- Delta-like 1, 42
- Delta-like 3, 42
- Delta-like 4, 42, 48–49
- DeMello, D.E., 36–37
- Desai, R., 198
- dexamethasone, 14, 211–212
- DHA (docosahexaenoic acid), 196
- DHCR7, 105–107
- DICER1, 111, 118
- diffusion front, 127–128
- DiGeorge syndrome, 102
- directed differentiation
 defined, 11
 deriving lung epithelium de-novo via, 11
- dismutase, 274–275
- Dkk1, 4
- docosahexaenoic acid (DHA), 196
- Dolly (cloned sheep), 10–11
- domain branching, 24
- dopamine, 226
- Down syndrome (trisomy 21), 97, 102, 105–107, 117
- ductus arteriosus (DA), 170
 changes in flow at birth, 172
 patent, 206–217
- Dunnill, M.S., 239
- duodenal atresia, 96–97
- EA (esophageal atresia), 6, 96–97, 129
- Eagle-Barret syndrome, 105–107
- ECFCs (endothelial colony-forming cells), 305–306
- echinoderm microtubule-associated protein-like 4 (EML4), 109
- E-cigarettes (electronic cigarettes), 83–84
- ECM. *See* extracellular matrix
- Edwards syndrome (trisomy 18), 97
- EGF (epidermal growth factor), 13, 41–42
- EGFR (epidermal growth factor receptor), 41–42, 67–69, 109
- EGLN1, 275
- elastase, 50, 213
- elastin, 190, 212–213, 292–294
 alveolarization, 134–137
 nicotine exposure, 78–79
 pulmonary arterial development, 46
- electronic cigarettes (E-cigarettes), 83–84
- Elliott, F.M., 128
- ELS (extralobular sequestrations), 111–115
- EMAPII (endothelial-monocyte activating polypeptide II), 44–45
- embryonic stage of lung
 development, 2–9, 23, 94–95, 128–129, 133, 187, 269–271
 dorsal-ventral patterning of foregut into trachea and esophagus, 6
 epithelial differentiation, 9
 formation and early anterior-posterior patterning of the endoderm, 2–4
 future directions, 15–16
 lung tissue from pluripotent stem cells, 9–15
 overview of, 2
 primary lung bud outgrowth, 6–8
 pulmonary vascular development, 34–35
 specification of Nkx2-1⁺ respiratory progenitors, 4–6
 embryonic stem cells (ESC), 10
 deriving lung epithelium de-novo via “directed differentiation” of ESC, 11
 functional assays of ESC-derived putative lung lineages, 15
 generating from mice, 10
 isolating from humans, 10
 lung specification from ESC-derived endoderm, 13
 proximal-distal patterning of ESC-derived lung lineages, 13–14

- EML4 (echinoderm microtubule-associated protein-like 4), 109
- emphysema, 261, 306, 308
- ENaCs (epithelial Na⁺ channels), 164, 166–167, 172, 198
- ENCODE, 66
- endoderm, 270
 - anterior foregut endoderm
 - induction in PSC, 12–13
 - formation and early
 - anterior-posterior patterning of, 2–4
 - lung specification from ESC-derived endoderm, 13
 - lung specification from ESC/iPSC-derived, 13
 - in vitro definitive endoderm
 - induction, 12
- endoglin, 42–43
- endomucin, 46
- endostatin, 44–45
- endothelial colony-forming cells (ECFCs), 305–306
- endothelial-monocyte activating polypeptide II (EMAPII), 44–45
- Engel, L.A., 258
- Ensembl, 66
- environmental air pollution, 84–85, 197–198
- environmental effects on lung morphogenesis and function, 77–88
 - climate change, 87
 - electronic cigarettes, 83–84
 - environmental air pollution, 84–85
 - future directions, 87–88
 - genetic susceptibility, 86–87
 - indoor air pollution, 85–86
 - tobacco product exposure, 78–83
 - clinical sequela of, 82
 - effects of, 78
 - mechanisms underlying effects of, 78–81
 - nicotine replacement therapy, 82–83
 - second hand tobacco smoke, 83
- EPCAM, 12
- Eph (ephrins), 44
 - EphA1, 44
 - EphB1, 44
 - EphB2, 42, 44, 47
 - EphB4, 42, 44
- epidermal growth factor (EGF), 13, 41–42
- epidermal growth factor receptor (EGFR), 41–42, 67–69, 109
- epigenetics, 58–60
- epinephrine, 226
- epithelial Na⁺ channels (ENaCs), 164, 166–167, 172, 198
- epithelium
 - branching morphogenesis, 24
 - deriving de-novo via “directed differentiation” of ESC/iPSC, 11
 - differentiation, 9, 129–131
 - origins of, 2
- ERK, 13–14
- erythropoietin administration, 209–210
- ESC. *See* embryonic stem cells
- esophageal atresia (EA), 6, 96–97, 129
- esophageal fistulas, 97
- esophagus
 - branching morphogenesis, 23–24
 - dorsal-ventral patterning of
 - foregut into, 6
- ESRRB, 11
- ETSF, 67
- Etv5, 67
- exome sequencing, 58–60
- extracellular matrix (ECM), 15, 129
 - influence on branching
 - morphogenesis, 28–29
 - pulmonary arterial development, 46
- extralobular sequestrations (ELS), 111–115
- extrinsic bronchial stenosis, 101
- extrinsic tracheal stenosis, 100
- Ezh2, 25
- FACS, 13
- FACS (fluorescence activated cell sorting), 63–64
- FAM13A, 290
- familial pulmonary arterial hypertension (FPAH), 43
- Fasudil, 216
- Fawke, J., 265, 292
- FBM (fetal breathing movements), 105–107, 130, 165–166, 198, 229–230
- Fedulov, A.V., 84–85
- Fehrenbach, H., 244–245
- Feingold syndrome, 96–97
- fenretinide, 308
- fetal breathing movements (FBM), 105–107, 130, 165–166, 198, 229–230
- fetal liver kinases (Flks). *See* VEGFRs
- fetal lung fluid, 144–147, 153, 198. *See also* airway liquid clearance
- FGFRs (fibroblast growth factor receptors), 4–5, 27
 - FGFR1, 40–41
 - FGFR2, 7–8, 99–100
 - FGFR2B, 6, 8, 27
 - FGFR4, 207
- FGFs (fibroblast growth factors), 2, 4–8, 12–13, 15–16, 23, 27, 40–41
 - FGF1, 40–41
 - FGF2, 4–5, 13–14, 40–41
 - FGF7 (KGF), 13–14, 40–41, 109, 210–211
 - FGF9, 5, 8, 27, 40–41, 109
 - FGF10, 5–8, 13–16, 26–27, 40–41, 45, 109, 190, 305
 - FGF18, 14, 40–41
- fibroblasts, 38–39, 62–63, 129
- fibronectin, 46
- fibulin-5, 44–45
- Filbrun, A.G., 292–293
- Firth, A.L., 14
- Fisher’s exact test, 66
- flagellin, 192–193
- Flks (fetal liver kinases). *See* VEGFRs
- flow meters, 254–257
- flow-volume loops, 254–255, 261–262, 286–287
- Flt-1, 44–45
- fluorescence activated cell sorting (FACS), 63–64
- foramen ovale (FO), 170
- forced expiratory flow-volume measurements, 255–256
- formaldehyde, 83–85
- Fox (Forkhead box) family, 24, 45–46
 - FOXA1, 9, 24
 - FOXA2, 3–4, 9, 12–14, 24, 26, 65, 67
 - FOXC1, 45–46, 48–49
 - FOXC2, 45–46, 48–49, 117
 - FOXF1, 26, 103–104, 118, 305
 - pulmonary arterial
 - development, 42, 45–46
 - pulmonary capillary system
 - development, 46–47
 - FOXJ1, 9, 13–14, 70–71
 - FOXM1, 67–69
 - FOXP1, 24
 - FOXP2, 24, 67
 - FOXP4, 24
- FPAH (familial pulmonary arterial hypertension), 43
- FRC (functional residual capacity), 164, 166–168, 173, 177, 225
- Friedrich, L., 292
- frizzled (Fz) family, 27, 43
 - Fzd2, 27
- Fuchs, H., 173
- Fujiwara, T., 141–142
- functional residual capacity (FRC), 164, 166–168, 173, 177, 225
- furosemide, 210
- Fusobacterium* spp., 188–189
- Fuzzy Heuristic Partition, 65
- Fz (frizzled) family, 27, 43
 - Fzd2, 27

- G alpha, 70
 G protein-coupled receptor 126 (*GPR126*), 290
 GABA (gamma-aminobutyric acid), 225–227, 233
 GABA_A receptors, 225–226
 GABA_B receptors, 225–226
 gas dilution techniques, 257–259
 gastrin-releasing peptide (GRP), 213
 gastrulation, 2–4, 12, 15–16
 GATA family, 46
 GATA1, 46
 GATA2, 46, 49
 GATA3, 46
 GATA6, 9, 26, 67
 GCs (glucocorticoids), 9, 72, 137, 152–153, 195–196
 Gene Ontology (GO) similarity analysis, 67
 GeneCards, 66
 genetic susceptibility, 86–87
 genome-wide association studies (GWAS), 58–60
 Gerber, H.P., 41
 GFP, 12–13
 Gharehbaghi, M.M., 195
 Gli family, 40
 Gli1, 193–194
 Gli2, 6
 Gli3, 6
 glomus (Type I) carotid body cells, 228–229
 Gluck, L., 153
 glucocorticoids (GCs), 9, 72, 137, 152–153, 195–196
 glutamate, 225–226, 228
 glutathione transferases (GST), 87
 GST M1, 87
 GST T1, 87
 glycine, 225–227
 glycogen, 146, 152
 GM-CSF, 146–147, 158, 191, 193
 GO (Gene Ontology) similarity analysis, 67
 goblet cells, 9, 13–14
 Goldenhar syndrome, 102
 GoMiner functional annotation tool, 66
 Gooseoid transcription factor, 3–4
GPR126 (G protein-coupled receptor 126), 290
 Gray, B., 78
 Green, M.D., 12–13
 Grhl2, 67
Group B streptococci, 187–188
 GRP (gastrin-releasing peptide), 213
 GSEA functional annotation tool, 66
 GST (glutathione transferases), 87
 GST M1, 87
 GST T1, 87
 GTP cyclohydrolase I, 216
 Guerra, S., 297
 Gurdon, Jim, 10–11
 GWAS (genome-wide association studies), 58–60
 Hall, S.M., 39, 47
 Hancox, R.J., 295
 Hanrahan, J.P., 78
 HDACs (histone deacetylases), 8, 25, 307
 HDAC1, 25
 HDAC2, 25, 307–308
 Hedgehog (HH), 2, 6–8, 27–28
 hedgehog interacting protein (*HHIP*), 290
³Helium magnetic resonance (³HeMR), 242–243, 245–246
 early studies using, 244
 merits and demerits of, 243–244
 Hennekam syndrome, 117
 heparin binding EGF, 41–42
 heparin proteoglycans, 41
 heparin sulfate, 13
 hepatocyte growth factor (HGF), 210–211
 Hes1, 9, 45–46
 Heyder, J., 243
 HGF (hepatocyte growth factor), 210–211
 HGMD (Human Gene Mutation Database), 66
 HH (Hedgehog), 2, 6–8, 27–28
 HHEX, 4, 12–13, 15–16
HHIP (hedgehog interacting protein), 290
 HIFs (hypoxia-inducible transcription factors), 45, 272–273
 HIF-1, 194
 HIF-1α, 45, 215, 272–273, 305–308
 HIF-1β, 272–273
 HIF-2, 272–273
 HIF-2α (EPAS1), 45, 194, 215, 275, 305
 Hippo, 8, 15–16
 Hislop, A., 36, 240
 histone deacetylases. *See* HDACs
 HO-1, 193–194
 Hollams, E.M., 297
 Hoo, A.F., 78
 Hopx, 67
 Howie, R.N., 141–142
 Hox family, 4, 6–7, 25, 45
 HOXA5, 4, 6–7, 25
 HOXB5, 4, 25, 45, 109
 HOXD13, 97
 Hsia, C.C., 244–245
³H-thymidine, 279
HTR4 (5-hydroxytryptamine receptor 4), 290
 Huang, S.X.L., 14
 Human Gene Mutation Database (HGMD), 66
 Husain, A.N., 246
 hyaline membranes, 150, 177–178
 Hyde, D.M., 241–242, 245
 hydrocortisone, 212
 5-hydroxytryptamine receptor 4 (*HTR4*), 290
 hyperbilirubinemia, 209, 216–217
 hypercapnia, 207–209, 229–230
 hyperoxemia, 174
 hyperoxia, 50, 165–166, 174–175, 178–179, 208–209, 211, 229, 273–275, 277–278
 hyperplasia, 48, 115–116, 198, 206
 hypoplasia, 46–47, 79, 105–109, 111–112, 130, 197–199, 206
 hypoxemia, 196–197
 hypoxia, 165–166, 172, 174–175, 194, 223, 229–230, 275–278.
 See also HIFs
 hypoxia-inducible transcription factors. *See* HIFs
 I (inspiratory) cells
 Iβ, 227
 Iγ, 227
 IBMX, 13
 ICA (Independent Components Analysis), 65
 Id1, 43
 Id2, 7–8, 15–16, 70–71
 IFNγ, 193
 IGFbPs (insulin-like growth factor binding proteins), 44
 IGFs (insulin-like growth factor receptors)
 IGFR1, 44
 IGFR2, 44
 IGFs (insulin-like growth factors), 44
 IGF-1, 44, 210
 IGF-2, 44
 Ihida-Stansbury, K., 45
 ILD (interstitial lung disease), 156
 ILs (interleukins)
 IL-1, 178, 189, 193, 212–213
 IL-1α, 188
 IL-1β, 188–190, 193–194, 213
 IL-4, 193
 IL-6, 178, 188, 191, 275–276
 IL-8, 178, 188, 193
 IL-10, 193
 IL-12, 193
 IL-13, 193
 ILS (intra-lobe sequestrations), 111–115
 immunofluorescence confocal microscopy, 61–63
 impulse oscillometry, 287
 Independent Components Analysis (ICA), 65

- indoor air pollution, 85–86
- induced pluripotent stem cells (iPSC), 10–11
- deriving lung epithelium de-novo via directed differentiation of, 11
 - functional assays of iPSC-derived putative lung lineages, 15
 - generating from humans, 11
 - lung specification from iPSC-derived endoderm, 13
 - master transcription factors, 11
 - nuclear transfer, 10–11
 - proximal-distal patterning of iPSC-derived lung lineages, 13–14
- infantile lobar emphysema. *See* congenital lobar overinflation
- Ingenuity knowledge base (IPA), 66
- initiation of breathing at birth, 164–179
- airway liquid clearance, 166–172
 - at birth, 167
 - after birth, 168–169
 - changes in ductus arteriosus flow at birth, 172
 - consequences of lung recoil, 169–170
 - hemodynamic consequences of lung aeration, 170–172
 - during labor, 166–167
 - before labor, 166
 - future directions, 179
 - respiratory drive, 165–166
 - support strategies for preterm
 - infants failing transition, 172–176
 - facilitating lung liquid clearance and lung aeration, 172–173
 - maintaining functional residual capacity, 173
 - use of oxygen, 174–176
 - ventilation induced injury, 176–179
 - during clearance of lung liquid and lung aeration, 176–177
 - following lung aeration, 177–178
 - hemodynamic consequences, 178
 - oxygen injury, 178–179
 - systemic consequences, 178
- innate immunity, 271
- inspiratory cells. *See* I cells
- insulin-like growth factor binding proteins (IGFBPs), 44
- insulin-like growth factor receptors (IGFRs)
- IGFR1, 44
 - IGFR2, 44
- insulin-like growth factors. *See* IGFs
- integrins, 28–29, 46
- α v integrins, 46
 - β 1 integrins, 46
 - β 3 integrins, 46
- interleukins. *See* ILs
- interstitial lung disease (ILD), 156
- intralobar sequestrations (ILS), 111–115
- intrauterine growth restriction (IUGR), 85–87, 196–197, 292–294
- intraventricular hemorrhage (IVH), 173, 178
- intrinsic bronchial stenosis, 101
- intrinsic tracheal stenosis, 99–100
- intussusceptive angiogenesis, 37
- IPA (Ingenuity knowledge base), 66
- iPSC. *See* induced pluripotent stem cells
- IRAK-M, 192–193
- IRF1, 67
- Isl1, 40, 47
- Ito, K., 307
- IUGR (intrauterine growth restriction), 85–87, 196–197, 292–294
- IVH (intraventricular hemorrhage), 173, 178
- Jagged 1, 42
- Jagged 2, 42
- Jedrychowski, W.A., 84
- Joad, J.P., 83
- Karlinsky, J.B., 245, 247
- Kasahara, Y., 306
- KEGG (Kyoto Encyclopedia of Genes and Genomes), 66
- KGF, 14
- Klf4, 11, 272–273
- Kovar, J., 245
- K-RAS, 67, 109
- Krasnow, Mark, 305–306
- Kreiner-Møller, E., 291
- Kyoto Encyclopedia of Genes and Genomes (KEGG), 66
- LacZ, 37–38
- lamellar bodies, 78–79, 131, 144–146, 151
- laminins (LNs), 28, 46, 130–131
- laryngeal atresia, 115–116
- LBW (low birth weight), 82–85, 197–198, 206
- LCI (lung clearance index), 257
- LEF, 4, 43
- Liggins, G.C., 141–142, 195–196
- Lin28, 11
- Lindner, W., 173
- lipofibroblasts, 62–63
- lipopolysaccharide (LPS), 188–196, 230–231, 273
- LL34, 26
- lncRNAs (long non-coding RNAs), 26, 71
- LNs (laminins), 28, 46, 130–131
- Loeb, J.S., 260–261
- long non-coding RNAs (lncRNAs), 26, 71
- Longmire, T.A., 14
- low birth weight (LBW), 82–85, 197–198, 206
- lower respiratory illnesses (LRI), 82, 85
- LPS (lipopolysaccharide), 188–196, 230–231, 273
- LRI (lower respiratory illnesses), 82, 85
- Lrp5, 27
- Lrp6, 27
- L-sepiapterin, 216
- lung cancer, 86–87, 277
- lung clearance index (LCI), 257
- lung diffusing capacity
 - measurements, 259–260
- lung function development, 286–298
- in animal models of premature birth and BPD, 294–295
 - asthma and, 287–288
 - COPD and, 289–290
 - future directions, 297–298
 - genetics of, 290–291
 - genetics of BPD, 293–294
 - intrauterine growth retardation and, 295–296
 - malnutrition and, 296–297
 - prematurity and, 291–293
 - tobacco product exposure and, 297
 - tracking with age, 288–289
 - wheezing and, 287–288
- lung function tests, 253–266, 286–287
- challenges of, 254, 260
 - clinical application of, 262–265
 - differences in FEV₁ between BPD and non BPD groups among preterm survivors, 264–265
 - differences in FEV₁ between preterm and control subjects, 262–264
 - forced expiratory flow-volume measurements, 255–256
 - future directions, 265–266
 - gas dilution techniques, 257–259
 - lung diffusing capacity, 259–260
 - published guidelines for, 253–257, 259–262
 - reference equations for, 262

- lung function tests (cont.)
 techniques for, 260–262
 tidal breathing measurements, 254–255
 whole-body plethysmography, 256–257
- lung lineages
 functional assays of ESC/iPSC-derived putative, 15
 proximal-distal patterning of ESC/iPSC-derived, 13–14
- lung structure, 126–137
 pre- and postnatal lung development, 128–137
 alveolarization, 134–137
 canalicular stage, 130–133
 embryonic stage, 128–129, 133
 pseudoglandular stage, 129–130, 133
 saccular stage, 132–134
 structural perspective on lung function, 126–128
 pulmonary airway tree, 127–128
 pulmonary vascular tree, 128
- lung structure maintenance program, 303–308
 copper, 307–308
 failure mechanisms, 306
 future directions, 308
 HDAC/HIF-1 α /VEGF axis, 306–307
 pulmonary vascular development, 305–306
 sphingolipids, 307–308
- LungGENS, 67
 LungMAP, 67
- lymphangiogenesis. *See* pulmonary lymphatic development
- lymphangiomatosis, 116–117
 lymphatic hyperplasia, 48
 Lys, 65
 lysyl-oxidase, 213, 307–308
- macrolides, 194–195
 macular degeneration, 11
 Mallory, 48
 mammalian target of rapamycin complex 1 (mTORC1), 305
- mammalian lung structure and air flow, 269
- MAPK, 4–5, 13–14, 70
 Marbach, D., 66–67
 Maritz, G.S., 78–79
 mask ventilation, 164–165, 175–177
 mass spectroscopy, 58–60
 Massaro, D., 245–247
 master transcription factors, 11
 MBW (multiple breath washout) tests, 257–258
- MCP-1, 193
 Mead, J., 141–142, 147, 238
- Meis, 6–7
 MEK1, 13
 MEK2, 13
 Menkes' disease, 307–308
 mesenchymal stem cells (MSCs), 15
 “mesendoderm” state, 3–4, 12
 methacholine, 80–81, 265
 methylxanthines, 232–234
 microcephaly, 96–97
 microRNAs. *See* miRNAs
 microvascular maturation phase, 134–136
- MIDI*, 97
 Miller, L.A., 84–85
 miRNAs (microRNAs), 8, 26, 45, 84–85
 miR17-92, 26
 miR-130, 44–45
 miR-130a, 26
 miR-149, 84–85
 miR-221, 26, 44–45
 miR302-367, 26
- misalignment of the pulmonary veins (MPV), 103–104
- Mizuno, S., 307
 MMP-9, 193–194
 MnSOD, 306
 Moessinger, A.C., 198
- morphogenesis. *See* branching morphogenesis; pulmonary vascular development
- Mortimer, K., 84
 MPV (misalignment of the pulmonary veins), 103–104
- MSCs (mesenchymal stem cells), 15
 mTORC1 (mammalian target of rapamycin complex 1), 305
- MUC1, 67
 MUC5AC, 9, 13–14, 277
 MUC5B, 277
- multiple breath washout (MBW) tests, 257–258
- Myb, 9
 MYCN, 97
Mycoplasma spp., 188–189
Mycoplasma hominis, 188–190
 MyoD, 11
- nAChRs (nicotinic acetylcholine receptors), 78–81, 197
 α 5 nAChR, 86–87
- NANCI, 26, 71
 Nanog, 11
 Narang, I., 247–248
 Narayanan, M., 245–246
 nasal tubes, 175–176
 NCBI Entrez Gene database, 66
 neuroendocrine cells, 9, 13–14
 NFATs (nuclear factor of activated T cells), 49
 NFATc1, 49
 NF- κ B, 190, 193–194, 208
- nicotine, 78–81, 196–197, 277
 nicotine replacement therapy (NRT), 82–83
 nicotinic acetylcholine receptors (nAChRs), 78–81, 197
 α 5 nAChR, 86–87
- nitric oxide (NO), 84–85, 197–198, 214–216, 273
- Nkx2-1 (thyroid transcription factor-1 [TTF-1]), 2, 8–9, 13–16, 23–26, 65, 67, 70–71, 105, 118, 156–157, 158
 endodermal precursors, 14
 haploinsufficiency, 156–157
 respiratory progenitors, 4–6, 11
- NMDA glutamate receptors, 225–226, 228
- NMR (nuclear magnetic resonance spectroscopy), 58–60
- N-myc proto-oncogene, 25
- NO (nitric oxide), 84–85, 197–198, 214–216, 273
- Nodal, 2–4, 15–16
 Noggin, 4, 6, 13
- Nonne-Milroy lymphedema syndrome, 117
- non-NMDA glutamate receptors, 227–228
- non-steroidal antiinflammatory drugs (NSAIDs), 211–212, 217
- Noonan syndrome, 117
 norepinephrine, 226
 Northway, W.H., 205, 265
- NOSIII, 190
- Notch system, 9
 Notch1, 42
 Notch2, 42, 45–46
 Notch3, 42
 Notch4, 42
 pulmonary arterial development, 42–43
 pulmonary venous development, 47
- NR3C1, 72
 NR5A2, 11
- NRT (nicotine replacement therapy), 82–83
- NSAIDs (non-steroidal antiinflammatory drugs), 211–212, 217
- NTS (nucleus of the solitary tract), 224, 226–228
- nuclear factor of activated T cells (NFATs), 49
 NFATc1, 49
- nuclear magnetic resonance spectroscopy (NMR), 58–60
- nuclear transfer, 10–11
- nucleus of the solitary tract (NTS), 224, 226–228
- NuRD, 25

- nutrition, 196, 296–297
 nutritional emphysema, 136–137
 Ochs, M., 247
 Oct4, 11
 oligohydramnios, 105–107, 134, 198, 206
 Oliver, G., 48
 Online Mendelian Inheritance in Man (OMIM), 66
 Onto-Express functional annotation tool, 66
 opioids, 226
 Opitz G/BBB syndrome, 97
 organogenesis. *See* embryonic stage of lung development
 orthogonal bifurcation, 24
 overinflation syndrome. *See* congenital lobar overinflation
 oxidative stress, 86
 oxygen toxicity, 207–208
 Ozdemir, R.O., 194–195
 ozone, 84–85, 87, 276–278

 p21, 136–137, 274–275
 p23, 275
 p27, 136–137
 p53, 111, 307
 p63, 13–14
 packed red blood cell transfusion, 209–210
 PAH (polycyclic aromatic hydrocarbons), 85–86
 Paiva, M., 258
 palmitoyl-protein thioesterase 2 (*PPT2*), 290
 PamCysK4, 188, 192–193
 PAMPs (pathogen-associated molecular patterns), 144
 Paracelsus, 279
 particulate matter, 84–87
 patched (*Ptc*), 27–28, 40
 PTCH1, 290
 patent ductus arteriosus (PDA), 206–217
 pathogen-associated molecular patterns (PAMPs), 144
 Patsari stoves, 85–86
 Pattle, R., 141–142
 PAX8, 4
 PAX9, 12–13
 PBF (pulmonary blood flow) at birth, 170–172
 Pbx, 6–7
 PCA (Principal Components Analysis), 65
 P-cells (pump cells), 227
 PD98059, 13
 PDA (patent ductus arteriosus), 206–217
 PDE (phosphodiesterase) inhibitors
 PDE-4, 215
 PDE-5, 215
 PDGFRs (platelet derived growth factor receptors)
 PDGFR-alpha, 43
 PDGFR-beta, 43
 PDGFs (platelet derived growth factors), 43–44, 305
 PDGF-A, 43–44
 PDGF-AA, 210
 PDGF-B, 43–44, 48–49
 PDGF-BB, 210
 Pdpn, 9
 PEDF (pigment epithelial derived factor), 44–45
 PEEP (positive end expiratory pressure), 173, 177–178
 Pena-Ahokeir syndrome, 105–107
 Peng, T., 39–40, 47
 perinatal disruptions of lung development, 269–280. *See also* environmental effects on lung morphogenesis and function
 environmental influences as antecedent of evolutionary novelty, 277–279
 future directions, 279–280
 new world pollutants, 276–277
 oxygen environment at birth, 272–276
 clinical trials, 273–274
 fluctuations in oxygen environment, 276
 hyperoxia, 273–275
 hypoxia, 275–276
 ozone, 276–277
 tobacco product exposure, 276–277
 perinatal insults to lung structure and function, 187–199
 bronchopulmonary dysplasia, 194–195
 chorioamnionitis, 187–193
 animal models of, 188–189
 effects on fetal lung development, 195–196
 human studies of, 194
 inflammation/injury of fetal lung, 190–191
 innate immune responses to, 191–193
 maturation of fetal lung and, 189
 fetal breathing and lung fluid, 198–199
 fetal growth restriction, 196
 future directions, 199
 glucocorticoids, 195–196
 lung growth stages, 187
 maternal nutrition, 196
 prenatal toxin exposure, 196–198
 signaling mediators modulating lung development after, 193–194
 Ureaplasma spp., 194–195
 periventricular leukomalacia (PVL), 230–231
 peroxisome proliferator-activated receptor gamma co-activator (PGC-1alpha), 305
 peroxisome proliferator-activated receptors. *See* PPARs
 peroxynitrite, 212–215
 persistent pulmonary disease (PPD), 273
 persistent pulmonary hypertension of the newborn (PPHN), 215–216
 Petrache, I., 308
 Pfeiffer syndrome, 99–100
 PFT (pulmonary function testing). *See* lung function tests
 PGC-1alpha (peroxisome proliferator-activated receptor gamma co-activator), 305
 PGEs (prostaglandins), 230
 PGE₂, 165
 PH (pulmonary hypertension), 42, 46, 107, 132, 197, 277–278
 PHD2, 275
 Phelan McDermid syndrome, 117
 phosphatidylcholine, 61–62, 142–148, 150–152, 154–155
 phosphatidylglycerol, 142–143, 153–155
 phosphatidylinositol, 142–144
 phosphodiesterase (PDE) inhibitors
 PDE-4, 215
 PDE-5, 215
 phosphodiesterase inhibitors, 215
 phosphotyrosine interaction domain containing 1 (*PID1*), 290
PHOX2B, 225
PID1 (phosphotyrosine interaction domain containing 1), 290
 pigment epithelial derived factor (PEDF), 44–45
 PIR (Protein Information Resource), 66
 PL (pulmonary lymphangiectasia), 48, 111–112, 116–118
 planar bifurcation, 24
 platelet derived growth factor receptors (PDGFRs)
 PDGFR-alpha, 43
 PDGFR-beta, 43
 platelet derived growth factors. *See* PDGFs
 pleuro-peritoneal membrane, 130
 pleuropulmonary blastoma (PPB), 104–111

- PLK1, 67–69
 Plopper, C.G., 84–85
 pluripotency, concept of, 10
 pluripotent stem cells (PSC), 9–15
 anterior foregut endoderm induction in PSC, 12–13
 deriving lung epithelium de-novo via the “directed differentiation” of ESC/iPSC, 11
 embryonic stem cells (ESC), 10
 functional assays of ESC/iPSC-derived putative lung lineages, 15
 induced pluripotent stem cells (iPSC), 10–11
 lung specification from ESC/iPSC-derived endoderm, 13
 proximal-distal patterning of ESC/iPSC-derived lung lineages, 13–14
 in vitro definitive endoderm induction, 12
 pneumonectomy, 51, 244–246
 pneumonia, 85
 pneumotachography, 254–255, 286–287
 podoplanin, 49
 polycomb repressive complex (PRC), 25
 PRC2, 25
 polycyclic aromatic hydrocarbons (PAH), 85–86
Pon1, 65
 positive end expiratory pressure (PEEP), 173, 177–178
 positive pressure ventilation (PPV), 164–165, 173, 176–178, 205
 Potter sequence, 105–107
 Potter’s syndrome (renal agenesis), 198
 PPARs (peroxisome proliferator-activated receptors)
 PPAR α , 275
 PPAR γ , 43, 67–69, 196, 211, 275
 PPB (pleuropulmonary blastoma), 104–111
 PPD (persistent pulmonary disease), 273
 PPHN (persistent pulmonary hypertension of the newborn), 215–216
 pPROM (preterm prelabor rupture of membranes), 198
PPT2 (palmitoyl-protein thioesterase 2), 290
 PPV (positive pressure ventilation), 164–165, 173, 176–178, 205
 PRC (polycomb repressive complex), 25
 PRC2, 25
 pre-Bötzinger complex (pre-BötC), 224
 preeclampsia, 194, 206
 preterm prelabor rupture of membranes (pPROM), 198
 primary lung bud outgrowth, 6–8
 primary pulmonary hypoplasia, 105
 primary pulmonary lymphangiectasia, 117–118
 Principal Components Analysis (PCA), 65
 progenitor cell therapy, 51
 progesterone, 165
 Proietti, E., 84
 prostaglandins (PGEs), 230
 PGE₂, 165
 Protein Information Resource (PIR), 66
 proteinacious pulmonary edema, 150
 Prox1
 pulmonary arterial development, 45
 pulmonary lymphatic development, 48–49
 PS (pulmonary sequestrations), 104–115, 130
 PSC. *See* pluripotent stem cells
 pseudoglandular stage of lung development, 2, 7, 23–24, 38, 41, 44–46, 48, 77–78, 94–95, 129–130, 133, 187, 269–270
 tight barrier function, 269, 271
 Ptc (patched), 27–28, 40
 PTCH1, 290
 PTEN (phosphatase encoding), 97
 PTPN11, 117
 PU.1, 191
 pulmonary agenesis, 102, 129
 pulmonary aplasia, 102, 129
 pulmonary arterial development (arteriogenesis), 34–35, 50
 cellular and molecular mechanisms in, 39–46
 angiopoietins and tie receptors, 42
 angiostatic factors, 44–45
 ephrins, 44
 epidermal growth factor receptor and ligands, 41–42
 extracellular matrix molecules, 46
 fibroblast growth factors, 40–41
 insulin-like growth factors, 44
 integrins, 46
 microRNAs, 45
 Notch system, 42
 platelet derived growth factors, 43–44
 Sonic hedgehog and patched, 40
 transcription factors, 45–46
 transforming growth factor, 42–43
 vascular endothelial growth factors, 41
 Wnts, 43
 origins of, 39
 overview of, 36–39
 patterning of, 38–39
 processes in, 36–38
 timing of, 36
 pulmonary blood flow (PBF) at birth, 170–172
 pulmonary capillary system development, 46–47, 50
 cellular and molecular mechanisms in, 46–47
 overview of, 46
 pulmonary dysplasia, 102–104
 pulmonary fibrosis, 41–42, 156, 205
 pulmonary function testing (PFT). *See* lung function tests
 pulmonary hyperplasia, 115–116, 198, 206
 pulmonary hypertension (PH), 42, 46, 107, 132, 197, 277–278
 pulmonary hypoplasia, 47, 79, 105–109, 111–112, 130, 197–199, 206
 pulmonary lymphangiectasia (PL), 48, 111–112, 116–118
 pulmonary lymphatic development (lymphangiogenesis), 47–49, 50
 cellular and molecular mechanisms in, 48–49
 overview of, 47–48
 pulmonary sequestrations (PS), 104–115, 130
 pulmonary valve stenosis, 129
 pulmonary vascular development, 34–51
 arteries, 34–35, 42
 angiostatic factors, 44–45
 cellular and molecular mechanisms, 39–46
 ephrins, 44
 epidermal growth factor receptor and ligands, 41–42
 extracellular matrix molecules, 46
 fibroblast growth factors, 40–41
 insulin-like growth factors, 44
 integrins, 46
 microRNAs, 45
 Notch system, 42
 platelet derived growth factors, 43–44
 Sonic hedgehog and patched, 40
 structure, origins, timing, and patterning, 36–39

- transcription factors, 45–46
transforming growth factor, 42–43
vascular endothelial growth factors, 41
vasculogenesis versus angiogenesis, 36–37
Wnts, 43
- capillary system, 46–47
cellular and molecular mechanisms, 46–47
structure, origins, timing, and patterning, 46
lymphatics, 47–49
cellular and molecular mechanisms, 48–49
structure, origins, timing, and patterning, 47–48
potential for regression and regeneration of normal lung vasculature, 51
pulmonary vascular function, 34–35
pulmonary vascular structure, 35
response to injury, 50
vasculogenesis versus angiogenesis, 49–50
veins, 47
cellular and molecular mechanisms, 47
structure, origins, timing, and patterning, 47
- pulmonary vein atresia, 47
pulmonary venous development, 47, 50
cellular and molecular mechanisms in, 47
overview of, 47
pump cells (P-cells), 227
PVL (periventricular leukomalacia), 230–231
- RA (retinoic acid), 2, 4, 6–8, 23, 105, 136–137, 196, 273, 305, 308
raised volume rapid thoracoabdominal compression (RVRTC), 255
Rajagopal, J., 13–14
Raldh2, 6
rapid thoracoabdominal compression (RTC), 255
rapidly adapting receptors (RARs), 224, 227–228
RDS (respiratory distress syndrome), 48, 141–142, 147–148, 151–153, 155, 194, 205
reactive oxygen species (ROS), 208
regional over-distension injury, 176–177
Rehan, V.K., 87
Reid, L.M., 128
renal agenesis (Potter's syndrome), 198
reptilian lung structure and air flow, 269
respiratory distress syndrome (RDS), 48, 141–142, 147–148, 151–153, 155, 194, 205
respiratory function monitoring (RFM), 176
respiratory inductive plethysmography, 254–255
respiratory morphogenesis, 2, 7–8.
See also branching morphogenesis; pulmonary vascular development
respiratory progenitors, 11
specification of, 2, 4–6
respiratory syncytial virus (RSV), 275–276
respiratory system overview, 1–2
respiratory zone, 127–128
retinoic acid (RA), 2, 4, 6–8, 23, 105, 136–137, 196, 273, 305, 308
retinoids, 137
retinopathy of prematurity (ROP), 273–274
retrotrapezoid nucleus (RTN), 224, 226, 228
Rett syndrome, 226
RFM (respiratory function monitoring), 176
RhoA, 216
Rho-kinase (ROCK), 216
RNA expression profiling, 63–69
data access, 64
data analysis, 64–65
differentially expressed genes, 65
pattern recognition and clustering analysis, 65
data output, 67
dynamic modeling of transcriptional programs, 67–69
transcriptional regulatory network regulating lung surfactant homeostasis, 67
data/knowledge integration, 65–67
biological knowledge incorporation, 66
driving force prediction, 67
gene set functional enrichment analysis, 66
network modeling, 66–67
RNA microarray, 63–64
RNA-Sequence analyses (RNA-Seq), 63–64
Robertson, B., 141–142
ROCK (Rho-kinase), 216
Rona, R.J., 295
ROP (retinopathy of prematurity), 273–274
ROS (reactive oxygen species), 208, 208
RSV (respiratory syncytial virus), 275–276
RTC (rapid thoracoabdominal compression), 255
RTN (retrotrapezoid nucleus), 224, 226, 228
RT-PCR, 63–64
RVRTC (raised volume rapid thoracoabdominal compression), 255
saccular stage of lung development, 2, 23–24, 38, 77–78, 94–95, 132–134, 187, 269–271, 275
alveolar formation, 60–61
expansion of gas-exchange area, 132–134
pulmonary arterial development, 41
Sapoval, B., 247
SARs (slowly adapting receptors), 224, 227
saturated phosphatidylcholine, 142–143, 146–148, 151
SB431542, 13
Scd1, 65
SCGB1A1, 9, 65, 70–71, 277
Schachtner, S.K., 37
Schelegle, E.S., 84–85
Schittny, J.C., 245
Scimitar syndrome, 105–107, 114–115
SCL34AC, 71
Scnn1g, 65
second hand smoke (SHS) exposure, 83
secondary pulmonary hypoplasia, 105–107
secondary pulmonary lymphangiectasia, 117
secretory cells, 13–14
secretory Club/Clara cells, 9
selective serotonin reuptake inhibitors (SSRIs), 197
semaxinib (SU5416), 306
sepsis, 209
serotonin, 130
sFlt-1, 42–45
Sfrps, 4–5
Sfrp1, 6
Sfrp2, 6
SFTPs (surfactant protein) family, 14, 69
nicotine exposure, 79
SFTPA, 14, 65, 71, 79, 142–143
SFTPA2, 158
SFTPB, 14, 65, 71, 79, 142–143, 153–154

- SFTPs (surfactant protein) family (cont.)
 SFTPC, 12–14, 28, 65, 70–71, 142–143, 155–156, 305
 SFTPD, 14, 65, 71, 142–143
 Shanbhag, D.D., 243
 Shh (Sonic hedgehog), 6–8, 14, 27–28, 40, 42, 67, 70–71, 97, 193–194
 Short Time-series Expression Miner (STEM), 67–69
 SHS (second hand smoke) exposure, 83
 SIDS (sudden infant death syndrome), 197, 226
 Sildenafil, 215
 Simpson, A., 291
 Sin3a, 25
 single nucleotide polymorphisms (SNPs), 207, 290, 293–294
 single-breath breath-holding technique, 259–260
 singular value decomposition, 65
 SIRP α , 143–144
Slc34a2, 65
 slowly adapting receptors (SARs), 224, 227
 Smad family, 5–6, 13
 Smad1, 5–6, 43
 Smad2, 3–4, 6, 193–194
 Smad3, 193–194
 Smad5, 5–6, 43
 Smad8, 5–6
 Smith-Lemli-Opitz syndrome, 105–107
 Smo (Smoothed), 27–28
 pulmonary arterial development, 40
 pulmonary venous development, 47
 Snider, Gordon, 306
 S-nitrosothiols, 215–216
 SNPs (single nucleotide polymorphisms), 207, 290, 293–294
 Sobotka, K.S., 175
 somatostatin, 226
 Sonic hedgehog (Shh), 6–8, 14, 27–28, 40, 42, 67, 70–71, 97, 193–194
 SOS1, 117
 Sox (sry-related high mobility group box) family, 46
 Sox F group, 46
 Sox2, 4–9, 11–16, 23, 25, 70–71, 97, 109, 118
 Sox7, 46
 Sox9, 7–9, 13–16, 25, 70–71
 Sox17, 3–4, 12, 36–38, 46
 Sox18, 46, 49
 SP (substance P), 226, 228
 Spedf, 9
 sphingomyelin, 153, 196
 sphingosine-1 phosphate, 308
 Spiegel, S., 308
 Spindel, E.R., 78–79
 spirometry, 255, 259–262, 287
 SPOCK2 (Testican-2), 293–294
 Sprouty, 8
 SPs (surfactant proteins), 61–62
 nicotine and, 197
 SP-A, 143–144, 146, 148–150, 189
 SP-B, 144, 146–150, 189
 SP-B deficiency, 153–154, 155
 SP-C, 144, 146–150, 189
 SP-C dysfunction, 155–156
 SP-D, 144, 146
 SREBP, 67
 sry-related high mobility group box family. *See* Sox family
 SSRIs (selective serotonin reuptake inhibitors), 197
 STAT1, 67–69
 STAT3, 193–194
 STEM (Short Time-series Expression Miner), 67–69
 Sterio, D.C., 242
 Stern, D.A., 288–289
 steroids, 206, 211–212
 stochastic mechanosensory stimulation, 229–232
 Stoddard, J.J., 78
 Strachan, D.P., 297
 SU5416 (semaxinib), 306
 substance P (SP), 226, 228
 sudden infant death syndrome (SIDS), 197, 226
 sulfur oxides, 84, 197–198
 supernumerary vessels, 36, 128
 Suresh, S., 295–296
 surfactant, 61–62, 65, 69, 131, 141–158. *See also* alveolar type 2 pneumocytes
 alveolar cycle of, 146
 defined, 141
 discovery of, 141–142
 future directions, 158
 genetic causes of abnormalities, 153–158
 ABCA3 deficiency, 154–155
 Nkx2-1 haploinsufficiency, 156–157
 pathology findings, 157–158
 SP-B deficiency, 153–154
 SP-C dysfunction, 155–156
 lung maturation, 151–153
 respiratory distress syndrome and induced lung maturation, 151–153
 tests for fetal lung maturation, 153
 physiology, 148–151
 inactivation, 150
 preterm surfactant-deficient lung, 149–150
 term fetus transitioning to air breathing, 148–149
 treatment responses, 151
 surfactant lipids, 142
 surfactant proteins, 142–144
 SP-A, 143–144
 SP-B, 144
 SP-C, 144
 SP-D, 144
 surfactant turnover, 146–148
 in adult lungs, 146–147
 in newborn lungs, 147–148
 synthesis and secretion of, 144–146
 transcriptional regulatory network regulating homeostasis, 67
 surfactant protein family. *See* SFTPs
 surfactant proteins. *See* SPs
 surfactant replacement therapy, 206
 Svanes, C., 297
 “systems biology” approach to study of epithelial maturation, 58–72
 advances in technologies, 58–60
 future directions, 72
 integration of gene expression data and lung structure, 63
 physiological adaptation to air-breathing and alveolar maturation, 61–63
 RNA expression profiling and functional genomics, 63–69
 data access, 64
 data analysis, 64–65
 data output, 67
 data/knowledge integration, 65–67
 role of mesenchymal-epithelial cross-talk, 72
 role of Nkx2-1, 71
 single cell genomics, 69–70
 structural dependency of lung ventilation at birth, 60–61
 transcriptional control of regulatory genetic circuits, 69
 T (brachyury), 3–4, 12
 T3 (triiodothyronine), 166–167
 Tager, I.B., 78, 82
 Takahashi, K., 11
 tamoxifen, 70–71
 TARCD syndrome, 97
 Taylor, B., 82
 TBX1, 12–13
 TBX4, 6–7
 TBX5, 6–7
 TCF, 4, 6, 43
 Te Pas, A.B., 173

- TEF (tracheoesophageal fistulas), 4, 6, 27–28, 96–97, 129
- tenascin-C (TN-C), 25, 43, 45–46, 136–137
- Tenney, S.M., 247
- teratocarcinomas, 10
- teratomas, 10
- Testican-2 (SPOCK2), 293–294
- tetracycline, 70–71
- tetrahydrobiopterin (BH4), 216
- TGFs (transforming growth factors)
- TGF- α , 41–42
 - TGF- β , 2–3, 6, 8, 12–13, 15–16, 28, 42–45, 50, 211, 275
 - TGF- β 1, 193–194, 211
- Thompson, B.R., 258
- thrombospondin, 46
- THSD4*, 290
 - TSP-1, 42–45
- Thurlbeck, W.M., 239–241
- thyrotropin-releasing hormone (TRH), 153, 226
- tidal breathing measurements, 254–255
- Tie1, 42
- Tie2, 42, 305–306
- tissue engineering, 15
- TLRs (Toll-like receptors)
- TLR2, 143–144, 193
 - TLR4, 193
 - TLR10, 207
- TN-C (tenascin-C), 25, 43, 45–46, 136–137
- TNFs (tumor necrosis factors), 178, 188
- TNF α , 188, 191, 193, 207, 275
- tobacco product exposure, 78–83, 196–197, 276–278
- clinical sequela of, 82
 - COPD and, 287–289
 - effects of, 78
 - intrauterine growth restriction and, 196
 - lung function development and, 297
 - mechanisms underlying effects of, 78
 - nicotine replacement therapy, 82–83
 - role of nicotine, 78–81
 - second hand tobacco smoke, 83
- Toll-like receptors. *See* TLRs
- ToppGene functional annotation tool, 66
- trachea, 1
- branching morphogenesis, 23–24
 - dorsal-ventral patterning of foregut into, 6
- tracheal agenesis, 97
- tracheal atresia, 6, 15, 115–116
- tracheal stenosis, 99–100
- tracheoesophageal fistulas (TEF), 4, 6, 27–28, 96–97, 129
- tracheomalacia, 100–101, 130
- transcriptional regulatory network (TRN) models, 67
- transforming growth factors. *See* TGFs
- transient tachypnoea (“wet lung”), 169
- TRH (thyrotropin-releasing hormone), 153, 226
- triiodothyronine (T3), 166–167
- trisomy 18 (Edwards syndrome), 97
- trisomy 21 (Down syndrome), 97, 102, 105–107, 117
- TRN (transcriptional regulatory network) models, 67
- Trp63, 9, 23, 25
- t-tests, 65
- TTF-1 (thyroid transcription factor-1). *See* Nkx2-1
- tuberculosis, 85
- tubular myelin, 143, 146, 150
- Turner syndrome, 117
- Type 0 CPAM (acinar agenesis; acinar dysplasia), 103, 108–109
- Type I (glomus) carotid body cells, 228–229
- Type II carotid body cells, 228
- tyrosine kinase, 304–305
- umbilical cord clamping, 171–172
- unilateral pulmonary agenesis, 102
- Ureaplasma* spp., 188–189, 194–195, 209
- Ureaplasma parvum*, 188–190, 192–193, 273
- VACTERL plus hydrocephalus, 97
- VACTERL syndrome, 96–97, 102
- vaginal squeeze, 166
- vascular endothelial growth factor receptors. *See* VEGFRs
- vascular endothelial growth factors. *See* VEGFs
- vasculogenesis, 36–37, 49–50, 60–61. *See* pulmonary vascular development
- VATER syndrome, 96–97
- VE (visceral endoderm), 3–4
- VEcadherin, 39
- pulmonary arterial development, 46
- VEGFRs (vascular endothelial growth factor receptors)
- VEGFR1 (Flt-1), 41, 44–45, 306
 - VEGFR2 (Flk-1; KDR), 36–37, 117, 190, 305–306
 - pulmonary arterial development, 40–41, 43–44, 46
 - pulmonary capillary system development, 46–47
 - pulmonary lymphatic development, 48
- VEGFR3 (Flt4), 117
- pulmonary lymphatic development, 48–49
- VEGFs (vascular endothelial growth factors), 103–104, 194, 207, 210–211, 215, 272–275, 305–306
- lung structure maintenance program, 303–305
 - pulmonary arterial development, 41, 45–46
 - pulmonary capillary system development, 46–47
 - pulmonary venous development, 47
- VEGF-1, 194
- VEGF-3, 41
- VEGF-120, 41
- VEGF-164, 41, 305
- VEGF-188, 41
- VEGF-206, 41
- VEGF-A, 67–69, 190, 291
- pulmonary arterial development, 41–44
 - pulmonary lymphatic development, 48
- VEGF-A₁₆₄, 41
- VEGF-A_{165a}, 291
- VEGF-A_{165b}, 291
- VEGF-C, 117
- pulmonary arterial development, 41, 45–46
 - pulmonary lymphatic development, 48–49
- VEGF-D, 41, 48
- vein varices, 46
- ventilation induced injury, 164–165, 176–179, 208–209
- during clearance of lung liquid and lung aeration, 176–177
 - following lung aeration, 177–178
 - hemodynamic consequences, 178
 - oxygen injury, 178–179
 - systemic consequences, 178
- ventral respiratory columns (VRC), 224, 226–227
- Vici syndrome, 102
- visceral endoderm (VE), 3–4
- Vitamin A, 196, 210, 216–217, 297
- Vitamin C, 82, 197
- Vitamin D, 193–194, 296–297
- von Willebrand factor, 46
- VRC (ventral respiratory columns), 224, 226–227
- Wadsworth, J., 82
- Wang, H., 294
- Weibel, E.R., 239–241

- “wet lung” (transient tachypnoea),
169
- wheezing, 82, 84–86, 197,
287–288
- whole genome sequencing,
58–60
- whole-body plethysmography,
256–257
- Wigglesworth, J.S., 198
- Wigle, J.T., 48
- Wnt family, 2, 4–8, 12–16,
23, 27
- pulmonary arterial development,
43
- Wnt2, 5–8, 27, 40, 43
- Wnt2b, 5–7, 27
- Wnt3a, 13
- Wnt5a, 27, 43
- Wnt7a, 43
- Wnt7b, 8, 27, 43
- Wnt10b, 43
- Wnt11, 27
- Wong, H.Y., 14
- wood smoke, 85–86
- xanthines, 233
- X-linked VACTERL with or without
hydrocephalus, 97
- Yablonskiy, D.A., 243
- Yamanaka, S., 11
- YAP, 8, 15–16
- Yasuo, M., 308
- Zeltner, T.B., 240
- Zeman, K.L., 244
- ZIC3, 97