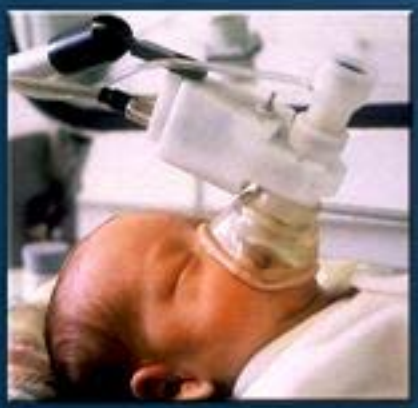
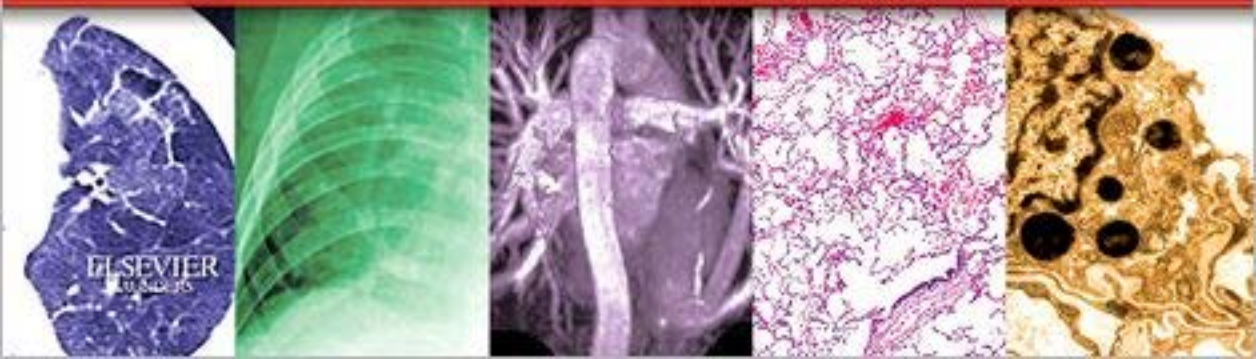
KENDIG AND CHERNICK'S Disorders OF THE Respiratory Tract IN Children



EIGHTH EDITION

Wilmott • Boat • Bush • Chernick • Deterding • Ratjen



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PREFACE

In editing this, the eighth edition of *Kendig and Chernick's Disorders of the Respiratory Tract in Children,* we are struck by how much has changed since the last edition. There have been remarkable new understandings of the basic mechanisms of lung disease in the last 7 years. We have recognized this by creating two new sections, each of which has a section editor: the section on Interstitial Lung Disease in Children edited by Robin Deterding and the Aerodigestive Section edited by Thomas Boat. Every chapter has been extensively updated and revised since the last edition, and there is an increased emphasis on the molecular mechanisms of disease and genetics. To save space we have limited the number of references in the paper version of the book, but the full reference lists are available in the online version.

There are now six editors who have enjoyed the collaboration on identification of authors, review of outlines, working with the individual chapter authors, and editing their work. With this edition we are joined by Robin Deterding of the University of Colorado and Felix Ratjen from the University of Toronto. Our plan is to add two new editors with each edition to establish a rotation that will allow some of us older ones to rotate off in the future. However, as you might have noticed, nobody has rotated off so far! However, we are delighted to recognize Dr. Victor Chernick's many years of contribution to the book with the change in its name.

There are 18 new chapters in this edition and 47 new authors have joined the team. Thirty-two authors have rotated off and we thank them all for their contributions. We particularly want to recognize Dr. Mary Ellen Wohl, who contributed several chapters to multiple editions of the book and who passed away in 2009.

Our goal in editing this book is to publish a comprehensive textbook of pediatric respiratory diseases for a wide audience: the established pediatric pulmonologist and intensivist, fellows in pediatric pulmonology or intensive care, pediatric practitioners, and residents. We also see this book as an important resource for pediatric radiologists, allergists, thoracic and cardiac surgeons, and others in the allied health specialties. We have covered both common and rare childhood diseases of the lungs and the basic science that relates to these conditions to allow for an understanding of pulmonary disease processes and their effect on pulmonary function. Edwin Kendig founded this book, which some say has become the bible of pediatric pulmonology, and we have strived to continue this tradition and this degree of authority and completeness.

The staff at Elsevier, especially Lisa Barnes and Judy Fletcher, have provided outstanding support for our work, and we are grateful for their organization, sound advice, attention to detail, and patience.

Finally, we must thank our families and partners for their patience during the writing of this book, which has been time consuming, and only their tolerance has made the work possible.

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General Basic Considerations

1 MOLECULAR DETERMINANTS OF LUNG MORPHOGENESIS

Jeffrey A. Whitsett, MD, and Susan E. Wert, PhD

OVERVIEW

The adult human lung consists of a gas exchange area of approximately 100 m^2 that provides oxygen delivery and carbon dioxide excretion required for cellular metabolism. In evolutionary terms, the lung represents a relatively late phylogenetic solution for the need to provide efficient gas exchange for terrestrial survival of organisms of increasing size, an observation that may account for the similarity of lung structure in vertebrates.[reviewed in 1,2] The respiratory system consists of mechanical bellows and conducting tubes that bring inhaled gases to a large gas exchange surface that is highly vascularized. Alveolar epithelial cells come into close apposition to pulmonary capillaries, providing efficient transport of gases from the alveolar space to the pulmonary circulation. The delivery of external gases to pulmonary tissue necessitates a complex organ system that (1) keeps the airway free of pathogens and debris, (2) maintains humidification of alveolar gases and precise hydration of the epithelial cell surface, (3) reduces collapsing forces inherent at air-liquid interfaces within the air spaces of the lung, and (4) supplies and regulates pulmonary blood flow to exchange oxygen and carbon dioxide efficiently. This chapter will provide a framework for understanding the molecular mechanisms that lead to the formation of the mammalian lung, focusing attention to processes contributing to cell proliferation and differentiation involved in organogenesis and postnatal respiratory adaptation. Where possible, the pathogenesis of congenital or postnatal lung disease will be considered in the context of the molecular determinants of pulmonary morphogenesis and function.

ORGANOGENESIS OF THE LUNG

Body Plan

Events critical to organogenesis of the lung begin with formation of anteroposterior and dorsoventral axes in the early embryo. The body plan is determined by genes that control cellular proliferation and differentiation and depends on complex interactions among many cell types. The fundamental principles determining embryonic organization have been elucidated in simpler organisms (e.g., *Drosophila melanogaster* and *Caenorhabditis elegans*) and applied to increasingly complex organisms (e.g., mouse and human) as the genes determining axial segmentation, organ formation, cellular proliferation, and differentiation have been identified. Segmentation and organ formation in the embryo are profoundly influenced by sets of master control genes that include various classes of transcription factors. Critical to formation of the axial body plan are the homeotic, or HOX, genes.[reviewed in 3–8] HOX genes are arrayed in clearly defined spatial patterns within clusters on several chromosomes. HOX gene expression in the developing embryo is determined in part by the position of the individual genes within these gene clusters, which are aligned along the chromosome in the same order as they are expressed along the anteroposterior axis. Complex organisms have more individual HOX genes within each locus and have more HOX gene loci than simpler organisms. HOX genes encode nuclear proteins that bind to DNA via a conserved homeodomain motif that modulates the transcription of specific sets of target genes. The temporal and spatial expression of these nuclear transcription factors, in turn, controls the expression of other HOX genes and their transcriptional targets during morphogenesis and cytodifferentiation.[reviewed in 9–14] Expression of HOX genes influences many downstream genes, such as transcription factors, growth factors, signaling peptides, and cell adhesion molecules,[13] that are critical to the formation of the primitive endoderm from which the respiratory epithelium is derived.[15]

Endoderm

The primitive endoderm develops very early in the process of embryogenesis (i.e., during gastrulation and prior to formation of the intraembryonic mesoderm, ectoderm,

and notochord—3 weeks postconception in the human).[16] Specification of the definitive endoderm and the primitive foregut requires the activity of a number of nuclear transcription factors that regulate gene expression in the embryo, including (1) forkhead box A2, or FOXA2 (also known as hepatocyte nuclear factor 3-beta, or HNF-3β), (2) GATA-binding protein 6, or GATA6, (3) sex-determining region Y (SRY)-related HMG-box (SOX) 17, or SOX17, (4) SOX2, and (5) β-catenin.[17–24] Genetic ablation of these transcription factors disrupts formation of the primitive foregut endoderm and its developmental derivatives, including the trachea and the lung.[22,24–29] Some of these transcription factors are also expressed in the respiratory epithelium later in development when they play important roles in the regulation of cell differentiation and organ function.[reviewed in 30–34]

Lung Morphogenesis

Lung morphogenesis is initiated during the embryonic period of fetal development (3 to 4 weeks of gestation in the human) with the formation of a small saccular outgrowth of the ventral wall of the foregut endoderm, a process that is induced by expression of the signaling peptide, fibroblast growth factor 10 (FGF10), in the adjacent splanchnic mesoderm (Figure 1-1).[16] This region of the ventral foregut endoderm is delineated by epithelial cells expressing thyroid transcription factor 1, or TTF1 (also known as NKX2.1, T/EBP, or TITF1), which is the earliest known marker of the prospective respiratory epithelium.[35] Thereafter, lung development can be subdivided into five distinct periods of morphogenesis based on the morphologic characteristics of the tissue (Table 1-1; Figure 1-2). While the timing of this process is highly species-specific, the anatomic events underlying lung morphogenesis are shared by all mammalian species. Details of human lung development are described in the following sections, as well as in several published reviews.[reviewed in 36–42]

The Embryonic Period (3 to 6 Weeks Postconception)

Relatively undifferentiated epithelial cells of the primitive foregut endoderm form tubules that invade the splanchnic mesoderm and undergo branching morphogenesis. This process requires highly controlled cell proliferation and migration of the epithelium to direct dichotomous branching of the respiratory tubules, which forms the main stem, lobar, and segmental bronchi of the primitive lung (see Table 1-1; Figure 1-2). Proximally, the trachea and esophagus also separate into two distinct structures at this time. The respiratory epithelium remains relatively undifferentiated and is lined by columnar epithelium. Experimental removal of mesenchymal tissue from the embryonic endoderm at this time arrests branching morphogenesis, demonstrating the critical role of mesenchyme in formation of the respiratory tract.[reviewed in 43] Interactions between epithelial and mesenchymal cells are mediated by a variety of signaling peptides and their associated receptors (signaling pathways), which regulate gene transcription in differentiating lung cells.[30–34,42,43] These epithelial-mesenchymal interactions involve both autocrine and paracrine signaling pathways that are critical

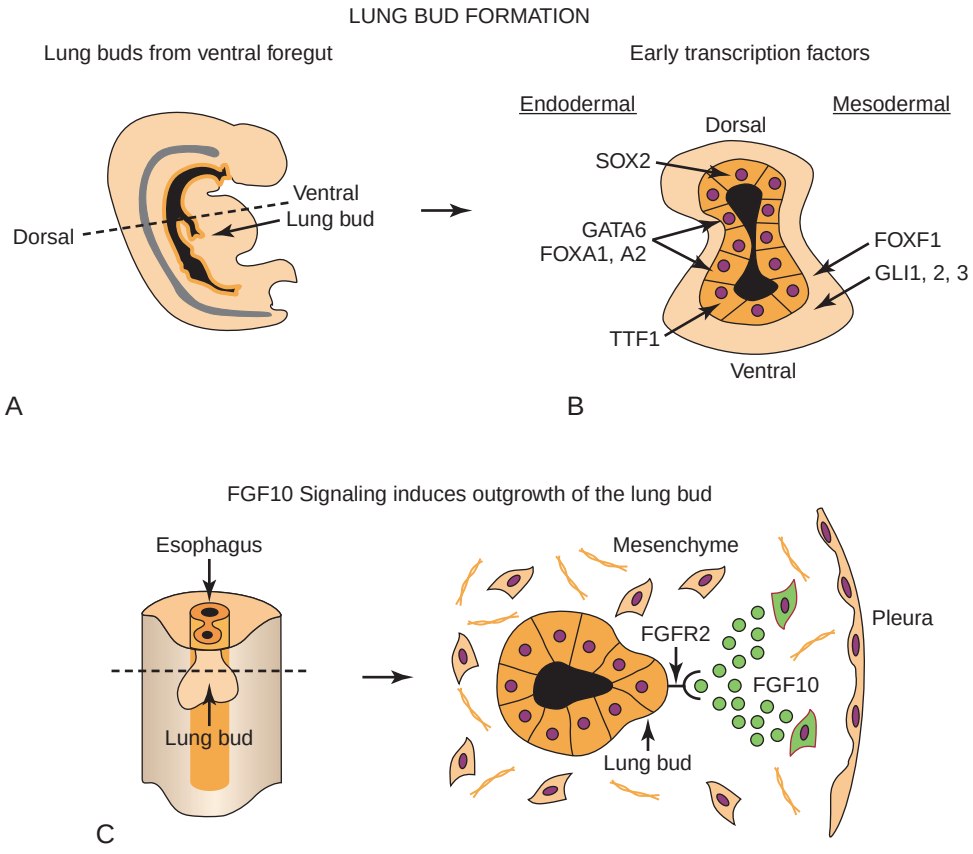

FIGURE 1-1. Lung bud formation. **A,** Lung development is initiated during the embryonic stage of gestation as a small, saccular outgrowth of the ventral foregut endoderm. **B,** Endodermal transcription factors critical for specification of the primitive respiratory tract include GATA6, FOXA1, and FOXA2, which are also expressed throughout the foregut endoderm. At this time, SOX2 expression is limited to the dorsal aspect (future esophagus) of the foregut endoderm, while TTF1 expression is limited to the ventral aspect (future trachea and lung) of the lung bud. Mesodermal transcription factors responsive to signaling peptides (e.g., SHH) released from the endoderm and critical for lung development include Gli1/2/3 and FOXF1. **C,** Expression of the signaling peptide, fibroblast growth factor 10 (FGF10), in the adjacent splanchnic mesoderm, induces outgrowth of the lung bud. FGF10 is secreted by mesenchymal cells and binds to its receptor, FGFR2, located on the endodermal cell surface, inducing formation of the lung bud.

TABLE 1-1 MORPHOGENETIC PERIODS OF HUMAN LUNG DEVELOPMENT

PERIOD	AGE (WEEKS)	STRUCTURAL EVENTS
Embryonic	3 to 6	Lung buds; trachea, main stem, lobar, and segmental bronchi; trachea and esophagus separate
Pseudoglandular	6 to 16	Subsegmental bronchi, terminal bronchioles, and acinar tubules; mucous glands, cartilage, and smooth muscle
Canalicular	16 to 26	Respiratory bronchioles, acinus formation, and vascularization; type I and II cell differentiation
Saccular	26 to 36	Dilation and subdivision of alveolar saccules, increase of gas-exchange surface area, and surfactant synthesis
Alveolar	36 to maturity	Further growth and alveolarization of lung; increase of gas-exchange area and maturation of alveolar capillary network; increased surfactant synthesis

for lung morphogenesis (Figure 1-3). Paracrine signaling pathways that are important for initial formation of the lung bud and the expansion and branching of the primitive respiratory tubules include: (1) fibroblast growth factor (FGF10/FGFR2), (2) sonic hedgehog (SHH/PTCH1), (3) transforming growth factor-beta (TGFβ/TGFβR2), (4) bone morphogenetic protein B (BMP4/BMPR1b), (5) retinoic acid (RA/RARα, β, γ), (6) WNT (WNT2/2b, 7b, 5a and R-spondin with their receptors Frizzled and LRP5/6), and (7) the β-catenin signaling pathways.[30–34,42–45] Nuclear transcription factors active in the primitive respiratory epithelium during this period include: TTF1, FOXA2, GATA6, and SOX2. Likewise, nuclear transcription factors active in the mesenchyme at this time include: (1) the HOX family of transcription factors (HOXA5, B3, B4); (2) the SMAD family of transcription factors (SMAD2, 3, 4) that are downstream transducers of the TGFβ/BMP signaling pathway; (3) the LEF/TCF family of transcription factors, downstream transducers of β-catenin; (4) the GLI-KRUPPEL family of transcription factors (GLI1, 2, 3), downstream transducers of SHH signaling; (5) the hedgehog-interacting protein, HHIP1, that binds SHH; and (6) FOXF1, another SHH target.[30–34,40,43,44,47] Disruption of many of these transcription factors and signaling pathways in experimental animals causes impaired morphogenesis, resulting in laryngotracheal

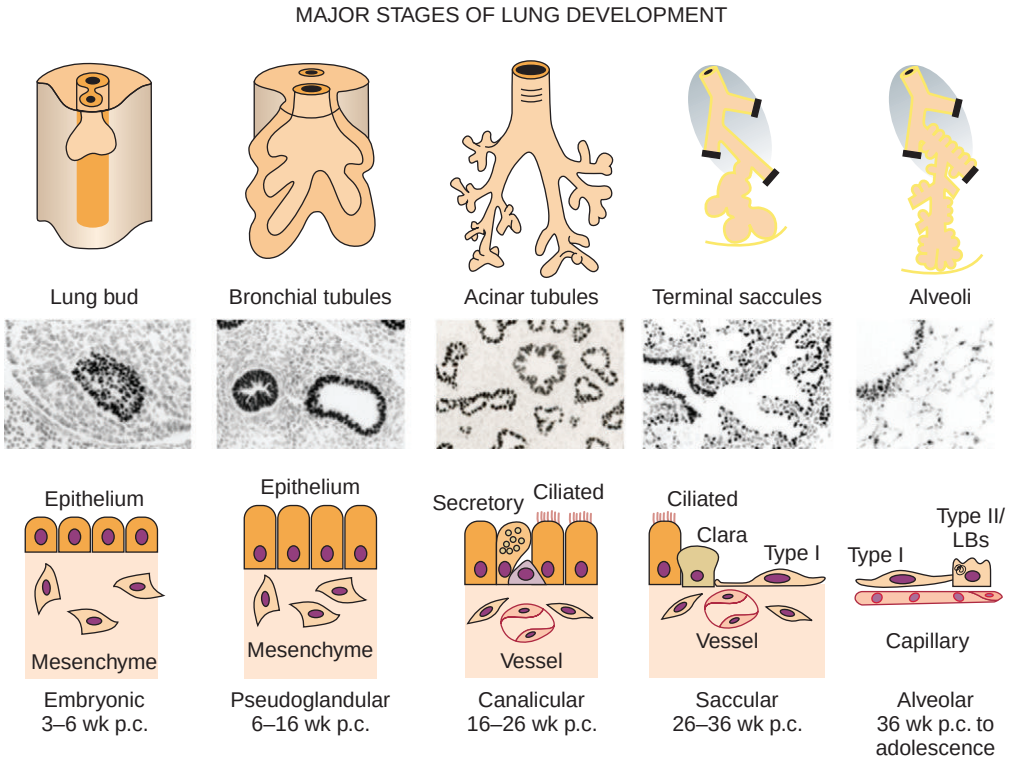

FIGURE 1-2. Major stages of lung development. The bronchi, bronchioles, and acinar tubules are formed by the process of branching morphogenesis during the pseudoglandular stage of lung development (6 to 16 weeks p.c.). Formation of the capillary bed and dilation/expansion of the acinar structures is initiated during the canalicular stage of lung development (16 to 26 weeks p.c.). Growth and subdivision of the terminal saccules and alveoli continue until early adolescence by septation of the distal respiratory structures to form additional alveoli. Cytodifferentiation of mature bronchial epithelial cells (secretory and ciliated cells) is initiated in the proximal conducting airways during the canalicular stage of lung development, while cytodifferentiation in the distal airways (ciliated and Clara cells) and alveoli (Type I and Type II cells) takes place later during the saccular (26 to 36 weeks p.c.) and alveolar (36 weeks p.c. to adolescence) stages of lung development. The alveolar stage of lung development extends into the postnatal period, during which millions of additional alveoli are formed and maturation of the microvasculature, or air-blood barrier, takes place, greatly increasing the surface area available for gas exchange.

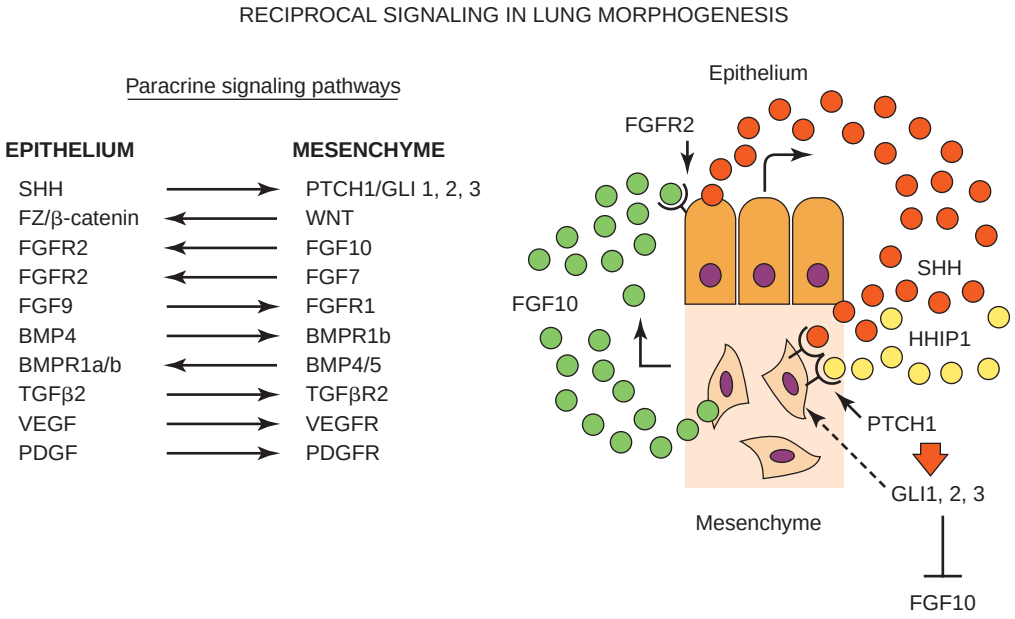

FIGURE 1-3. Reciprocal signaling in lung morphogenesis. Paracrine and autocrine interactions between the respiratory epithelium and the adjacent mesenchyme are mediated by signaling peptides and their respective receptors, influencing cellular behaviors (e.g., proliferation, migration, apoptosis, extracellular matrix deposition) that are critical to lung formation. For example, FGF10 is secreted by mesenchymal cells and binds to its receptor, FGFR10, on the surface of epithelial cells (paracrine signaling). SHH is secreted by epithelial cells and binds to its receptor, PTCH1, on mesenchymal cells (paracrine signaling), while HHIP1 is upregulated by SHH in mesenchymal cells, secreted, and binds back to receptors on cells in the mesenchyme (autocrine signaling). Binding of SHH to mesenchymal cells activates the transcription factors, GLI1, GLI2, and GLI3, which, in turn, inhibit FGF10 expression (negative feedback loop). In contrast, the binding of HHIP1 to mesenchymal cells attenuates or limits the ability of SHH to inhibit FGF10 signaling. Together, these complex, interacting, signaling pathways control branching morphogenesis of the lung, differentially influencing bronchial tubule elongation, arrest, and subdivision into new tubules.

malformations, tracheoesophageal fistulae, esophageal and tracheal stenosis, esophageal atresia, defects in pulmonary lobe formation, pulmonary hypoplasia, and/or pulmonary agenesis.[30–34,40,43–45]

Although formation of the larger, more proximal, conducting airways, including segmental and subsegmental bronchi, is completed by the 6th week postconception (p.c.), both epithelial and mesenchymal cells of the embryonic lung remain relatively undifferentiated. At this stage, trachea and bronchial tubules lack underlying cartilage, smooth muscle, or nerves, and the pulmonary and bronchial vessels are not well developed. Vascular connections with the right and left atria are established at the end of this period (6 to 7 weeks p.c.), creating the primitive pulmonary vascular bed.[39] Human developmental anomalies occurring during this period of morphogenesis include laryngeal, tracheal, and esophageal atresia, tracheoesophageal fistulae, tracheal and bronchial stenosis, tracheal and bronchial malacia, ectopic lobes, bronchogenic cysts, and pulmonary agenesis.[40,46] Some of these congenital anomalies are associated with documented mutations in the genes involved in early lung development, such as GLI3 (tracheoesophageal fistula found in Pallister-Hall syndrome), FGFR2 (various laryngeal, esophageal, tracheal, and pulmonary anomalies found in Pfeiffer, Apert, or Crouzon syndromes), and SOX2 (esophageal atresia and tracheoesophageal fistula found in anophthalmia-esophageal-genital, or AEG, syndrome).[40,46]

Pseudoglandular Period (6 to 16 Weeks' Postconception)

The pseudoglandular stage is so named because of the distinct glandular appearance of the lung from 6 to 16 weeks of gestation. During this period, the lung consists primarily of epithelial tubules surrounded by a relatively thick mesenchyme. Branching of the airways continues, and formation of the terminal bronchioles and primitive acinar structures is completed by the end of this period (see Table 1-1; Figure 1-2). During the pseudoglandular period, epithelial cell differentiation is increasingly apparent and deposition of cellular glycogen and expression of a number of genes expressed selectively in the distal respiratory epithelium is initiated. The surfactant proteins (SP), SP-B and SP-C, are first detected at 12 to 14 weeks of gestation.[48,49] Tracheobronchial glands begin to form in the proximal conducting airways; and the airway epithelium is increasingly complex, with basal, mucous, ciliated, and nonciliated secretory cells being detected.[36,38] Neuroepithelial cells, often forming clusters of cells, termed *neuroepithelial bodies* and expressing a variety of neuropeptides and transmitters (e.g., bombesin, calcitonin-related peptide, serotonin, and others), are increasingly apparent along the bronchial and bronchiolar epithelium.[50] Smooth muscle and cartilage are now observed adjacent to the conducting airways.[51] The pulmonary vascular system develops in close relationship to the bronchial and bronchiolar tubules between the 9th and 12th weeks of gestation. Bronchial arteries arise from the aorta and form along the epithelial tubules, and smooth muscle actin and myosin can be detected in the vascular structures.[39]

During this period, FGF10, BMP4, TGFβ, β-catenin, and the WNT signaling pathway continue to be important for branching morphogenesis, along with several other signaling peptides and growth factors, including: (1) members of the FGF family (FGF1, FGF2, FGF7,

FGF9, FGF18); (2) members of the TGFβ family, such as the SPROUTYs (SPRY2, SPRY4), which antagonize and limit FGF10 signaling, and LEFTY/NODAL, which regulate left-right patterning; (3) epithelial growth factor (EGF) and transforming growth factor alpha (TGFα), which stimulate cell proliferation and cytodifferentiation; (4) insulin-like growth factors (IGFI, IGFII), which facilitate signaling of other growth factors; (5) platelet-derived growth factors (PDGFA, PDGFB), which are mitogens and chemoattractants for mesenchymal cells; and (6) vascular endothelial growth factors (VEGFA, VEGFC), which regulate vascular and lymphatic growth and patterning.[30–34,40,42,43] Many of the nuclear transcription factors that were active during the embryonic period of morphogenesis continue to be important for lung development during the pseudoglandular period. Additional transcription factors important for specification and differentiation of the primitive lymphatics in the mesenchyme at this time include: (1) SOX18, (2) the paired-related homeobox gene, PRX1, (3) the divergent homeobox gene, HEX, and (4) the homeobox gene, PROX1.[40,42]

A variety of congenital defects may arise during the pseudoglandular stage of lung development, including bronchopulmonary sequestration, cystic adenomatoid malformations, cyst formation, acinar aplasia or dysplasia, alveolar capillary dysplasia with or without misalignment of the pulmonary veins, and congenital pulmonary lymphangiectasia.[40] The pleuroperitoneal cavity also closes early in the pseudoglandular period. Failure to close the pleural cavity, often accompanied by herniation of the abdominal contents into the chest (congenital diaphragmatic hernia), leads to pulmonary hypoplasia.

Canalicular Period (16 to 26 Weeks Postconception)

The canalicular period is characterized by formation of acinar structures in the distal tubules, luminal expansion of the tubules, thinning of the mesenchyme, and formation of the capillary bed, which comes into close apposition to the dilating acinar tubules (see Table 1-1; Figure 1-2). By the end of this period, the terminal bronchioles have divided to form two or more respiratory bronchioles, and each of these have divided into multiple acinar tubules, forming the primitive alveolar ducts and pulmonary acini. Epithelial cell differentiation becomes increasingly complex and is especially apparent in the distal regions of the lung parenchyma. Bronchiolar cells express differentiated features, such as cilia, and secretory cells synthesize Clara cell secretory protein, or CCSP (also known as CC10 or segretoglobin 1A1, SCGB1A1).[49,52–54] Cells lining the distal tubules assume cuboidal shapes and express increasing amounts of surfactant phospholipids[55] and the associated surfactant proteins, SP-A, SP-B, and SP-C.[48,49,56–60] Lamellar bodies, composed of surfactant phospholipids and proteins, are seen in association with rich glycogen stores in the cuboidal pre–type II cells lining the distal acinar tubules.[61–64] Some cells of the acinar tubules become squamous, acquiring features of typical type I alveolar epithelial cells. Thinning of the pulmonary mesenchyme continues; and the basal lamina of the epithelium and mesenchyme fuse. Capillaries surround the distal acinar tubules, which together will ultimately form the gas exchange region of the lung. By the end of the canalicular period in the human infant (26 to 28 weeks p.c.), gas exchange can be supported after birth, especially when surfactant is provided by administration of exogenous surfactants. Surfactant synthesis and mesenchymal thinning can be accelerated by glucocorticoids at this time,[60,65–67] which are administered to mothers to prevent respiratory distress syndrome (RDS) after premature birth.[68,69] Abnormalities of lung development occurring during the canalicular period include acinar dysplasia, alveolar capillary dysplasia, and pulmonary hypoplasia, the latter caused by (1) diaphragmatic hernia, (2) compression due to thoracic or abdominal masses, (3) prolonged rupture of membranes causing oligohydramnios, or (5) renal agenesis, in which amniotic fluid production is impaired. While postnatal gas exchange can be supported late in the canalicular stage, infants born during this period generally suffer severe complications related to decreased pulmonary surfactant, which causes RDS and bronchopulmonary dysplasia, the latter a complication secondary to the therapy for RDS.[70,71]

Saccular (26 to 36 Weeks' Postconception) and Alveolar Periods (36 Weeks' Postconception through Adolescence)

These periods of lung development are characterized by increased thinning of the respiratory epithelium and pulmonary mesenchyme, further growth of lung acini, and development of the distal capillary network (see Table 1-1; Figure 1-2). In the periphery of the acinus, maturation of type II epithelial cells occurs in association with increasing numbers of lamellar bodies, as well as increased synthesis of surfactant phospholipids,[55,61] the surfactant proteins, SP-A, SP-B, SP-C, and SP-D,[48,49,56–60,72] and the ATP-binding cassette transporter, ABCA3, a phospholipid transporter important for lamellar body biogenesis.[73] The acinar regions of the lung increase in surface area, and proliferation of type II cells continues. Type I cells, derived from differentiation of type II epithelial cells, line an ever-increasing proportion of the surface area of the distal lung. Capillaries become closely associated with the squamous type I cells, decreasing the diffusion distance for oxygen and carbon dioxide between the alveolar space and pulmonary capillaries. Basal laminae of the epithelium and stroma fuse; the stroma contains increasing amounts of extracellular matrix, including elastin and collagen; and the abundance of smooth muscle in the pulmonary vasculature increases prior to birth.[37] In the human lung, the alveolar period begins near the time of birth and continues through the first decade of life, during which the lung grows primarily by septation and proliferation of the alveoli,[74] and by elongation and luminal enlargement of the conducting airways. Pulmonary arteries enlarge and elongate in close relationship to the increased growth of the lung.[37] Pulmonary vascular resistance decreases, and considerable remodeling of the pulmonary vasculature and capillary bed continues during the postnatal period.[37] Lung growth remains active until early adolescence, when the entire complement of approximately 300 million alveoli has been formed.[74]

Signaling pathways that are critical for growth, differentiation, and maturation of the alveolar epithelium and capillary bed during these periods include the FGF, PDGF,

VEGF, RA, BMP, WNT, β-catenin, and NOTCH signaling pathways.[30–34,42,43] For example, FGF signaling is critical for alveologenesis during these periods. Targeted deletion of the FGF receptors, *Fgfr3* and *Fgfr4,* blocks alveologenesis in mice. Likewise, targeted deletion of *Pdgfa,* another growth factor critical for alveologenesis, interferes with myofibroblast proliferation and migration, resulting in complete failure of alveologenesis and postnatal alveolar simplification in mice.[30–34,42,43]

Nuclear transcription factors found earlier in lung development (i.e., FOXA2, TTF1, GATA6, and SOX2) continue to be important for maturation of the lung, influencing sacculation, alveolarization, vascularization, and cytodifferentiation of the peripheral lung. Transcription factors associated with cytodifferentiation during these periods include: (1) FOXJ1 (ciliated cells), (2) MASH1 (or HASH1) and HES1 (neuroendocrine cells), (3) FOXA3 and SPDEF (mucus cells), and (4) ETV5/ERM (alveolar type II cells).[32] Morphogenesis and cytodifferentiation are further influenced by additional transcription factors expressed in the developing respiratory epithelium at this time, including: (1) several ETS factors (ETV5/ERM, SPDEF, ELF3/5); (2) SOX genes (SOX-9, SOX11, SOX17); (3) nuclear factor of activated T cells/calcineurin-dependent 3, or NFATC3; (4) nuclear factor-1, or NF-1; (5) CCAAT/enhancer binding protein alpha, or CEBPα; and (6) Krüppel-like factor 5, or KLF5; as well as the transcription factors, GLI2/GLI3, SMAD3, FOXF1, POD1, and HOX (HOXA5, HOXB2 to B5), all of which are expressed in the mesenchyme.[30–34]

Control of Gene Transcription During Lung Morphogenesis

Numerous regulatory mechanisms influence cell commitment, proliferation, and terminal differentiation required for formation of the mammalian lung. These events must be precisely controlled in all organs to produce the complex body plan characteristic of higher organisms. In the mature lung, approximately 40 distinct cell types can be distinguished on the basis of morphologic and biochemical criteria.[75] Distinct pulmonary cell types arise primarily from subsets of endodermal and mesodermal progenitor cells. Pluripotent or multipotent cells receive precise temporal and spatial signals that commit them to differentiated pathways, which ultimately generate the heterogeneous cell types present in the mature organ. The information directing cell proliferation and differentiation during organogenesis is derived from the genetic code contained within the DNA of each cell in the organism. Unique subsets of messenger RNAs (mRNAs) are transcribed from DNA and direct the synthesis of a variety of proteins in specific cells, ultimately determining cell proliferation, differentiation, structure, function, and behavior for each cell type. Unique features of differentiating cells are controlled by the relative abundance of these mRNAs, which, in turn, determine the relative abundance of proteins synthesized by each cell. Cellular proteins influence morphologic, metabolic, and proliferative behaviors of cells, characteristics that traditionally have been used to assign cell phenotype by using morphologic and cytologic criteria. Gene expression in each cell is also determined by the structure of DNA-protein complexes that comprise the chromatin within the nucleus of each cell. Chromatin structure, in turn, influences the accessibility of individual genes to the transcriptional machinery. Diverse extracellular and intracellular signals also influence gene transcription, mRNA processing, mRNA stability and translation—processes that determine the relative abundance of proteins produced by each cell.

Only a small fraction of the genetic material present in the nucleus represents regions of DNA that direct the synthesis of mRNAs encoding proteins. There is an increasing awareness that sequences in the noncoding regions of genes influence DNA structure and contain promoter and enhancer elements (usually in flanking and intronic regions of each gene) that determine levels of transcription.[76] Nucleotide sequencing and identification of expressed complementary DNA (cDNA) sequences encoded within the human genome have provided insight into the amount of the genetic code used to synthesize the cellular proteins produced by each organ.[77] At present, nearly all of the expressed cDNAs have been identified and partially sequenced for most human organs. Analysis of these mRNAs reveals distinct, and often unique, subsets of genes that are expressed in each organ, as well as the relative abundance and types of proteins encoded by these mRNAs. Of interest, proteins bearing signaling and transcriptional regulatory information are among the most abundant of various classes of proteins in human cells. Organ complexity in higher organisms is derived, at least in part, by the increasingly complex array of signaling molecules that govern cell behavior. Regulatory mechanisms controlling transcription are listed in Figure 1-4.

Transcriptional Cascades/Hierarchies

Gene transcription is modulated primarily by the binding of transcription factors (or trans-acting factors) to DNA. Transcription factors are nuclear proteins that bind to regulatory motifs consisting of ordered nucleotides, or specific nucleotide sequences. The order of these specific nucleotide sequences determines recognition sites within the DNA (*cis*-acting elements) that are bound by these nuclear proteins. The binding of transcription factors to these *cis*-acting elements influences the activity of RNA polymerase II, which binds to sequences near the transcription start site of target genes, initiating mRNA synthesis.[76,78] Numerous families of transcription factors have been identified, and their activities are regulated by a variety of mechanisms, including posttranslational modification and interactions with other proteins or DNA, as well as by their ability to translocate or remain in the nucleus.[78] Transcription factors also activate the transcription of other downstream nuclear factors, which, in turn, influence the expression of additional trans-acting factors. The number and cell specificity of transcription factors have proven to be large and are represented by diverse families of proteins categorized on the basis of the structural motifs of their DNA binding or trans-activating domains.[76,78] These interacting cascades of factors comprise a network with vast capabilities to influence target gene expression. The HOX family of transcription factors (homeodomain,

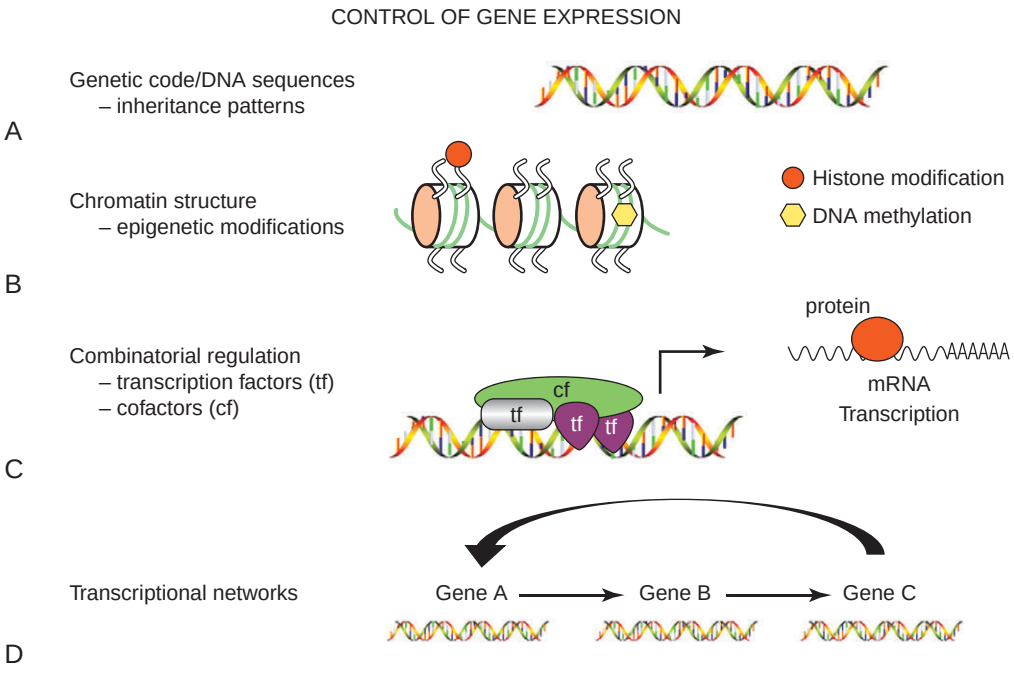

FIGURE 1-4. Control of gene expression. Diverse cellular mechanisms regulate varying levels of gene transcription that, in turn, control messenger RNA and protein synthesis governing cell differentiation and function during lung development. Inherited patterns of each individual's genetic code (**A**) are modified by epigenetic mechanisms that modify chromatin structure through methylation of DNA and/or modification of histone proteins (**B**). Binding of nuclear transcription factors to specific structural motifs (*cis*-acting elements) in DNA sequences is modified by associated cofactors and other transcription factors (**C**). Protein expression is often controlled by transcriptional networks, in which several genes are activated in series to induce or inhibit expression of downstream targets and/or other proteins (**D**).

helix-turn-helix-containing family of DNA-binding proteins) represents an example of such a regulatory motif. A series of HOX genes are located in arrays containing large numbers of distinct genes arranged 3' to 5' in distinct loci within human chromosomes.[7] HOX genes bind to and activate other downstream HOX gene family members that, in turn, bind to and activate the transcription of additional related and unrelated transcription factors, altering their activity and interactions at the transcriptional level.[10] Such cascades are now well characterized in organisms such as in *D. melanogaster*[74] and *C. elegans*.[79–81] Mammalian homologues exist for many of these genes, and their involvement in similar regulatory cascades influences gene expression and organogenesis in more complex organisms.[3–15] In the mammalian lung, TTF1 and FOX family members are involved in regulatory cascades that determine organogenesis and lung epithelial–specific gene expression. In addition, many other nuclear transcription factors, such as β-catenin, GATA6, POD1, FOXA2, NF1, FOXF1, GLI family members, ETS factors, N-MYC, CEBP family members, retinoic acid receptors (RAR), estrogen receptors, and glucocorticoid receptors, influence lung growth, cytodifferentiation, and function.[30–34]

Combinatorial Regulation of Gene Transcription and Expression

Advances in understanding mRNA expression profiles, genomics, chromatin structure, and mechanisms regulating gene expression are transforming current concepts regarding the molecular processes that control gene expression. Bioinformatics and advances in computational and systems biology are providing new insights into the remarkable interactions among genes that control other cellular processes. To influence gene expression, genes function in complex networks, which are dependent on each individual's inherited DNA sequences (genes) and on epigenetic mechanisms independent of genetic constitution. Changes in chromatin structure (packaging of DNA, histones, and other associated proteins) influence the accessibility of DNA to the regulatory actions of various transcriptional complexes (proteins) and is dependent upon posttranslational modification of histone proteins by methylation or acetylation. The regulatory regions of target genes in eukaryotes are highly complex, containing numerous *cis*-acting elements that bind various nuclear transcription proteins to influence gene expression. Nuclear proteins may bind DNA as monomers or oligomers, or form homo- or hetero-oligomers with other transcriptional proteins. Furthermore, many transcriptional proteins are modified by posttranslational modifications that are induced by receptor occupancy or by phosphorylation and/or dephosphorylation events. Binding of transcription factors influences the structural organization of DNA (chromatin), making regulatory sites more or less accessible to other nuclear proteins, which, in turn, positively or negatively regulate gene expression. Numerous *cis*-acting elements and their cognate *trans*-acting proteins interact with the basal transcriptional apparatus to regulate mRNA synthesis. The precise stoichiometry and specificity of the occupancy of various DNA-binding sites also influence the transcription of specific target genes, either positively or negatively. This mode of regulation is characteristic of most eukaryotic cells, including those of the lung. For example, in pulmonary epithelial cells, a distinct set of transcription factors, including TTF1, GATA6, activator protein 1 (AP1), FOX family members, RARs, STAT3, NF1, and specificity protein 1 (SP1), act together to regulate expression of surfactant protein genes, which influence postnatal respiratory adaptation.[32,82–84]

Influence of Chromatin Structure on Gene Expression

The structure of chromatin is a critical determinant of the ability of target genes to respond to regulatory information influencing gene transcription. The abundance and

organization of histones and other chromatin-associated proteins, including nuclear transcriptional proteins, influence the structure of DNA at genetic loci. The accessibility of regulatory regions within genes or groups of genes for binding and regulation by transcription factors is often dependent on chromatin structure. Changes in chromatin structure are likely determined by the process of cell differentiation during which target genes become available or unavailable to the regulatory influences of transcription factors.[85] Thus, the activity of a transcription factor at one time in development may be entirely distinct from that at another time. Chemical modification of DNA (e.g., methylation of cytosine) is also known to modify the ability of *cis*-active elements to bind and respond to regulatory influences. For example, cytosine-guanine (CG)–rich islands are found in transcriptionally active genes, and methylation of these regions may vary developmentally or in response to signals that influence gene transcription. Chromatin structure, in turn, is influenced by post-transcriptional modification of histones and other DNA-associated proteins by biochemical processes, including acetylation, methylation, demethylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation, which then influence the binding of transcriptional complexes and coactivator proteins that interact with the basal transcriptional machinery via polymerase II to alter gene transcription.[86]

Non-Transcriptional Mechanisms

While regulation of gene transcription is an important factor in organogenesis, numerous regulatory mechanisms, including control of RNA expression, mRNA stability, and protein synthesis and degradation are also known to provide further refinement in the abundance of mRNAs and proteins synthesized by a specific cell, which ultimately determine its structure and function.[87] For example, microRNAs (miRNAs) have been implicated recently in the regulation of proliferation, differentiation, and apoptosis of epithelial progenitor cells in the lung.[86] miRNAs are small (19 to 25 nucleotides), single-stranded, non-coding RNAs that regulate protein expression by binding to the 3' untranslated region of target mRNAs, which results in degradation or inhibition of protein translation in the cytoplasm. mi/RNAs are transcribed initially as very long primary transcripts (pri-miRNAs) that contain hundreds to thousands of nucleotides. This primary transcript is cleaved to release a much smaller 70 to 100 nucleotide fragment (pre-miRNA), which is then exported to the cytoplasm. Once in the cytoplasm, this fragment is further cleaved by an RNA polymerase II (DICER) to release a 19- to 25- nucleotide fragment, which is then incorporated into an miRNA-induced silencing complex (miRISC) that guides the miRNA to its target mRNA, where it binds to the mRNA affecting its translation and/or stability.[88] High expression levels of at least three members of the miR-17-92 cluster are present in the embryonic lung, but decline as lung development progresses.[89] Mice deficient in the miR-17-92 cluster exhibited hypoplasia of the lung,[90] while targeted deletion of DICER in the lung resulted in abnormal lung development with increased apoptosis and abnormal branching morphogenesis.[91] Overexpression of the miR-17-92 cluster during lung development resulted in the absence of normal terminal (alveolar) saccules, which were replaced with respiratory tubules lined by highly proliferative, undifferentiated epithelium, suggesting that downregulation of the miR-17-92 cluster is critical for normal cellular growth and differentiation.[92]

Receptor-Mediated Signal Transduction

Receptor-mediated signaling is well recognized as a fundamental mechanism for transducing extracellular information. Such signals are initiated by the occupancy of membrane-associated receptors capable of initiating additional signals (known as secondary messengers), such as cyclic adenosine monophosphate, calcium, and inositide phosphates, which influence the activity and function of intracellular proteins (e.g., kinases, phosphatases, proteases). These proteins, in turn, may alter the abundance of transcription factors, the activity of ion channels, or changes in membrane permeability, which subsequently modify cellular behaviors. Receptor-mediated signal transduction, induced by ligand-receptor binding, mediates endocrine, paracrine, and autocrine interactions on which cell differentiation and organogenesis depend. For example, signaling peptides and their receptors, such as FGF, SHH, WNT, BMP, VEGF, PDGF, and NOTCH have been implicated in organogenesis of many organs, including the lung.[30–34,42,43]

Gradients of Signaling Molecules and Localization of Receptor Molecules

Chemical gradients within tissues, and their interactions with membrane receptors located at distinct sites within the organ, can provide critical information during organogenesis. Polarized cells have basal, lateral, and apical surfaces with distinct subsets of signaling molecules (receptors) that allow the cell to respond in unique ways to focal concentrations of regulatory molecules. Secreted ligands (e.g., FGFs, TGFβ/BMPs, WNTs, SHH, and HHIP1) function in gradients that are further influenced by binding of the ligand to basement membranes or proteoglycans in the extracellular matrix.[30,33,34,43] Spatial information is established by gradients of these signaling molecules and by the presence and abundance of receptors at specific cellular sites. Such systems provide positional information to the cell, which influences its behavior (e.g., shape, movement, proliferation, differentiation, and polarized transport).

Transcriptional Mechanisms Controlling Gene Expression During Pulmonary Development

While knowledge of the determinants of gene regulation in lung development is rudimentary at present, a number of transcription factors and signaling networks that play critical roles in lung morphogenesis have been identified.[30–34,42,43] Lung morphogenesis depends on formation of definitive endoderm, which, in turn, receives signals from the splanchnic mesenchyme to initiate organogenesis along the foregut, forming thyroid, liver, pancreas, lung, and portions of the gastrointestinal tract.[17] The ventral plate of the endoderm in mammals forms under the direction of FOXA2, a transcription factor that is known to play a critical role in

committing progenitor cells of the endoderm to form the primitive foregut.[17] FOXA2 is member of a large family of nuclear transcription factors, termed the *winged helix* family of transcription factors, that are involved in cell commitment, differentiation, and gene transcription in a variety of organs, such as the central nervous system and derivatives of the foregut endoderm, including the gastrointestinal tract, lung, and liver.[93] FOXA2 is required for the formation of foregut endoderm, from which the lung bud is derived, and plays a critical role in organogenesis of the lung. While FOXA2 plays a critical role in formation and commitment of progenitor cells to form the foregut endoderm, FOXA2 also influences the expression of specific genes in the respiratory epithelium later in development.[94–100] Conditional deletion of *Foxa2* after birth caused goblet cell metaplasia, airspace enlargement, and inflammation during the postnatal period,[101] while deletion of *Foxa2* prior to birth resulted in delayed pulmonary maturation, associated with decreased surfactant lipid and protein expression and the development of a respiratory distress-like syndrome.[100] Thus, FOXA2 plays a critical role in specification of foregut endoderm in the early embryo, and is used again in the perinatal and postnatal period to direct surfactant production, alveolarization, postnatal lung function, and homeostasis (Figure 1-5).

TTF1 (TITF1) is a 38-kd nuclear protein, containing a homeodomain DNA-binding motif, that is critical for formation of the lung and for regulation of a number of highly specific gene products produced only in the respiratory epithelium.[84,102,103] TTF1 is also expressed in the thyroid and in specific regions of the developing central nervous system.[35,102] In the lung, TTF1 is expressed in the respiratory epithelium of the primitive lung bud (see Figure 1-2).[35,102,103] Ablation of *Titf1* in the mouse impaired lung morphogenesis, resulting in tracheoesophageal fistula and hypoplastic lungs lined by a poorly differentiated respiratory epithelium and lacking the distal, alveolar, gas exchange regions.[102,103,106,107] Substitution of a mutant *Titf1* gene, which lacked phosphorylation sites, restored lung development in the *Titf1* knockout mouse.[108] Expression of a number of genes, including those regulating surfactant homeostasis, fluid and electrolyte transport, host defense, and vasculogenesis, is regulated by TTF1 phosphorylation prior to birth. TTF1 regulates the expression of a number of genes in a highly specific manner in the respiratory epithelium, including surfactant proteins, SP-A, SP-B, and SP-C, and CCSP.[109–112] TTF1 functions in concert with other transcription factors, including FOXA2, GATA6, NF1, ERM, PARP2, SP1/SP3, TAZ, NFAT, and RARs to regulate lung-specific gene transcription.[32,113–123] TTF1 gene transcription itself is modulated by the activity of FOXA2, which binds to the promoter enhancer region of the TTF1 gene, thus creating a transcriptional network.[99] A combinatorial mode of regulation is evidenced by the apposition of clustered TTF1 *cis*-active elements and FOXA2 binding sites in target genes, such as the SP-B and CCSP genes.[96,116] The stoichiometry, timing, and distinct combinations of transcription factor binding, as well as posttranscriptional modification of TTF1 by phosphorylation, are involved in differential gene expression throughout lung development. TTF1 and other transcription factors are recruited to nuclear complexes at regulatory sites of target genes that influence respiratory epithelial cell differentiation, providing and translating spatial information required for the formation of the highly diverse epithelial cell types lining distinct regions of the respiratory tract (see Figure 1-5).

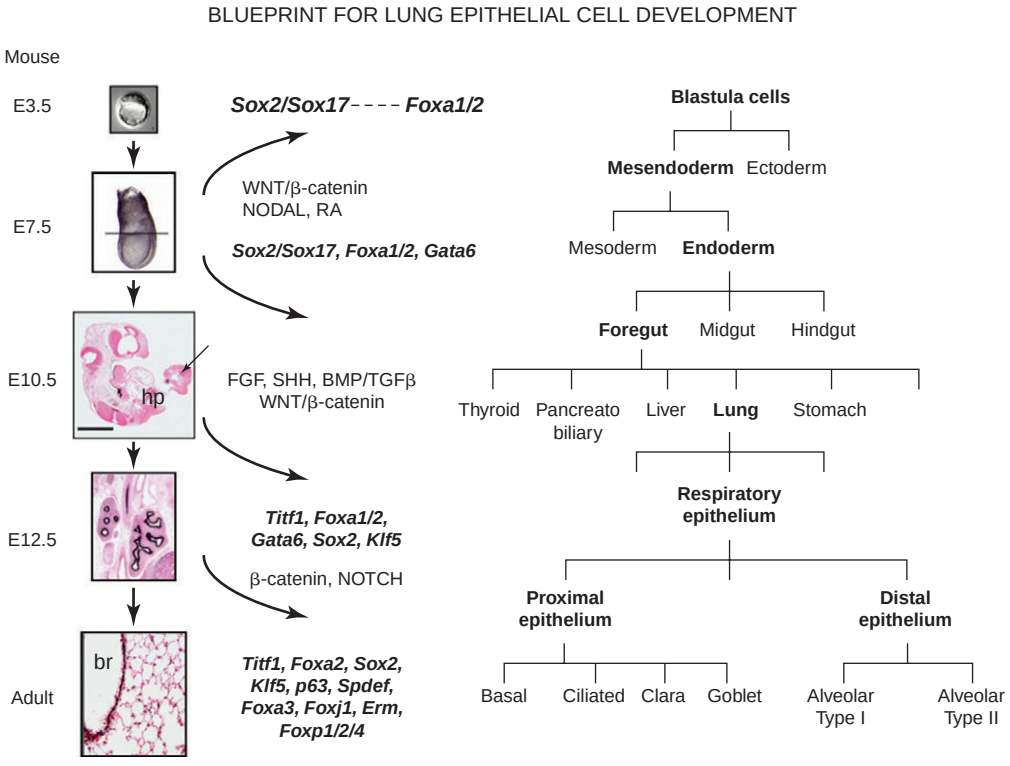

FIGURE 1-5. A blueprint for lung epithelial cell development. Cytodifferentiation of the respiratory epithelium is controlled by transcriptional networks of genes (highlighted) that are expressed throughout lung development, in conjunction with autocrine and paracrine signaling pathways that control structural morphogenesis of the lung. Additional transcription factors are induced or repressed later in development, and in the adult organ, to influence the differentiation of specific cell types.

Epithelial-Mesenchymal Interactions and Lung Morphogenesis

In vivo and *in vitro* experiments support the concept that branching morphogenesis and differentiation of the respiratory tract depends on reciprocal signaling between endodermally derived cells of the lung buds and the pulmonary mesenchyme or stroma.[30–34,43] This interdependency depends on autocrine and paracrine interactions that are mediated by the various signaling mechanisms governing cellular behavior (see Figure 1-3). Similarly, autocrine and paracrine interactions are known to be involved in cellular responses of the postnatal lung, generating signals that regulate cell proliferation and differentiation necessary for its repair and remodeling following injury. The splanchnic mesenchyme produces a number of signaling peptides critical for migration and proliferation of cells in the lung buds, including FGF10, FGF7, FGF9, BMP5, and WNT 2/2b, which activate receptors found on epithelial cells. In a complementary manner, epithelial cells produce WNT7b, WNT5a, SHH, BMP4, FGF9, VEGF, and PDGF that activate receptors and signaling pathways on target cells in the mesenchyme.[30,33,34,42,43]

Branching Morphogenesis, Vascularization, and Sacculation

Two distinct processes, branching and sacculation, are critical to morphogenesis of the mammalian lung. The major branches of the conducting airways of the human lung are completed by 16 weeks (p.c.) by a process of dichotomous branching, initiated by the bifurcation of the main stem bronchi early in the embryonic period of lung development. Epithelial-lined tubules of ever-decreasing diameter are formed from the proximal to distal region of the developing lung. Pulmonary arteries and veins form along the tubules and ultimately invade the acinar regions, where capillaries form between the arteries and veins, completing the pulmonary circulation.[37,42] The bronchial vasculature arises from the aorta, providing nutrient supply predominantly to bronchial and bronchiolar regions of the lung. In contrast, the alveolar regions are supplied by the pulmonary arterial system. Lymphatics and nerves form along the conducting airways, the latter being prominent in hilar, stromal and vascular tissues, but lacking in the alveolar regions of the lung.[124] A distinct period of lung sacculation and alveolarization begins in the late canalicular period (16 weeks p.c. and thereafter), which will result in the formation of the adult respiratory bronchiole, alveolar duct, and alveoli. During sacculation, a unique pattern of vascular supply forms the capillary network surrounding each terminal saccule, providing an ever-expanding gas exchange area that is completed in adolescence. Both vasculogenesis and angiogenesis contribute to formation of the pulmonary vascular system.[37,42] Signaling via SHH, VEGFA, FOXF1, NOTCH, Ephrins, and PDGF plays important roles in pulmonary vascular development.[30,33,34,42] For example, VEGFA and its receptors (VEGFR1, VEGFR2) are critical factors for vasculogenesis in many tissues. Targeted inactivation of *Vegf* and *Vefgfr1* in mice results in impaired angiogenesis,[125] while overexpression of the VEGFA 164 isoform disrupts pulmonary vascular endothelium in newborn conditional transgenic mice, causing pulmonary hemorrhage.[126] PROX1, a homeo domain transcription factor, is induced in a subset of venous endothelial cells during development and upregulates other lymphatic-specific genes, such as *VEGFR3* and *LYVE1,* which are critical for development of the lymphatic network in the lung.[124] Growth factors important for lymphatic development include VEGFC and its receptor, VEGFR3, as well as the angiopoietins, ANG1 and ANG2, and their receptors, TIE1 and TIE2.[124] Insufficiency or targeted deletion of these factors in mice impairs lymphatic vessel formation.[127,42]

Control of Lung Proliferation During Branching Morphogenesis

Dissection of the splanchnic mesenchyme from the lung buds arrests cell proliferation, branching, and differentiation of the pulmonary tubules *in vitro*.[43] Both *in vitro* and *in vivo* experiments strongly support the concept that the mesenchyme produces signaling peptides and growth factors critical to the formation of respiratory tubules.[43] In addition, lung growth is influenced by mechanical factors, including the size of the thoracic cavity and by stretch. For example, complete occlusion of the fetal trachea *in utero* enhances lung growth, while drainage of lung liquid or amniotic fluid causes pulmonary hypoplasia.[128,129] Regional control of proliferation is required for the process of dichotomous branching: division is enhanced at the lateral edges of the growing bud and inhibited at branch points.[130] Precise positional control of cell division is determined by polypeptides derived from the mesenchyme (e.g., growth factors or extracellular matrix molecules) that selectively decrease proliferation at clefts and increase cell proliferation at the edges of the bud. Proliferation in the respiratory tubule is dependent on a number of growth factors, including the FGF family of polypeptides. *In vitro,* FGF1 and FGF7 (also known as keratinocyte growth factor, KGF) partially replace the requirement of pulmonary mesenchyme for continued epithelial cell proliferation and budding.[131,132] FGF polypeptides are produced by the mesenchyme during lung development and bind to and activate a splice variant of FGFR2 (FGFR2IIIb) that is present on respiratory epithelial cells, completing a paracrine loop.[133,134] Blockade of FGFR2 signaling in the epithelium of the developing lung bud *in vivo,* using a dominant-negative FGF receptor mutant, completely blocked dichotomous branching of all conducting airway segments except the primary bronchi in mice.[135] FGF10 produced at localized regions of mesenchyme near the tips of the lung buds creates a chemoattractant gradient that activates the FGFR2IIIb receptor in epithelial cells of the lung buds, inducing cell migration, differentiation, and proliferation required for branching morphogenesis.[136] Deletion of *Fgf10* or *Fgfr2IIIb* in mice blocked lung bud formation, resulting in lung agenesis.[137,138] Increased expression of FGF10 or FGF7 in the fetal mouse lung caused severe pulmonary lesions with all of the histologic features of cystic adenomatoid malformations.[139,140] FGF7 is also mitogenic for mature respiratory epithelial cells *in vivo,* enhancing proliferation of bronchiolar and alveolar cells when administered intratracheally to the lungs of adult rats or by conditional targeted overexpression in mice.[141,142] Since FGF7 is

BOX 1-1 Secreted Polypeptides That Influence Lung Morphogenesis and Differentiation

Sonic hedgehog (SHH)
β-catenin
WNT family members (WNT2/2b, 7b, 5a, and R-spondin)
Fibroblast growth factors (FGF1, FGF7, FGF9, FGF10)
Bone morphogenetic proteins (BMP4)
Transforming growth factor-beta (TGFβ)
Vascular endothelial growth factor (VEGFA, VEGFC)
Platelet-derived growth factor (PDGFA, PDGFB)
Epidermal/transforming growth factors (EGF/TGFα)
Hepatocyte growth factor (HGF)
Insulin-like growth factors (IGFI, IGF2)
Granulocyte-macrophage colony-stimulating factor (GM-CSF)

produced during lung injury, it is likely that FGF signaling molecules mediate cell proliferation or migration to influence repair.[143] FGF7 and FGF1 increase expression of surfactant proteins *in vitro* and *in vivo,* suggesting that these factors enhance type II cell differentiation.[144,145] Signaling polypeptides known to influence branching morphogenesis and differentiation of the respiratory tract are listed in Box 1-1.

Role of Extracellular Matrix, Cell Adhesion, and Cell Shape

The pulmonary mesenchyme is relatively loosely packed, and there is little evidence that cell type is specified during the early embryonic period of lung development. However, with advancing gestation, increasing abundance of extracellular matrix molecules, including laminin, fibronectin, collagens, elastin, and proteoglycans, is readily detected in the mesenchyme adjacent to the developing epithelial structures.[146–152] Variability in the presence and abundance of various matrix molecules within the mesenchyme influences structural development, cytodifferentiation, and cell interactions *in vivo*. *In vitro,* inhibitors of collagen, elastin, and glycosaminoglycan synthesis, as well as antibodies to various extracellular and cell attachment molecules, alter cell proliferation and branching morphogenesis of the embryonic lung. Mesenchymal cells differentiate to form vascular elements (endothelium and smooth muscle) and distinct fibroblastic cells (myofibroblasts and lipofibroblasts), which all arise from the relatively undifferentiated progenitor cells of the splanchnic mesenchyme. While little is known regarding the factors influencing differentiation of the pulmonary mesenchyme, the development of pulmonary vasculature is dependent on VEGFs.[42] VEGFA is secreted by respiratory epithelial cells, stimulating pulmonary vasculogenesis via paracrine signaling to receptors that are expressed by progenitor cells in the mesenchyme.[153–156] PDGFA, another growth factor secreted by the respiratory epithelium, influences proliferation and differentiation of myofibroblasts in the developing lung by binding to the PDGF alpha receptor, and deletion of *Pdgfa* caused pulmonary malformation in transgenic mice.[157] The organization of both mesenchyme and epithelium is further modulated by cell adhesion molecules of various classes, including the cadherins, integrins, and polypeptides forming cell-cell junctions, which contribute to cellular organization and polarity of various tissues during pulmonary organogenesis. Furthermore, the surrounding extracellular matrix contains adhesion molecules that interact with attachment sites at cell membranes, influencing cell shape and polarity.[147,149] Cell shape is determined, at least in part, by the organization of these cell attachment molecules to the cytoskeleton. Cell shape, polarity, and mobility are further influenced by cytoskeletal proteins that interact with the extracellular matrix, as well as neighboring cells. Recently, the planar cell polarity (PCP) pathway and its downstream effector, Rho kinase, have been shown to be critical for branching morphogenesis *in vivo* through their effects on cytoskeletal remodeling and organization, which influence apical-basal polarity within epithelia.[158,159] Mutations in the genes, *Celsr1* and *Vangl2* that are key components of the PCP pathway, disrupted the actin-myosin cytoskeleton during mouse lung development, resulting in hypoplastic lungs with fewer branches and terminal buds, thickened mesenchyme, and highly disorganized epithelia with narrow or absent lumina.[160]

Cell shape also influences intracellular routing of cellular proteins and secretory products, determining sites of secretion. *In vitro,* epithelial cells grown on extracellular matrix gels at an air-liquid interface form a highly polarized cuboidal epithelium that maintains cell differentiation and polarity of secretions *in vitro*. Loss of cell shape is associated with the loss of differentiated features, such as surfactant protein and lipid synthesis, demonstrating the profound influence of cell shape on gene expression and cell behavior.[161–163]

Autocrine-Paracrine Interactions in Lung Injury and Repair

As in lung morphogenesis, autocrine-paracrine signaling plays a critical role in the process of repair following lung injury. The repair processes in the postnatal lung, as in lung morphogenesis, require the precise control of cell proliferation and differentiation and, as such, are likely influenced by many of the signaling molecules and transcriptional mechanisms that mediate lung development. Events involved in lung repair may recapitulate events occurring during development, in which progenitor cells undergo proliferation and terminal differentiation after lung injury. While many of the mechanisms involved in lung repair and development may be shared, it is also clear that fetal and postnatal lung respond in distinct ways to autocrine-paracrine signals. Cells of the postnatal lung have undergone distinct phases of differentiation and may have different proliferative potentials, or respond in unique ways to the signals evoked by lung injury. For example, after acute or chronic injury, increased production of growth factors or cytokines may cause pulmonary fibrosis or pulmonary vascular remodeling in neonatal life, mediated by processes distinct from those occurring during normal lung morphogenesis.[164–169] The role of inflammation and the increasing activity of the immune system that accompanies postnatal development also distinguishes the pathogenesis of disease in fetal and postnatal lungs.

Host Defense Systems

Distinct innate and adaptive defense systems mediate various aspects of host responses in the lung. During the postnatal period, the numbers and types of immune cells present in the lung expand markedly.[170] Alveolar macrophages,

dendritic cells, lymphocytes of various subtypes, polymorphonuclear cells, eosinophils and mast cells each have distinct roles in host defense. Immune cells mediate acute and chronic inflammatory responses accompanying lung injury or infection. Both the respiratory epithelium and inflammatory cells are capable of releasing and responding to a variety of polypeptides that induce the expression of genes involved in (1) cytoprotection (e.g., antioxidants, heat shock proteins); (2) adhesion, influencing the attraction and binding of inflammatory cells to epithelial and endothelial cells of the lung; (3) cell proliferation, apoptosis, and differentiation that follow injury or infection; and (4) innate host defense. An increasing array of cytokines and chemokines have now been identified that contribute to host defense following lung injury.[171,172]

The adaptive immune system includes both antibody and cell-mediated responses to antigenic stimuli. Adaptive immunity depends on the presentation of antigens by macrophages, dendritic cells, or the respiratory epithelium to mononuclear cells, triggering the expansion of immune lymphocytes and initiating antibody production and cytotoxic activity needed to remove infected cells from the lung. The lung contains active lymphocytes (natural killer cells, helper and cytotoxic T cells) that are present within the parenchyma and alveolus. Organized populations of mononuclear cells are also found in the lymphatic system along the conducting airways, termed the *bronchiolar-associated lymphocytes*. Cytokines and chemokines, including (1) interleukin (IL) 1, or IL1, (2) IL8, (3) tumor necrosis factor-α, or TNFα, (4) regulated on activation, normal T-expressed and secreted protein, or RANTES, (5) granulocyte-macrophage colony-stimulating factor, or GM-CSF, and (6) macrophage inflammatory protein-1α, or MIP-1α, are produced by cells in the lung and provide proliferative and/or differentiative signals to inflammatory cells that, in turn, amplify these signals by releasing additional cytokines or other inflammatory mediators within the lung.[172] Receptors for some of these signaling molecules have been identified in pulmonary epithelial cells. For example, GM-CSF plays a critical role in surfactant homeostasis. Genetic ablation of GM-CSF or GM-CSF-IL3/5β chain receptor in mice causes alveolar proteinosis associated with macrophage dysfunction and surfactant accumulation.[173–177] Pulmonary alveolar proteinosis in adult human patients is associated with high-affinity autoantibodies against GM-CSF that block receptor activation required for surfactant catabolism by alveolar macrophages.[178,179] Inherited defects in the GM-CSF receptor, including both the GM-CSF receptor alpha and beta chains, have been associated with alveolar proteinosis in children.[178,179] GM-CSF stimulates both differentiation and proliferation of Type II epithelial cells, as well as activating alveolar macrophages to increase surfactant catabolism. Thus, GM-CSF acts in an autocrine and paracrine fashion as a growth factor for both the respiratory epithelium and for alveolar macrophages. A number of additional growth factors, including FGFs, EGF, TGFα, PDGF, IGFs, TGFβ, and others, are released by lung cells following injury. These polypeptide growth factors likely play a critical role in stimulating proliferation of the respiratory epithelial cells required to repair the injured respiratory epithelium.[169,172] For example, intratracheal administration of FGF7 causes marked proliferation of the adult respiratory epithelium and protects the lung from various injuries.[141]

Innate Defenses

The lung also has innate defense systems that function independently of those provided by the mesodermally derived immune system. The respiratory epithelium and other lung cells secrete a variety of polypeptides that serve defense functions, including bactericidal polypeptides (lysozyme and defensins), collectins (surfactant proteins, SP-A and SP-D), and other polypeptides that enhance macrophage activity involved in the clearance of bacteria and other pathogens. SP-A and SP-D, both members of the collectin family of mammalian lectins,[158] are secreted by the respiratory epithelium and bind to pathogenic organisms, enhancing their phagocytosis by alveolar macrophages.[180–183] Polypeptide factors with bactericidal activity, such as the defensins, are produced by various cells in response to inflammation within the lung, and are likely to play roles in host defense.[184] Thus, the immune system and accompanying production of chemokines and cytokines serve in an autocrine-paracrine fashion to modulate expression of genes mediating innate and immune-dependent defenses, as well as cell growth, critical to the repair of the parenchyma after injury. Uncontrolled proliferation of stromal cells leads to pulmonary fibrosis, just as uncontrolled growth of the respiratory epithelium produces pulmonary adenocarcinoma. Chronic inflammation, whether through inhaled particles, infection, or immune responses, may therefore establish ongoing proliferative cascades that lead to fibrosis and abnormal alveolar remodeling associated with chronic lung disease.[185]

Gene Mutations in Lung Development and Function

Knowledge of the role of specific genes in lung development and function is expanding rapidly, extending our understanding of the role of genetic mutations that cause lung malformation and disease. Mutations in the DNA code may alter the abundance and function of encoded polypeptides, causing changes in cell behavior that lead to lung malformation and dysfunction. While poorly understood at present, a congenital malformation, termed *acinar dysplasia*, is associated with decreased or absent levels of TTF1, FOXA2, and surfactant proteins; lungs from these infants are severely hypoplastic and lack peripheral airways at birth.[186] Such findings implicate the transcription factors TTF1 and FOXA2, or their upstream regulators, in acinar dysplasia. Mutations in TTF1 cause lung hypoplasia, hypothyroidism, and neurologic disorders.[187–195] Mutations in SOX9 influence the growth of the chest wall and cause lung hypoplasia in campomelic dwarfism,[196–200] while mutations in SOX2 have been associated with tracheoesophageal fistula, anophthalmia, microphthalmia, and central nervous system defects.[201] Similarly, defects in SHH and FGF signaling have been associated with lung and tracheobronchial malformations in human infants.[202,203] Mutations in the transcription factor FOXF1 have been causally linked to the lethal congenital malformation, alveolar capillary dysplasia with misalignment of the pulmonary veins.[204,205] Thus, it

is increasingly apparent that mutations in genes influencing transcriptional and signaling networks that control lung morphogenesis cause pulmonary malformations in infants. Likewise, it is highly likely that allelic diversity in genes influencing lung morphogenesis will impact postnatal lung homeostasis and disease pathogenesis. Findings that SOX2 and TTF1 are frequently amplified in adults with squamous and non–small cell adenocarcinoma, respectively, links the processes controlling morphogenesis with those regulating epithelial cell proliferation and transformation in the respiratory tract.[206–208]

Postnatally, mutations in various genes critical to lung function, host defense, and inflammation are associated with genetic disease in humans. Hereditary disorders affecting lung function include: (1) cystic fibrosis, caused by mutations in the cystic fibrosis transmembrane conductance regulator protein; (2) emphysema, caused by mutations in α_1-antitrypsin; (3) lymphangioleiomyomatosis, caused by mutations in tuberous sclerosis complex 1 and 2; (4) alveolar proteinosis, caused by mutations in the GM-CSF receptor; and (5) respiratory distress, interstitial lung disease, and pulmonary fibrosis caused by mutations in the surfactant proteins, SP-B and SP-C, and in the phospholipid transporter, ABCA3.[209–214] In addition, mutations in polypeptides controlling neutrophil oxidant production lead to bacterial infections associated with chronic granulomatous disease.[215,216] The severity of disease associated with these monogenetic disorders is often strongly influenced by other inherited genes or environmental factors (e.g., smoking) that ameliorate or exacerbate underlying lung disease. The identification of "modifier genes" and the role of gene dosage in disease susceptibility will be critical in understanding the pathogenesis and clinical course of pulmonary disease in the future.

SUMMARY

The molecular and cellular mechanisms controlling lung morphogenesis and function provide a fundamental basis for understanding the pathogenesis and therapy of pulmonary diseases in children and adults. Future advances in pulmonary medicine will depend on the identification of genes and their encoded polypeptides that play critical roles in lung formation and function. Knowledge regarding the complex signaling pathways that govern lung cell behaviors during development and after injury will provide the basis for new diagnostic and therapeutic approaches that will influence clinical outcomes. Diagnosis of pulmonary disease will be facilitated by the identification of new gene mutations that cause abnormalities in lung development and function. Since many of the events underlying lung morphogenesis are likely to be involved in the pathogenesis of lung disease postnatally, elucidation of molecular pathways governing lung development will provide the knowledge to understand the cellular and molecular basis of lung diseases. Advances in recombinant DNA technology and the ability to synthesize bioactive polypeptides, and to add or delete genes via DNA transfer, are likely to influence the therapy of pulmonary disease in the future.

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2 BASIC GENETICS AND EPIGENETICS OF CHILDHOOD LUNG DISEASE

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BACKGROUND

During childhood, long-term respiratory illnesses occur at a higher prevalence than all other chronic conditions combined.[1] Among the respiratory illnesses, asthma is the single most common acute disease of childhood affecting an estimated 300 million individuals worldwide.[2] The most common lethal inherited disease of childhood is cystic fibrosis, which occurs in approximately 1 in 3000 births in Northern European populations. Both diseases are considered to have significant heritable components underlying disease etiology.[3–6] Cystic fibrosis is inherited, with heritable factors accounting for 54% to 100% of inter-individual variation in disease presentation and severity.[3] Estimates indicate that asthma, on the other hand, is 36% to 79% heritable.[4–6] Despite consistent evidence of strong heritability and high levels of investment in the genetic characterization of these diseases, to date only a fraction of the total heritability of asthma has been accounted for, as compared with cystic fibrosis. The basis for this polarity lies in the type and number of underlying disease-causing factors.

Cystic fibrosis is a classic Mendelian disease. This means that its transmission follows a simple pattern of inheritance set forth by *Gregor Mendel* in the 1800s and is now recognized as characteristic of single-gene autosomal recessive disorders. Attempts to model the causation of asthma, on the other hand, indicate that the heritable proportion of disease risk is composed of multiple effects, each of moderate size (a so-called "complex" or "multifactorial" etiology). Cystic fibrosis and asthma have therefore required somewhat different approaches toward their genetic dissection, and this has influenced how successful disease gene identification has been.

In this chapter, we will outline the approaches taken to identify individual sources of disease heritability for respiratory illnesses of childhood, using cystic fibrosis and bronchial asthma as examples. In addition, we will also consider potential explanations for missing heritability (i.e., the proportion of heritability that remains unaccounted for by known genetic factors). We will highlight current shortfalls in research paradigms (e.g., genetic factors that are not amenable to detection via existing technologies and study designs), and we will discuss alternative sources of heritability inseparable from genetics during the early phase of heritability estimation (i.e., epigenetic inheritance and gene × environment interactions).

CYSTIC FIBROSIS: STRATEGIES FOR THE MAPPING OF A SINGLE GENE DISORDER

Cystic fibrosis (CF) follows a characteristic autosomal recessive pattern of inheritance, requiring two copies of a risk allele to be present for the expression of the disease phenotype. *De novo* mutation coupled with the inheritance of a single risk allele from one apparently disease-free (heterozygous carrier) parent are infrequent.[7] This relatively simple pattern of disease transmission can be considered indicative of single gene involvement and large-effect, highly penetrant alleles. These represent ideal conditions for the application of linkage mapping; a technique that traces allele and disease transmission in families. By using the patterns of allele sharing in individuals concordant for disease, it is possible to identify gross genomic intervals that contain disease-causing genetic lesions. This technique was successfully applied to CF across a series of experiments in the 1980s and resulted in the identification of a large contiguous interval located on the long arm of human chromosome 7 (7q31).[8–14] This locus was found to contain at least four transcribed sequences, three of which could be excluded following recombination mapping[15] and chromosome walking/jumping techniques.[16]

Recombination mapping directly compares the frequency and distribution of cross-over events within a defined interval between cases and controls, and chromosome walking uses each end of a DNA fragment to screen a library of DNA clones for the identification of adjoining sequences, the most distal elements of which become new probes. This technique allows the researcher to effectively "walk" along a DNA sequence of interest, while jumping impassable regions (e.g., those that are highly repetitive or rich in G and C nucleotides) by the omission of bases between defined intervals. Through a combination of DNA sequence analysis and interrogation of overlapping cDNA clones derived from cultured epithelial cell libraries with a genomic DNA segment obtained from the putative CF locus, Riordan and colleagues successfully cloned the fourth transcribed sequence in 1989.[16] The consensus region from the isolated overlapping cDNA clones revealed an Open Reading Frame (ORF) encoding a 1480 amino acid polypeptide (the *Cystic Fibrosis Transmembrane Conductance Regulator* or *CFTR*). Within the ORF, loss of a single phenylalanine residue at position 508 was observed in 68% of cystic fibrosis chromosomes as compared with 0% of disease-free controls. This mutation, now known as F508del, can be traced back at least 2300 years to Iron Age Europeans.[17] It is hypothesized to have persisted due to a heterozygote selective advantage possibly in terms of resistance to infectious pathogens such as the chloride-secreting diarrheas *(Vibrio cholerae and Escherichia coli)*,[18] or alternatively as a reproductive advantage.[19,20]

CFTR represents the first human disease gene to be cloned exclusively through position-based methods, collectively termed *positional cloning*, without guidance from

cytogenetic aberrations (i.e., rearrangements or deletions) as had been the case for previously cloned disease genes such as *Dystrophin (DMD)* in Duchenne muscular dystrophy.[21] The *CFTR* gene encodes an ABC protein that acts both as a chloride channel, regulating the flow of chloride anions and therefore water across cellular membranes. It also regulates the activity of several other substrate transporter pathways (e.g., chloride-coupled bicarbonate). These activities are required for normal fluid transport in the secretory epithelia of the lungs, gastrointestinal tract, pancreas, sweat glands, and testes; impairments lead to slowed epithelial surface fluid secretion, dehydration of epithelial surface materials, congestion, obstruction, and, ultimately, recurrent bacterial infections.

CYSTIC FIBROSIS: FINE-SCALE HETEROGENEITY IN DISEASE CAUSATION

Today almost 1900 disease-causing mutations have been documented in *CFTR (www.genet.sickkids.on.ca/cftr/StatisticsPage.html)*, although the majority of these are infrequent or specific to individual populations. F508del remains the most common mutation, with only five variants carrying frequencies above 1%.[22] *CFTR* mutations are now classified into five functional groups: (I) complete absence of *CFTR* protein production, (II) *CFTR* protein trafficking defects (with low or absent protein production), (III) defective regulation, (IV) defective chloride transport through *CFTR*, and (V) defective *CFTR* splicing with diminished production of wild-type *CFTR* (reviewed in [23]). These groupings have broad clinical implications, with mutation classes I to III associated with a more severe form of the disease and pancreatic insufficiency, the latter being a common feature of CF. With the exception of this crude heuristic, a marked variability in the clinical presentation and organ involvement of patients carrying identical *CFTR* alleles has been observed. As such, efforts are now focused on dissection of the genotype-phenotype relationship and the identification of factors capable of its modification.

Although environmental factors such as nutrition and exposure to infection undoubtedly influence clinical presentation and disease severity, evidence is also now accumulating in favor of a genetic contribution, suggesting that the condition may not in fact be a single gene disorder. Early experiments have shown that mice deficient for *CFTR* vary in disease severity (in particular the degree of intestinal obstruction), as a function of genetic background (i.e., strain).[16] Similar effects have also been documented in humans, although with varying degrees of replication. A number of potential genes with modifier effects have been proposed based on existing knowledge of CF disease biology (a candidate gene approach) and tested for association with various parameters of clinical presentation including disease severity, rate of pulmonary function decline, and survival. Many of these studies have relied, however, on small phenotypically and genetically diverse populations, thereby limiting the interpretation of the results. Two of the more consistent effects reported in the literature include *TGFβ1* (Transforming Growth Factor β1) and *MBL2* (Mannose binding lectin 2). *TGFβ1* is a pro-fibrotic cytokine involved in a variety of cellular processes such as growth, proliferation, differentiation, and apoptosis. Variants at the 5' terminus of this gene have been associated with lung disease severity in CF (determined through Forced Expiratory Volume in 1 second [FEV_1]) with odds ratios of around 2.2.[20] MBL2 is an antigen recognition molecule that is capable of binding a range of pathogens and symbionts including bacteria, fungi, viruses, and parasites, and it is involved in the complement-mediated (innate immune) host defense response. MBL2 protein deficiencies caused by prevalent mutations in both the promoter and exon 1 of the gene appear to moderate susceptibility to infectious diseases across a wide range of populations, in particular the critically ill, immunocompromised, and young (6 to 18 months).[24] Early research associated these low MBL-producing genotypes with poor lung function and survival in CF.[25,26] Recent research has implicated that the genotypes are involved in early bacterial infection,[27,28] providing a potential mechanism for MBL-deficiency–related pulmonary decline. Not all such experiments[29] support this observation, but this might be attributable to variation in sample size and consequently power of the studies.

NOVEL METHODS FOR THE IDENTIFICATION OF GENETIC MODIFIERS

Recent advances in technology have enabled a shift away from candidate gene, knowledge-driven approaches toward the identification of genetic modifiers. New high throughput techniques allow the simultaneous interrogation of all known genes in the human genome irrespective of their hypothesized role in disease. To date, only a handful of studies have applied such techniques to CF, and they focus predominantly on determining the global gene expression profile of the respiratory epithelium and its response to CF disease–causing mutations. Zabner and colleagues recently performed a systematic comparison between gene expression patterns of non-CF (wild type) and CF (F508del homozygous) primary human airway epithelial cell cultures under resting conditions.[30] Expression patterns were assayed across a total of 22,283 genes and examined for significant differences. Minimal changes were observed, with only 24 genes reaching a 1% False Discovery Rate (FDR) threshold; 18 were found to have increased expression in CF, and the remaining 6 genes had decreased expression. The 24 genes included *SLC12A4* (Solute Carrier family 12, member 4, a potassium and chloride transporter) and *IL21R* (Interleukin 21 Receptor, a type I cytokine receptor for interleukin 21), both genes of relevance to CF.

Data from these types of study provide potential clues into the biological pathways involved in CF and insights into the possible sources of inter-individual variability. The quality of data and the conclusions that can be drawn are, however, inextricably linked to the degree of stringency applied to the study design. Extraneous, uncontrolled sources of variation that originate from factors such as sample cell type composition, sample treatment prior to RNA extraction, and distribution of age, gender, and environmental exposures

TABLE 2-1 FACTORS PREVIOUSLY IDENTIFIED TO HAVE AN IMPACT ON GENE EXPRESSION DATA

VARIABLE	REFERENCES	SPECIES
Age	95, 96	Human, mouse, rat, dog
Sex	97–100	Human, drosophila, mouse, nematode
Ethnicity	101, 102	Human
Environment (lifestyle/geography)	103	Human
Diet	96, 104	Mouse, dog
Time of day	105–107	Human, rat, arabidopsis
Sample cellular composition	105	Human
Agonal factors (postmortem tissue)	108	Human
Method of sample preservation	109	Human
Platform	110–112	Human, mouse, rat
Cell culture conditions	113, 114	Human
Laboratory	111	Human

across sample groups can have profound effects on the transcriptional profile. This consequently can lead to anomalous differential expression results (Table 2-1).

ASTHMA

The term *asthma* is derived from the identical Greek word meaning "noisy breathing."[31] The disease manifests as periods of reversible airflow obstruction accompanied by bronchoconstriction and inflammation. Symptoms are variable but include wheeze, cough, chest tightness, and shortness of breath. While associated with normal life expectancy, unlike CF sufferers, asthma is still estimated to be responsible for approximately 1 in 250 deaths worldwide; and each death is viewed to be preventable.[2] Buoyed by the successes in Mendelian disease gene identification, genome-wide linkage methods were first applied to asthma in 1996[32] and were subsequently repeated across a variety of different population collections. These experiments led to the identification of numerous putative disease loci, only a proportion of which were found to replicate consistently between cohorts. While this failure to reproduce may reflect cryptic gene × environment interactions or ancestry-related variation in linkage disequilibrium (LD) patterns, the likelihood is that a proportion of the unreplicated linkage peaks actually represent false positives. Interestingly, a recent meta-analysis of genome-wide linkage studies for asthma involving more than 2000 families and 5000 affected individuals identified only one region—chromosome 5 (141 to 169 centimorgans [cM])—that in all families attained genome-wide significance, and two regions—2p21-14 and 6p21—that attained significance only in families of European ancestry.[33]

Once identified, and replicated in more than one population, a small number of linkage intervals have been pursued by positional cloning (identification of underlying disease gene[s] by position-based methods). Relative to Mendelian diseases, this has proven to be an expensive and lengthy undertaking, typically requiring many successive rounds of fine-mapping in order to reduce the size of the linkage interval to a tractable number of genes. To date, six loci have been positionally cloned; *ADAM33* chromosome 20p13,[34] *DPP10* chromosome 2q14,[35] *PHF11* chromosome 13q14,[36] *NPSR1* (previously known as *GPRA*) chromosome 7p14,[37] *HLA-G* chromosome 6p21,[38] and *CYFIP2* chromosome 5q33.[39] The proteins encoded by these genes are engaged in a variety of distinct processes, including airway remodeling *(ADAM33),* T-cell adhesion and differentiation *(CYFIP2),* and transcriptional regulation *(PHF11).*

Prior to these genes being identified, historical concepts of disease causation had been founded on simple observations such as efficacy of pharmacologic therapies (e.g., β2-adrenergic receptor agonists, see[40] for an excellent review). Positional cloning has consequently extended our knowledge of the biological systems underlying asthma, but the genes identified account for relatively little of the estimated 36% to 79% heritability of asthma, as the effect size of each locus is comparatively small. A recent meta-analysis of *ADAM33* variants and haplotypes found a maximum odds ratio of 1.46 (95% CI 1.21 to 1.76)[41], while a large German case-control study of *NPSR1* observed a maximum single-marker odds ratio of 1.40 (95% CI 1.04 to 1.88).[42] There are a number of potential explanations for why such a small amount of asthma heritability has been identified so far.

The Common Disease, Common Variant (CDCV) hypothesis, postulated in the late 1990s,[43] suggests that common diseases such as asthma and diabetes are caused by many prevalent alleles of small effect acting in concert to generate the disease phenotype. This model of causation provides a viable explanation for the shortfalls of linkage mapping. Linkage mapping possesses relatively low power in such scenarios being better designed for the identification of loci harboring recessive, highly penetrant effects, and situations of allelic heterogeneity in which multiple individually rare alleles co-localize to a common locus. A more appropriate technique for CDCV identification is genetic association. This approach directly compares allele frequencies between cases and controls, seeking sites at which allele frequency correlates with case status.

GENOME-WIDE ASSOCIATION

Genome-Wide Association (GWA) applies the power of genetic association across the entire genome simultaneously. The technique relies upon the prevalence of Single Nucleotide Polymorphisms (SNPs) occurring approximately once every 100 to 300 bases. Due to knowledge of linkage disequilibrium (LD) patterns in different populations available through the HapMap project *(http://*

snp.cshl.org/), it is possible to have near-complete coverage of common variation (minor allele frequency [MAF] ≥5%) via a SNP "tagging" method (Figure 2-1). Implementing the use of tag SNPs results in a reduction in the genotyping burden of high-density mapping experiments by defining a non-overlapping, fully informative marker set, omitting those markers the genotypes of which can be inferred from other proximal positions.

The first GWA scan for asthma was published in 2007.[44] It involved the genotyping of 317,000 genome-wide tag SNPs in a cohort of 2200 individuals, achieving approximately 79% coverage of common SNPs (MAF ≥5%), assuming an r^2 of 0.8 (where r^2 is a measure of the extent of LD between genotyped and un-genotyped markers). More than half of all markers that were significant at a 1% FDR threshold were located in a single locus on chromosome 17q21. This locus was found to possess *cis*-acting regulatory potential; in other words, it has the potential to moderate the activity of genes positioned in close proximity to it. Loci that operate on genes located distally, even on different chromosomes, are referred to as *trans-acting*. The 17q21 locus was initially observed to modulate the expression of *ORMDL3 (Orosomucoid-1-like `3);* an endoplasmic reticulum (ER)–based transmembrane protein involved in calcium signaling, cellular stress, and sphingolipid homeostasis.[45,46] The locus has since been shown to additionally regulate the expression of two other proximal genes—*ZPBP2* and *GSDMB*—in an allele-specific manner, achieving domain-wide *cis*-regulation through chromatin remodeling (specifically changes in insulator protein CTCF binding and nucleosome occupancy).[47] Contrary to a large proportion of early linkage and candidate gene association data, the relationship between 17q21 genotypes and asthma appears to be very robust, and a high level of replication across a diverse range of populations has been reported.[48–55] These studies have also shown that the 17q21 association may be driven by a subset of cases with early disease onset,[49] and both subject to environmental influences (early exposure to environmental tobacco smoke)[49] and capable of calibrating environmental influence (amplifying the association between early respiratory infections and asthma).[56]

Since the publication of this first asthma GWA study in 2007, 14 additional screens have been published investigating not only the genetic etiology of asthma[57–62] but also a diverse array of related quantitative traits,[63–69] e.g. FEV_1. The most recent and largest of these screens included over 10,000 cases and 16,000 controls (all of whom were matched for ancestry), resulting in the generation of approximately 15 billion genotypes for analysis and the identification of 7 loci of genome-wide significance.[62] This represents an unprecedented leap forward in our understanding of disease biology, enabling the identification of more genes involved in the etiology of asthma within a single study than it has been possible to achieve in fourteen years of positional cloning. The results of all the GWA studies detailing the 33 loci identified are outlined in Table 2-2. With the exception of *DPP10,* none of the genes previously identified by the positional cloning approach for asthma have been found by the GWA studies. These positionally cloned genes have, however, replicated successfully across a number of prior focused experiments. This failure, therefore, by GWA to reaffirm their involvement is not necessarily an indication of error, but likely a reflection of differences in the types of effect amenable to detection via these two contrasting techniques as well as the phenotypes (traits) examined by the two methods.

A small number of the observed GWA effects confirm previously equivocal candidate genes, for example the alpha polypeptide of the Fc fragment of the high-affinity IgE receptor *(FCER1A)* association with total serum Immunoglobulin E (IgE). Others highlight distinct components of common biological pathways (e.g., *Interleukin [IL]33* and its receptor *IL1RL1)* or identify alternative members of previously implicated gene families to be of importance in disease etiology. An example of the latter is a GWA analysis of an FEV_1/FVC phenotype (the proportion of the forced vital capacity exhaled in the first second of expiration, which acts as an index of airway obstruction that controls for restrictive lung disease)

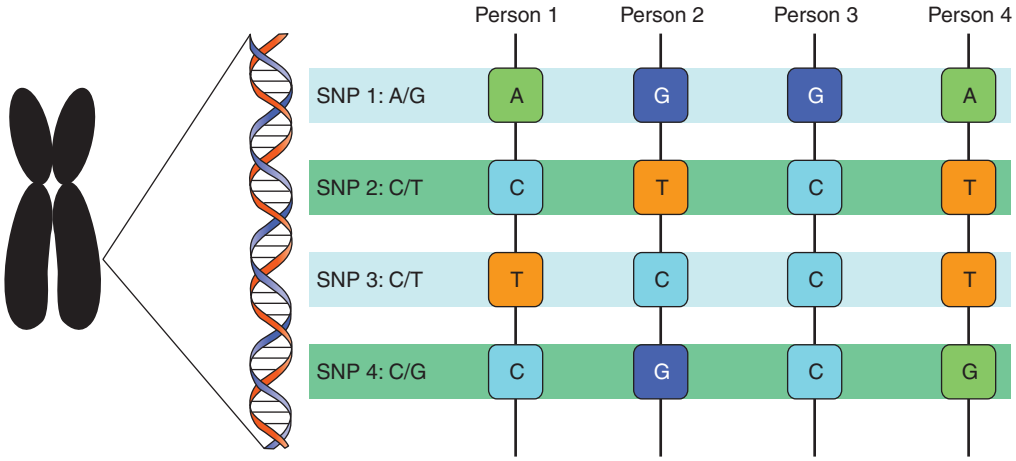

FIGURE 2-1. A haplotype tagging approach to SNP selection. Haplotypes are shown across four single nucleotide polymorphisms (SNPs) at a single chromosomal locus in four separate individuals (haplotypes are shown on the vertical). It can be seen that the allele at SNP 1 is perfectly predictive of the allele at SNP 3 (both SNP 1 and SNP 3 are highlighted in pale blue). An *A* allele at SNP 1 is always accompanied by a *T* allele at SNP 3 and the alternative allele *G* at SNP 1 is always accompanied by a *C* at SNP 3. A similar situation is seen for SNPs 2 and 4 (highlighted in green), where each is perfectly predictive of the other in terms of alleles present. The SNPs are therefore said to exhibit strong levels of linkage disequilibrium with one another, meaning that they are frequently co-inherited. Consequently, it is not necessary to genotype an individual for all four SNPs in this region. To gain complete genetic coverage, only two SNPs are required.

TABLE 2-2 SUMMARY OF GENOME-WIDE ASSOCIATION (GWA) FINDINGS FOR ASTHMA AND ITS RELATED TRAITS (AS OF OCTOBER 1, 2010)

PROPOSED GENE(S)	STUDY	CYTOGENETIC POSITION	PEAK MARKER	CHR	BP POSITION (HG19)	PEAK P-VALUE	PHENOTYPE
FCER1A	Weidinger et al. 2008	1q23	*rs2251746*	1	159,272,060	1.85×10^{-20}	IgE
DENND1B	Sleiman et al. 2010	1q31	*rs2786098*	1	197,325,908	8.55×10^{-9}	Asthma
CHI3L1	Ober et al. 2008	1q32.1	*rs4950928*	1	203,155,882	1.10×10^{-13}	Serum YKL-40 levels
IL1RL1	Gudbjartsson et al. 2009	2q12.3-q14.2	*rs1420101*	2	102,957,716	5.30×10^{-14}	Eosinophil count
IL18R1 / IL1RL1	Moffatt et al. 2010	2q12.1	*rs3771166*	2	102,986,222	3.4×10^{-9}	Asthma
DPP10	Mathias et al. 2010	2q12.3-q14.2	*rs1435879*	2	115,492,887	3.05×10^{-6}	Asthma
IKZF2	Gudbjartsson et al. 2009	2q13	*rs12619285*	2	213,824,045	5.40×10^{-10}	Eosinophil count
TNS1	Repapi et al. 2010	2q35	*rs2571445*	2	218,683,154	1.11×10^{-12}	FEV_1
GATA2	Gudbjartsson et al. 2009	3q21	*rs4857855*	3	128,260,550	8.60×10^{-17}	Eosinophil count
NPNT / INTS12 / FLJ20184 / GSTCD	Hancock et al. 2010	4q24	*rs11727189*	4	106,619,140	4.66×10^{-17}	FEV_1
GSTCD	Repapi et al. 2010	4q24	*rs10516526*	4	106,688,904	2.18×10^{-23}	FEV_1
HHIP	Repapi et al. 2010	4q31	*rs12504628*	4	145,436,324	6.48×10^{-13}	FEV_1 / FVC
HHIP	Hancock et al. 2010	4q31.21	*rs1980057*	4	145,485,738	3.21×10^{-20}	FEV_1 / FVC
PDE4D	Himes et al. 2009	5q12	*rs1588265*	5	59,369,794	4.30×10^{-7}	Asthma
IL5	Gudbjartsson et al. 2009	5q31	*rs4143832*	5	131,862,977	1.20×10^{-10}	Eosinophil count
RAD50 / IL13	Li et al. 2010	5q31.1	*rs2244012*	5	131,901,225	3.04×10^{-7}	Asthma
RAD50	Weidinger et al. 2008	5q31	*rs2040704*	5	131,973,177	4.46×10^{-8}	IgE
HTR4	Hancock et al. 2010	5q32	*rs11168048*	5	147,842,353	1.08×10^{-11}	FEV_1 / FVC
HTR4	Repapi et al. 2010	5q33.1	*rs3995090*	5	147,845,815	4.29×10^{-9}	FEV_1
ADAM19	Hancock et al. 2010	5q33.3	*rs2277027*	5	156,932,376	9.93×10^{-11}	FEV_1 / FVC
ADRA1B	Mathias et al. 2010	5q33	*rs10515807*	5	159,364,998	3.57×10^{-6}	Asthma
AGER	Repapi et al. 2010	6p21.32	*rs2070600*	6	32,151,443	3.07×10^{-15}	FEV_1 / FVC
AGER / PPT2	Hancock et al. 2010	6p21.32	*rs2070600*	6	32,151,443	3.15×10^{-14}	FEV_1 / FVC
HLA-DQ	Moffatt et al. 2010	6p21.32	*rs9273349*	6	32,625,869	7.0×10^{-14}	Asthma
HLA-DR/DQ	Li et al. 2010	6p21.3	*rs1063355*	6	32,627,714	9.55×10^{-6}	Asthma
GPR126	Hancock et al. 2010	6q24.1	*rs3817928*	6	142,750,516	1.17×10^{-9}	FEV_1 / FVC
-	Himes et al. 2009	8p12	*rs11778371*	8	27,319,905	8.10×10^{-7}	Asthma
IL33	Moffatt et al. 2010	9p24.1	*rs1342326*	9	6,190,076	9.2×10^{-10}	Asthma
TLE4	Hancock et al. 2009	9q21.31	*rs23783823*	9	82,039,362	7.10×10^{-6}	Asthma

TABLE 2-2 SUMMARY OF GENOME-WIDE ASSOCIATION (GWA) FINDINGS FOR ASTHMA AND ITS RELATED TRAITS (AS OF OCTOBER 1, 2010)—CONT'D

PROPOSED GENE(S)	STUDY	CYTOGENETIC POSITION	PEAK MARKER	CHR	BP POSITION (HG19)	PEAK P-VALUE	PHENOTYPE
STAT6	Weidinger et al. 2008	12q13.3	*rs12368672*	12	57,512,470	1.52×10^{-5}	IgE
SH2B3	Gudbjartsson et al. 2009	12q24	*rs3184504*	12	111,884,608	6.50×10^{-19}	Eosinophil count
SMAD3	Moffatt et al. 2010	15q22.33	*rs744910*	15	67,446,785	3.9×10^{-9}	Asthma
THSD4	Repapi et al. 2010	15q23	*rs12899618*	15	71,645,120	7.24×10^{-15}	FEV_1 / FVC
ORMDL3 / GSDMB	Moffatt et al. 2010	17q12	*rs2305480*	17	38,062,196	9.6×10^{-8}	Asthma
ORMDL3 (GSDMB, ZPBP2)	Moffatt et al. 2007	17q21	*rs7216389*	17	38,069,949	9.00×10^{-11}	Asthma
GSDMA / ORMDL3	Soranzo et al. 2009	17q21	*rs17609240*	17	38,110,689	9.40×10^{-9}	Total white blood cell count
GSDM1	Moffatt et al. 2010	17q21.1	*rs3894194*	17	38,121,993	4.6×10^{-9}	Asthma
PSMD3-CSF3	Okada et al. 2010	17q21.1	*rs4794822*	17	38,156,712	6.30×10^{-10}	Neutrophil count
PRNP	Mathias et al. 2010	20pter-p12	*rs6052761*	20	4,657,017	2.27×10^{-6}	Asthma
PLCB4	Okada et al. 2010	20p12	*rs2072910*	20	9,365,303	3.10×10^{-10}	Neutrophil count
IL2RB	Moffatt et al. 2010	22q12.3	*rs2284033*	22	37,534,034	1.2×10^{-8}	Asthma

CHR, Chromosome; *bp*, base pair; *FEV1*, forced expiratory volume in 1 second; *FVC*, forced vital capacity.

that identified a significant association with variants in the gene encoding *ADAM metallopeptidase domain 19* (*ADAM19*).[67] *ADAM19* is a member of the same gene family as *ADAM33*—a gene positionally cloned for asthma in 2002.[34] Both these genes are expressed in the human lung, with *ADAM19* localized to the apical part of the epithelium and *ADAM33* to the basal epithelial cells.[70] The genes have similar functional effects on integrin-mediated cell migration.[71] The remainder of the GWA findings for asthma relate to novel factors located in previously unsuspected genes or functional non-coding regions.

In some instances, more than one disease-specific screen has implicated the same locus. The 17q21 site including the gene *ORMDL3* has shown association not only for asthma but also total leukocyte cell count phenotypes. Interestingly, a GWA study for ulcerative colitis, a chronic disease involving inflammation of the gut epithelium, also found association with the chromosome 17q21 site.[72] The concordance between these GWA studies for the 17q21 locus indicates that the site may form an integral part of the inflammatory response, most notably within epithelial tissues. Consistent with this hypothesis, *ORMDL3* appears to be expressed across a broad range of immune tissues including peripheral blood leukocytes, bone marrow and lymph nodes, as well as several epithelial disease-relevant tissues including the lung and colon.[72] Experimental modulation of *ORMDL3* expression in epithelial cells has been shown to produce downstream effects on the ER stress–induced unfolded protein response (UPR),[72] a mechanism of attenuating endogenous sources of cellular stress resulting from the accumulation of misfolded proteins in the ER, and a signaling pathway of relevance to the normal functioning of the mammalian immune system.[73]

As may be predicted by the CDCV theory, the asthma loci identified through GWA are characteristically of high frequency and low magnitude. The risk allele for the 17q21 marker most significantly associated with disease is present in 62% of asthmatics and 52% of non-asthmatics. Although genotypes at this site explain a large proportion of variance in gene expression phenotypes (29.5% of the variance in *ORMDL3* expression in lymphoblastoid cell lines),[44] the effect size for asthma is relatively small (an odds ratio of 1.45 in the original study[44] and 1.44 in a subsequent meta-analysis of nine populations[74]). Similarly, protective minor alleles in the asthma-associated gene *PDE4D* (*Phosphodiesterase 4D, cAMP-specific*, a modulator of smooth muscle contractility) yield an odds ratio of just 0.85 in Caucasian and Hispanic populations, and are present in approximately 28% of affected and 32% of unaffected individuals.[58] Consistent with these

observations, the most highly powered GWA study of asthma to date recorded odds ratios ranging from just 0.76 to 1.26 for the seven disease loci identified. [62] Together these data suggest that GWA represents a productive tool for the identification of novel, common, low-magnitude effects involved in the etiology of multifactorial disease, but that collectively these factors are unlikely to account for the full heritability of asthma.

MISSING HERITABILITY

While GWA studies have led to the identification of numerous previously unrecognized factors involved in the etiology of asthma, these factors are of only moderate effect size, leaving a large proportion of disease heritability as yet unaccounted for. Clues as to the source(s) of this so-called "missing heritability" can be gleaned from direct comparisons between linkage and genome-wide association data. Several replicated linkage peaks show a complete absence of overlap with existing GWA data (e.g., the asthma susceptibility locus on human chromosome 19q13).[75–77] This is not only true of asthma, but also the majority of so-called complex traits. Simultaneous application of both linkage and GWA methods to large overlapping obesity cohorts recently demonstrated a complete lack of co-incidence between regions of linkage and association.[78] One reason for this may lie in the "common disease common variant" premise. GWA studies are typically powered to detect common effects of low magnitude. Coverage is calculated as the proportion of known variants (e.g., in the HapMap database) with a minor allele frequency above 5% captured at an r^2 of 0.8. Power rapidly declines when the degree of Linkage Disequilibrium (LD) between genotyped and un-genotyped variants decreases. Rare variants and situations of allelic heterogeneity are therefore not adequately captured by existing GWA strategies.

Allelic heterogeneity is a phenomenon whereby multiple disease-causing variants exist at the same locus. Sites harboring numerous individually rare, highly penetrant alleles of large effect are more amenable to detection via linkage (since these variants still lie within in the same region) rather than association (in which the signal may be diluted by alternative disease-causing variants exhibiting different levels of LD with the genotyped marker). There are now known cases of rare, highly penetrant alleles contributing to common diseases (e.g., the 16p11.2 deletions that occur in ~0.5% of children with severe early-onset obesity)[79] and well-described cases of allelic heterogeneity (e.g., the broad spectrum of disease-causing variants in the filaggrin *[FLG]* gene located within the 1q21 linkage peak for atopic dermatitis, a chronic inflammatory disease of the skin).[80]

The *FLG* gene has been shown to harbor an array of both prevalent and rare variants, including two loss-of-function alleles with odds ratios between 2.8 and 13.4[81] and a population attributable risk of around 11%.[82] The *FLG* mutations were identified via an exon resequencing strategy in a series of kindreds segregating for a related monogenic disease, Ichthyosis vulgaris, also known to exhibit linkage to chromosome 1q21.

Similar phenomena including situations of allelic heterogeneity and/or multiple rare allele genetic risk composition may as yet be found to contribute toward the pathophysiology of asthma. Indeed there is some evidence that the *FLG* loss of function alleles associate with asthma in the presence of AD. Recently developed "next generation" sequencing technologies that provide unprecedented depths and speeds of DNA sequence analysis will undoubtedly assist in answering this question (*www.illumina.com/technology/sequencing_technology.ilmn* and *www.genome-sequencing.com/*).

HERITABLE AND *GENETIC* ARE NOT INTERCHANGEABLE TERMS

Another potential explanation for the so-called "missing heritability" is the erroneous assumption that all sources of heritability must be genetic in origin. Estimates of heritability represent an amalgam of factors that can be transmitted down the germ line. Genetic sources of causation cannot be effectively separated from gene × environment interactions and epigenetic sources of heritability in standard twin study designs. As such, it remains feasible that residual heritability can be accounted for, at least in part by epigenetic factors and interactions between alleles and environments. The latter may vary between populations, depending on the prevailing environmental milieu and allele frequencies.

The term *epigenetic* refers to sources of inter-individual variation that can be transmitted down the germ line but is not due to change in the underlying DNA sequence. This includes DNA methylation; addition of a methyl group to the 5' carbon of cytosine residues, typically at CpG (Cytosine-phosphate-Guanine) dinucleotides, and various modifications of histones (e.g., methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP-ribosylation); histones being the scaffold around which DNA is wound. Evidence suggests that these epigenetic marks may be environmentally malleable,[83] tissue specific,[83,84] subject to influences such as age[83–85] and sex,[84,85] and capable of maintenance across both the lifespan and across generations.

The role of DNA methylation in asthma has not yet been systematically explored in humans on a genome-wide basis. A number of small-scale focused studies have produced evidence consistent with environmentally determined patterns of DNA methylation. For example, a study following transplacental exposure to traffic-related polycyclic aromatic hydrocarbons identified individual loci at which the extent of methylation appears to associate with disease.[86] Likewise, a recent genome-wide survey of DNA methylation in a model organism (the mouse) revealed an array of sites at which the extent of DNA methylation was (a) subject to environmental influence, exhibiting a consistent relationship with the availability of methyl donors in the prenatal maternal diet, (b) correlated with gene transcription, (c) associated with various asthma-related traits in the offspring including airway hyperreactivity, serum IgE and lung lavage eosinophilia, and (d) demonstrated a trans-generational pattern of inheritance.[87]

Histone modifiers, in particular histone acetyltransferases (HAT) and deacetylases (HDAC), are also thought to play a role in the pathogenesis of asthma. HATs and HDACs are classes of enzyme that selectively add (acetylate) or remove (deacetylate) acetyl groups from conserved lysine amino acids in core histone proteins. Thus they dynamically control gene expression by altering the potential for histones to bind DNA. These antagonistic enzymes have been implicated in a variety of different processes from cell survival and proliferation to DNA repair and gene transcription.[88,89] Both their expression and activity have been found to differ in asthma[90] as well as chronic obstructive pulmonary disease (COPD),[91] another inflammatory disease of the lung.

Together these data suggest that epigenetic effects have the potential to contribute toward the etiology of asthma. Further systematic surveys will be required in order to specify the extent of this contribution; both in terms of the number and type of contributory loci, and proportion of phenotypic variance accounted for. Such approaches have already begun to be applied to a small number of alternative common, non-Mendelian diseases. A recent genome-wide scan for differential CpG methylation in diabetes mellitus, for example, identified a small number of both novel and known loci (i.e., loci overlapping with previously defined genetic susceptibility sites) that associate with presence or absence of diabetic nephropathy, which is a serious complication. The most significant of these sites achieved a *P*-value of 3.27×10^{-6}, and an odds ratio of just 1.88. This is an effect of comparable proportions to previously documented genetic factors.

ENVIRONMENTS: AN ADDITIONAL LAYER OF COMPLEXITY

Like epigenetic effects, current estimates of heritability also include interactions between genetic factors (G) and environments (E). These interactions are commonly referred to as Gene × Environment (G×E) interactions, but in reality they are not limited to genes but include sequence variants located in any portion of the genome (e.g., promoters, transcription factor binding sites, transcriptional enhancers). These sources of heritability have been extensively studied in asthma using a candidate gene approach. They have primarily focused on genes and variants already implicated in disease and identified through alternative techniques (positional cloning), or based on existing knowledge of gene or variant functionality (i.e., involvement in phenotypically relevant biological pathways such as pathogen detection or antimicrobial response). A small number of significant interactions have been observed. These include interactions between *TNF* genotypes and ozone exposure in childhood asthma and wheeze, and interactions between microbial exposure and variants in innate immunity genes (in particular *CD14* and the toll-like receptors *TLR4* and *TLR2*) in the determination of atopy phenotypes (e.g., serum IgE, eczema, and allergic sensitization) (reviewed by Vercelli [92]).

Since the majority of genes studied to date as potential sources of G×E in asthma were initially pursued following direct evidence of gene involvement, these results do not provide original information regarding new genetic risk factors. Instead they allow a redistribution of heritability between G and G×E. As yet there has been no systematic genome-wide association analysis of G×E in humans, although supplementary analyses of loci implicated by direct (G only) GWA indicate that a proportion of these sites may be subject to environmental moderation.[49] A recent unguided analysis of G×E effects in mice showed that, depending on the specific type of interaction occurring, a proportion of G×E effects may prove undetectable when G×E interaction is ignored.[93] As such, studies powered to detect effects of G alone may not be capable of identifying the full complement of latent G×E interactions. Consistent with this, a recent genome-wide G×E linkage analysis for asthma resulted in the identification of several previously unsuspected genomic sites, all of which proved undetectable in the same dataset when the interaction term (early life passive smoke exposure) was not included in the analysis.[94] (See Chapter 3 for a further discussion of G×E interactions in the context of the lung.)

IMPLICATIONS FOR THE HERITABILITY OF ASTHMA

Since the first genome-wide association of asthma was published three years ago, there has been a rapid and dramatic shift in our concepts of disease causation and the factors underlying it. Until recently, the most productive approach toward disease gene identification was positional cloning, a technique that interrogated the entire genome (using a relatively sparse marker set) for regions of disease and marker co-transmission in families. This approach was highly successful for Mendelian traits such as cystic fibrosis, but has been less productive in the field of multifactorial (complex) traits. The positional cloning technique did nonetheless result in the identification of six genes contributing toward the etiology of asthma. These genes, however, were only found to explain a relatively small proportion of the total disease heritability, leaving the source (or sources) of residual heritability unknown. Founded on the premise that common diseases are likely to be caused by common alleles, the research emphasis has now shifted from genome-wide linkage to genome-wide association, using dense haplotype tagging marker panels containing many hundreds of thousands of markers to effectively capture virtually all common variation in the human genome.

Since the first genome-wide association study for asthma in 2007, the approach has been applied to asthma or asthma-related traits a total of 14 times, and has led to the identification of more than 30 disease-relevant loci; almost all of which have been successfully resolved to individual genes (Figure 2-2). Like positionally cloned genes however, these loci appear to exert relatively small effects.

The origin(s) of missing heritability has become a topic of considerable interest and debate. In this chapter, we have discussed several possible sources, including rare

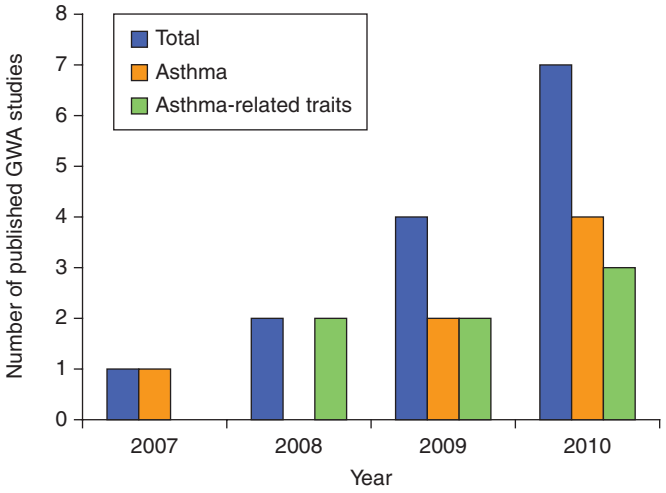

FIGURE 2-2. Publication of genome-wide association studies of asthma and related traits from 2007 to October 2010.

variants, situations of allelic heterogeneity, epigenetic effects, and G×E interactions. Systematic exploration of these sources is now required in order to determine their relative contribution to phenotypic variance, with the ultimate aim of specifying factors of sufficient size and penetrance to offer predictive or prognostic value in a clinical setting. Similar approaches (including GWA) may now usefully be applied to Mendelian traits such as cystic fibrosis in order to support the identification of cryptic modifier loci (altering disease progression or clinical presentation). Indeed the CF Modifier Gene Consortium has now completed a GWA study of CF, and the results will be available soon. Thus the genetic analysis of heritable chronic lung disease traits has come full circle, with techniques that were originally developed for exploration of multifactorial traits and diseases now being applied to single gene disorders for identification of new contributory factors including so-called gene modifiers.

Suggested Reading

Moffatt M, Gut I, Demenais F, et al. A large-scale genome-wide association study of asthma. *N Engl J Med*. 2010;363(13):1211–1221. This paper reports the findings of the largest GWA for asthma to date conducted by the GABRIEL Consortium *(www.gabriel-fp6.org/)*.

Moffatt MF, Kabesch M, Liang L, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature*. 2007;448(7152):470–473. This paper reports the findings of the first GWA study for asthma.

O'Sullivan BP, Freedman SD. Cystic fibrosis. *Lancet*. 2009;373(9678):1891–1904. A comprehensive review of cystic fibrosis.

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Vercelli D. Gene-environment interactions in asthma and allergy: the end of the beginning? *Curr Opin Allergy Clin Immunol*. 2010;10(2):145–148. This review provides a detailed description of gene environment interactions in asthma.

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The complete reference list is available online at www.expertconsult.com

3 GENE BY ENVIRONMENT INTERACTION IN RESPIRATORY DISEASES

CHIH-MEI CHEN, MD, AND MICHAEL KABESCH, MD

THE DEFINITION OF GENE BY ENVIRONMENT INTERACTION

In recent years, the term *gene by environment interaction* has become popular, but the meaning of the term varies considerably in different disciplines. When clinicians talk to statisticians and biologists, all may have their own view on gene by environment interactions. Gene by environment interactions need to be assessed by statisticians in large datasets, but they need to be proven experimentally in biological settings (e.g., by manipulating the presence of an environmental factor). Gene by environment interactions are only of clinical importance when they affect medicine and clinical practice. It is also important to note that the effect of gene by environment interaction may change with the age of the study subject. Environmental stimuli start to affect our health *in utero*. Throughout life, humans are exposed to different levels of environmental stimuli. Some exposures may have long-term effects (and the timing of the exposure is crucial for the effect size), while others may only cause strong short-term reactions (Figure 3–1).

In general, gene by environment interaction indicates some sort of interplay between genetic and environmental factors. The term may be misused in situations in which several independent risk factors (including genetic and environmental) contribute to the development or worsening of the diseases (so called *complex* or *multifactorial* diseases), while the dependence between these factors was not evaluated statistically or biologically.[1]

A statistical interaction is established only when the effect of one disease risk factor depends on another risk factor. A simple example is the interaction between the genetically determined expression of a detoxifying enzyme and the exposure to a toxic substance (environmental factor) on the occurrence of a disease. Disease will occur only when both factors are present. In epidemiology, the term *effect modification* is also commonly used to denote the existence of statistical interaction. When there is no interaction, the effects of each risk factor are consistent across the level of the other risk factor. Statistical interaction (or heterogeneity of effects) is usually defined as "departure from additivity of effects" as effects are not independent. In other words, the effect of a genetic risk factor is "multiplied" by the presence of an additional environmental risk factor. If the two risk factors are independent, they only "add up" but do not multiply. A simple example is shown in Table 3–1. It is helpful to draw such a table if one is to judge the presence of (claimed) gene by environment interaction. If combined effects are not multiplicative (but additive), gene by environment interaction is not present.

As indicated in an excellent review by Dempfle and colleagues,[1] interactions can be divided into removable and nonremovable types (Figure 3–2). An interaction is removable if a monotone transformation (e.g., taking logarithms or square roots of quantitative phenotypes) exists that removes the interaction. This implies that there is an additive relationship between the variables, just on a different scale. Therefore, nonremovable interactions are usually of greater interest. Nonremovable interaction effects are also called *crossover effects* or *qualitative interactions* (as opposed to *quantitative, removable interactions*).[1]

Confounding needs to be distinguished from interaction. *Confounding* refers to a mix of effects where a risk factor leads to a noncausative association. In gene by environment interactions, this relates to a correlation between genetic and environmental effects, which could be misinterpreted as interaction. This could be the case in a population with population stratification where unknowingly different ethnic groups are included in one study population and genetic as well as environmental factors depend on ethnicity.

Biological interaction is defined as the joint effect of two factors that act together in a direct physical or chemical reaction and the co-participation of two or more factors in the same casual mechanism of disease development.[1] In other words, genetic and environmental factors are acting directly on the same pathway. A gene by environment interaction can only be firmly ascertained when it is confirmed both statistically and biologically.[2] An observed statistical interaction does not necessarily imply interaction on the biological or mechanistic level.

In a statistical test, there is always the possibility of a false-positive finding or type I error (denoted as α). In studies of genetic effects on a specific health endpoint, it is common for numerous genetic loci to be considered simultaneously, especially in the case of genome-wide association studies. In these cases, statistical tests are used repeatedly, which results in multiple comparisons and an increase in type I errors. Nowadays, corrections for multiple testing are commonly applied in genetic studies, however there is still the possibility that the observed associations were random. Therefore, it is crucial to establish the biological plausibility and clinical relevance of the positive finding. *A priori* knowledge of biological interaction can facilitate the investigation of gene by environment interaction because correction of multiple testing strongly reduces the power. The power of statistical analysis also decreases with discrete outcomes. Therefore, unnecessary categorization or using cut-off values should be avoided.

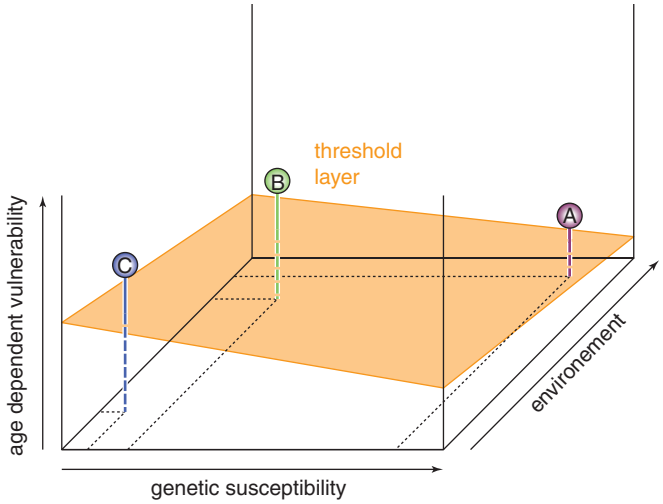

FIGURE 3–1. An asthma phenotype may result from an interaction between strong genetic and environmental effects independent of the timing of these effects *(A)*. However, contrary genetic predisposition and environmental factors may oppose each other, leading to no clinical expression of disease. Asthma may also result from strong environmental factors in the absence of a strong genetic predisposition *(B)*. Weak genetic susceptibility and relatively mild environmental risk may still lead to an asthma phenotype when risk occurs at a vulnerable time for disease development (e.g., the first year of life) *(C)*. (From Kabesch M. Gene by environment interactions and the development of asthma and allergy. *Toxicol Lett*. 2006;162(1):43–48.) Used with permission.

On the other hand, when an empirical gene by environment interaction is indicated (e.g., the association between exposure to certain carcinogens and the risk of disease development seems to be restricted to the subpopulation having the dysfunctional alleles), the observed interactions also need to be tested statistically to confirm whether the gene by environment interaction exists and the magnitude of it.

In this chapter, we will focus on asthma to illustrate how to investigate the effects of gene by environment interaction and how to interpret the clinical values, as most data on interactions in childhood respiratory diseases are available in that field.

TABLE 3–1 RELATIVE RISKS (RR) FOR EXAMPLES OF ADDITIVE AND MULTIPLICATIVE MODELS OF ENVIRONMENTAL AND GENETIC RISK FACTOR INTERACTIONS

	ENVIRONMENTAL RISK FACTOR		GENETIC RISK FACTOR	
	Additive Model		*Multiplicative Model*	
	Absent	**Present**	**Absent**	**Present**
Absent	1	2	1	2
Present	1.5	2.5	1.5	3

(From Dempfle A, Scherag A, Hein R, et al., 2008. Gene-environment interactions for complex traits: definitions, methodological requirements and challenges. *Eur J Hum Genet*. 2008;16:1164–1172. Used with permission.)

GENE BY ENVIRONMENT INTERACTION IN ASTHMA

Asthma is a complex syndrome, and no standard method can be used to identify the disease with certainty. Based on a large-scale international study—the International Study

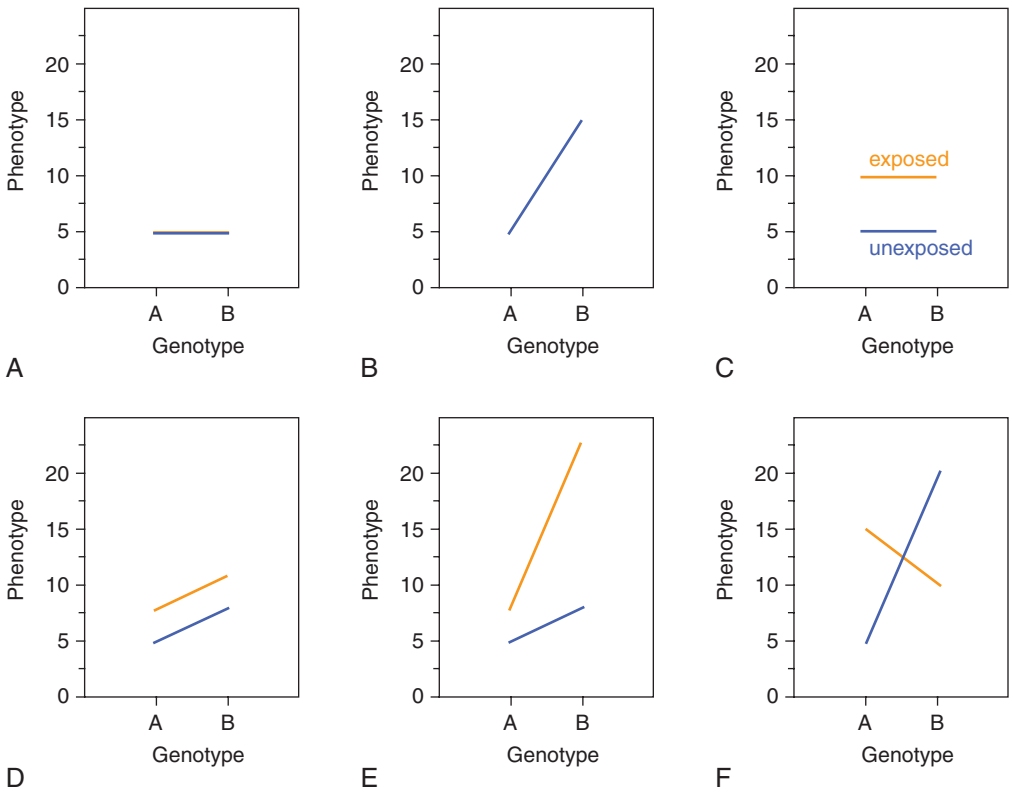

FIGURE 3–2. Examples of main and interaction effects. Phenotypic values depending on genotype G (two groups, e.g., under a dominant genetic model) and exposure E (also two groups, exposed *[yellow line]* and unexposed *[blue line]*). (**A**) Neither G nor E have a main effect and there is no interaction; (**B**) G has a main effect, E has no main effect, and there is no interaction; (**C**) E has a main effect, G has no main effect, and there is no interaction; (**D**) both G and E have main effects, and there is no interaction; (**E**) G and E have main effects, and there is an interaction (which can be removed by changing the phenotype scale, e.g., to a logarithmic scale); (**F**) G and E have main effects, and there is an interaction (which cannot be removed by any monotone transformation). (From Dempfle A, Scherag A, Hein R, et al. Gene-environment interactions for complex traits: definitions, methodological requirements and challenges. *Eur J Hum Genet*. 2008;16:1164–1172. Used with permission.)

on Asthma and Allergy in Childhood (ISAAC)—the prevalence of asthma symptoms in 13- to 14-year-olds reached 31% in the United Kingdom and 17.5% in Germany in 2003.[3] Observational and interventional studies demonstrated that the development of asthma is a result of multiple genetic and environmental factors.[4,5] Family history is a long-established risk factor for asthma development with a positive predictive value ranging from 11% to 37% between different study populations, which underlines the importance of genetics in asthma etiology.[6] However, genetic variation does not fully explain asthma pathogenesis or epidemiologic findings. Numerous environmental factors have been examined in epidemiologic and experimental studies, including domestic and occupational chemical and microbiological exposure, diet, and lifestyle in general. However, no conclusive explanation was found for the development of asthma caused by environmental factors alone, and prevention strategies based on epidemiologic association findings are still lacking. Instead, genetic as well as environmental factors contribute to the complex disease as shown by segregation analyses.[7] In recent years, studies have attempted to investigate if gene by environment interaction effects truly exist in asthma, and thus better understand the development and course of the disease.

ENVIRONMENTAL TOBACCO SMOKE

Negative effects of environmental (passive) tobacco smoke (ETS) exposure on children's health are well documented. For asthma, ETS exposure is the single most prominent environmental risk factor for the development of childhood asthma worldwide.[8] Smoking during pregnancy and exposure to tobacco smoke in the home reduces children's lung function and increases the lifelong risk of asthma.[9] Tobacco smoke contains over 4000 chemical compounds, which include about 50 to 60 carcinogens, several mutagens, and many irritating or toxic substances. It has been noted that susceptibility to ETS exposure varies between individuals, thus a genetic component is suspected.

Genes may exist that increase the susceptibility to develop asthma specifically in the presence of tobacco smoke exposure.[10] Linkage studies that took smoking and passive smoking status into account differed significantly in their results from unstratified analyses. It was noted that some chromosomal regions that showed strong linkage with asthma and bronchial hyperresponsiveness (e.g., 1p, 3p, 5q, 9q) may harbour genes that exert their effects, mainly in combination with ETS exposure.[11,12] However, other linkage peaks for asthma or other allergic diseases seem not to be influenced by passive smoke exposure status. Thus, it may be speculated that a gene by environment interaction between passive smoking and genetic susceptibility may be causally involved in the development of asthma in some but not all children with asthma. Genes responsible for these linkage peaks in combination with ETS exposure have not yet been identified by positional cloning.

In addition to this systematic approach, specific candidate genes (selected by their putative function to be involved in a gene by environment interaction with ETS) have been investigated. Glutathione S-transferase genes (GST) are likely candidates as they contribute to biotransformation of xenobiotics and protection against oxidative stress.[13] GST enzymes, which are divided into classes such as alpha (A), mu (M), pi (P), and theta (T), may thus play a role in the detoxification of components found in passive (and active) smoke and also in the detoxification of other air pollutants. Conversely, genetic variations of GST can change an individual's susceptibility to carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs. For GST classes T1 and M1, common gene deletions leading to a complete absence of the respective enzymes have been described. Approximately 50% of the Caucasian population show a deletion of GSTM1, and 15% to 20% show a deletion of GSTT1. In GSTP1, polymorphisms putatively influencing gene function and expression were detected.

It has been suggested that GSTM1-deficient children may have impaired lung growth in general.[14,15] The effect of genetic alterations in the GST system and smoke exposure on lung function seems not to be limited to childhood but may well extend into later life. Also, adult smokers with GSTT1 deficiency were shown to have a faster decline in lung function than those with functional GSTT1 enzymes.[16] In the same study, carriers of the GSTP1 allele 105Val showed lower lung function values, but an interaction between smoking and GSTP1 polymorphisms was not observed in this study or other studies.

In a study of more than 3000 children, the interaction of the genetically determined deficiency of the GST isoenzymes mu (GSTM1) and theta (GSTT1) with *in utero* and current ETS exposure was investigated specifically to assess gene by environment interaction models.[17] When ETS exposure was not included in the analysis, neither GSTM1 nor GSTT1 deficiency had an effect on the development of asthma. In children lacking GSTM1 who were exposed to current ETS, the risk for asthma and asthma symptoms was significantly elevated compared to GSTM1-positive individuals without ETS exposure. *In utero* smoke exposure in GSTT1-deficient children was associated with significant decrements in lung function compared to GSTT1-positive children who were not exposed to ETS. These findings indicate that environmental exposure to toxic substances is necessary to unravel the effect of genetically determined deficiencies in GST-dependent detoxification processes. Interaction models showed an overall trend for a positive interaction effect, above the expected multiplicative interaction between GSTM1 and GSTT1 deficiency or ETS exposure alone.

Experimental data support the observations from population genetics: In the lung tissue of GSTM1-deficient individuals, higher levels of aromatic DNA adducts have been found,[19] and cytogenetic damage to lung cells caused by smoke exposure increases with GSTM1 deficiency.[20] This indicates an increased damage to DNA and the destruction of tissue due to diminished GSTM1 function. Also, GSTT1-negative individuals showed significantly higher levels of DNA damage than GSTT1-positive individuals in experimental *in vitro* settings.[21] Furthermore, recent data indicate that GSTM1 may modify the adjuvant effect of diesel exhaust particles on allergic inflammation.[22] These observations may help to explain why

GST deficiency seems to exert a stronger effect on atopic asthma than on non-atopic asthma in population genetic studies.[17,23] Furthermore, a dosage effect for GSTT1 and GSTM1 alleles on the occurrence of atopic asthma was observed.[24] Studies have also investigated how polymorphisms of oxidative stress pathway–associated genes modify the effect of exposure to ETS on asthma; further evidence is needed to confirm the positive results observed in some of these studies.[25]

The modifying effects of genes involved in innate immune pathways on the association between ETS and asthma were also investigated because endotoxin is one component of cigarette smoke. Several genes were studied as potential effect modifiers, including *CD14, IL-10, IL-13,* and *IL-1 receptor antagonist (IL-1RA),* however, it is too early to draw any conclusions.[5,25]

AIR POLLUTION AND OXIDATIVE STRESS RESPONSE PATHWAYS

Previous studies showed that air pollutants, especially ozone and fine particles, are associated with the exacerbation of asthma symptoms.[26,27] A recent review assessing evidence from prospective cohort studies concluded that exposure to traffic exhaust contributes to the development of respiratory symptoms in healthy children.[28] Because oxidative stress was suggested as the major underlying mechanism of the toxic reactions induced by air pollutants,[29] studies have investigated modifications of the effect of exposures to air pollution by common polymorphisms with known functions related to the oxidative stress response. As noted earlier in the chapter, the most commonly studied genes include Glutathione S-transferase M1 (GSTM1) and Glutathione S-transferase P1 (GSTP1). GSTP1 polymorphisms are expressed in the respiratory tract and are also associated with an individual's susceptibility to oxidant defenses, xenobiotic metabolism, and detoxification of hydroperoxides. Studies from Mexico City showed that GSTM1 deficiency in children with a high level of ozone exposure increased the risk for asthma in an interactive manner.[30] In addition, it was reported that children who were homozygous for the GSTP1 Ile105 allele and were exposed to high levels of air pollution in China had a significantly higher risk of developing asthma.[31] While adverse effects of air pollutant exposures are mainly observed in individuals having a GSTM1-null genotype, evidence of the interaction effect between GSTP1 and exposures to air pollutants on respiratory diseases is not consistent.[25]

MICROBIAL EXPOSURES AND PATTERN RECOGNITION RECEPTOR

Microorganisms are ubiquitous in the environment, and there is a wide geographical variation of the quantities of different species and their compounds. Recent research has linked different levels of microbial exposures to asthma in support of the hygiene hypothesis. It was observed that children who were born in farm environments and continued to spend their early childhood in such environments had a lower risk of developing allergic respiratory diseases.[32,33] One of the major characteristics of the farm environment is the high level of microbial exposure. The effects of exposures to *endotoxin,* a constituent of the outer membrane of Gram-negative bacteria, were studied both in farm environments as well as inner-city homes. Studies investigating the effect of endotoxin exposure on asthma and allergy, however, do not always reproduce the protective effect observed in farm studies.[34] So far, it remains uncertain as to whether the protective effect observed in children from a farm environment was caused by exposure to one specific agent or exposures to an extensive variety of microbes.

Pattern recognition receptors identify pathogen-associated molecular patterns as part of the innate immune defense system. Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)1 and NOD2, and CD14 are the most prominent examples of human pattern recognition receptors. CD14, a receptor molecule involved in the recognition of bacterial cell wall components (e.g., endotoxin) was the first to be studied in the context of environmental exposure. A promoter polymorphism was associated with serum levels of soluble CD14 protein in some studies and phenotypes of allergy in others. The CD14 polymorphism, which was identified in the promoter region of the gene, alters CD14 gene expression *in vitro*. Intriguingly, its effect seems to be dependent on the level of microbial exposure. This first was suggested by Donata Vercelli in her "endotoxin-switch theory"[35] and later was shown by association studies.[36,37] These data indicated that a polymorphism in the CD14 promoter modified IgE levels, depending on endotoxin load (which were measured in the children's mattresses as an indicator of microbial exposure). In farmer[36] and non-farmer[37] populations of children exposed to high levels of endotoxin, the polymorphic C allele is associated with lower IgE levels[36] and less allergy.[37] An opposite association is seen in individuals who are exposed to low endotoxin levels. However, the results are not consistent in the direction of the effect,[25,34] which may be caused by high variability of environment exposure levels and small sample sizes.

Genetic variations in TLRs may also predispose to allergies and asthma. In farm children (but not those growing up in rural environments without farm exposure), a polymorphism in the TLR2 promoter significantly modified the risk for developing allergic sensitization, hay fever, and asthma.[38] However, these data are derived from a subgroup analysis, and the prevalence of asthma and other atopic diseases is extremely low in farm children. Therefore, these data must be viewed as preliminary until they are replicated in an independent population with similar exposure characteristics showing the same direction of association.

GENOME-WIDE INTERACTION STUDIES (GWIS)

In 2007, the first genome-wide association study on asthma was published, and many more of these studies followed. In these studies, hundreds of thousands of

common polymorphisms are genotyped per individual covering large areas of the genome. These data can be the basis for genome-wide interaction studies in which a systematic approach on gene by environment interaction analysis can be performed. A first study of this kind was published. It focused on genome by farming effect interaction,[39] and further studies on genome by smoking (active and passive smoke exposure) are in progress. In the first published GWIS, none of the previous suggested polymorphisms in candidate genes for genome by farming (or microbial) exposure interaction effects were found to be significant. However, a number of rare variants in genes so far unrelated to asthma were identified to show gene by environment interaction with farm exposure. Replication of GWIS as well as identification of biological plausibility for the statistical interactions need to be established before conclusions are possible.

EPIGENETICS: GENETIC AND ENVIRONMENTAL FACTORS

Epigenetics describes the fact that environmental factors can imprint on DNA without changing the nucleotide sequence of the genome by modifying the tertiary structure of DNA. A variety of molecular mechanisms are involved in epigenetic regulation, including posttranscriptional histone modifications, histone variants, ATP-dependent chromatin remodeling complexes, polycomb/trithorax protein complexes, small and other non-coding RNAs (siRNA and miRNAs), and DNA methylation. Epigenetic mechanisms seem to be important in cancer development, but very little is known about these effects in complex diseases such as asthma. Epigenetic studies in asthma are still at a very early stage. It would be surprising if epigenetic regulation was not involved in the development of asthma, which is driven by environmental as well as genetic susceptibility factors. However, existing epigenetic data is sparse, and to study epigenetics in asthma is a daunting task for the future.[40] Table 3–2 provides an overview on environmental factors related to asthma that may influence the mechanisms and genes involved in its development.

CONCLUSION

Based on previous research, there are reasons to believe that many genetic and environmental factors interact to cause asthma. However, genetic studies have generally ignored environmental factors, and environmental studies have generally ignored genetics. Thus, there are relatively few examples of specific gene by environment interactions in relation to asthma. Genetic studies assuming equal environmental exposure lead to false-negative findings when the effect of the specific gene is small. Environmental studies neglecting the effect of genetics lead to inconsistency. One of the major difficulties in gene-related research is the lack of statistical power, which can only be overcome by international collaborations. Furthermore, environmental exposure measurements need to be standardized. For example, exposures to air pollutants may be measured by personal monitoring or stationary monitoring, which are hardly comparable. Finally, for complex diseases, the power of detecting gene by environment interactions can also be enhanced by better-defined health endpoints, a challenge for such a vague entity as asthma.

Thus, while there is good reason to believe that gene by environment interactions play a role in asthma and other respiratory diseases in childhood, the evidence for such interactions is still slim and controversial. However, further investigations into gene by environment interactions are valuable as they could provide insight into mechanisms leading to asthma development and open the door for personalized medicine and true prevention of respiratory diseases in childhood.

TABLE 3–2 ENVIRONMENTAL FACTORS THAT HAVE BEEN REPORTED TO INFLUENCE ASTHMA AND EVIDENCE FOR CONSEQUENCES AT THE LEVEL OF EPIGENETIC MODIFICATIONS INDUCED BY THE SAME ENVIRONMENTAL FACTORS

ENVIRONMENTAL FACTOR	GENES PUTATIVELY INVOLVED IN GENE-ENVIRONMENT EFFECTS	EPIGENETIC MODIFICATIONS INDUCED BY THE ENVIRONMENTAL FACTOR	TARGET OF EPIGENETIC EFFECTS	TISSUE ANALYZED/DISEASE CONTEXT
Passive smoking	*IL1R*	DNA methylation	Global	Mouse lung tissue/lung cancer
In utero smoking	*GSTM1/GSTP1*	DNA methylation	Global + *AXL PTPRO*	Human buccal cells/effects of *in utero* tobacco smoke
Ozone/oxidative stress	*TNF*[41]	DNA methylation	Global	Murine melanocytes/cancer
Farm exposure	Innate immunity receptors	No data available	No data available	No data available
Endotoxin	*CD14*	Chromatin remodeling	*TNF/IL-1*β	Human promonocytic cells (THP-1), blood leukocytes/ systemic inflammation

(From Kabesch M, Michel S, tost J. Epigenetic mechanisms and the relationship to childhood asthma. *Eur Respir J*. 2010;36(4):950–961.) Used with permission.

Suggested Reading

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4 THE SURFACTANT SYSTEM

Paul Kingma, MD, PhD, and Alan H. Jobe, MD, PhD

Pulmonary surfactant is a complex substance with multiple functions in the microenvironments of the alveoli and small airways.[1] The traditional functions of surfactant are biophysical activities to keep the lungs open, to decrease the work of breathing, and to prevent alveolar edema. Most of the components of surfactant also contribute to innate host defenses and injury responses of the lung. Surfactant deficiency states occur with prematurity and with severe lung injury syndromes. Recent studies in humans and in mice are defining an expanding number of genetic and metabolic abnormalities that disrupt surfactant and cause lung diseases that range from lethal respiratory failure at birth to chronic interstitial lung disease in later life. This chapter summarizes those aspects of surfactant biology that are relevant to children.

SURFACTANT COMPOSITION

Metabolism

Surfactant recovered from lungs by bronchoalveolar lavage contains about 80% phospholipids, about 8% protein, and about 8% neutral lipids, primarily cholesterol (Figure 4-1).[2] The phosphatidylcholine species of the phospholipids contribute about 70% by weight to surfactant. The phospholipids in surfactant are unique relative to the lipid composition of lung tissue or other organs. About 50% of the phosphatidylcholine species have two palmitic acids or other saturated fatty acids esterified to the glycerol-phosphorylcholine backbone, resulting in "saturated" phosphatidylcholine, which is the principal surface-active component of surfactant. About 8% of surfactant is the acidic phospholipid phosphatidylglycerol. Surfactant from the immature fetus contains relatively large amounts of phosphatidylinositol, which then decreases as phosphatidylglycerol appears with lung maturity.[3]

Four primary surfactant proteins have been identified: surfactant proteins A, B, C, and D.[4,5] Initial analyses of these proteins suggested that the hydrophilic surfactant protein A (SP-A) and surfactant protein D (SP-D) are primarily involved in pulmonary innate immunity, whereas the hydrophobic surfactant protein B (SP-B) and surfactant protein C (SP-C) facilitate surfactant lipid physiology. However, we now know that the surfactant proteins often cross these lines of functional classification.

SP-A and SP-D are members of the collectin family of innate defense proteins. Collectins are defined by four structural domains shared by all family members: a short amino-terminal cross-linking domain, a triple helical collagenous domain, a neck domain, and a carbohydrate recognition (CRD) domain.[4–8] Three neck domains combine and facilitate the formation of a collagen-like triple helix that then aggregates to form larger multimers of the collectin trimer. SP-A is a 24-kd monomer that further assembles to a bouquet of six trimers with a molecular size of 650 kd.[9–11] SP-A is encoded by two genes *(Sftpa)* located within a "collectin locus" on the long arm of chromosome 10 that also includes the genes for SP-D *(Sftpd)* and mannose binding protein.[12] In humans, SP-A synthesis begins during the second trimester of gestation and occurs primarily in the alveolar type II epithelial cells, Clara cells, and in cells of tracheobronchial glands. SP-A is required for the formation of tubular myelin and has several roles in pulmonary host defense.

SP-D is a 43-kd hydrophilic collectin with a monomer structure that is similar to SP-A, although the collagen domain of SP-D is much longer.[11,13] Structural studies demonstrate that SP-D trimers further combine into larger multimeric complexes through N-terminal interactions that are stabilized by disulfide bonds.[6,10,14] Although larger, more complex forms have been identified, SP-D exists predominately as a tetramer of trimeric subunits (dodecamer) assembled into a cruciform. SP-D is synthesized by type II cells and by Clara cells, as well as other epithelial cells. Like the other surfactant proteins, SP-D expression is developmentally regulated and induced by glucocorticoids and inflammation.[15] In addition to the complex roles of SP-D in pulmonary host defense, SP-D also influences surfactant structure and is required for surfactant reuptake and the regulation of pulmonary surfactant pool sizes.[16,17]

SP-B is a small hydrophobic protein that contributes about 2% to the surfactant mass.[1,4] The SP-B gene is on human chromosome 2 and is expressed in a highly cell-specific manner. The primary translation product is 40 kd, but the protein is clipped within the type II cell to become an 8-kd protein prior to associating with phospholipids during the formation of lamellar bodies. SP-B facilitates surface absorption of lipids into the expanding alveolar surface film and enhances their stability during the movements of the respiratory cycle. A genetic lack of SP-B causes a loss of normal lamellar bodies in type II cells, a lack of mature SP-C, and the appearance of incompletely processed SP-C in the airspaces.[18]

The SP-C gene is located on chromosome 8, and its primary translation product is a 22-kd protein that is processed to an extremely hydrophobic 35 amino acid peptide rich in valine, leucine, and isoleucine.[19] The SP-C gene is expressed in cells lining the developing airways from early gestation. With advancing lung maturation, SP-C gene expression becomes localized only to type II cells. SP-B and SP-C are packaged together into lamellar bodies and function cooperatively to optimize rapid adsorption and spreading of phospholipids. Surfactants prepared by organic solvent extraction of natural surfactants or from lung tissue contain SP-B and SP-C. Such surfactants are similar to natural surfactants when evaluated for *in vitro* surface properties or for function *in vivo*.

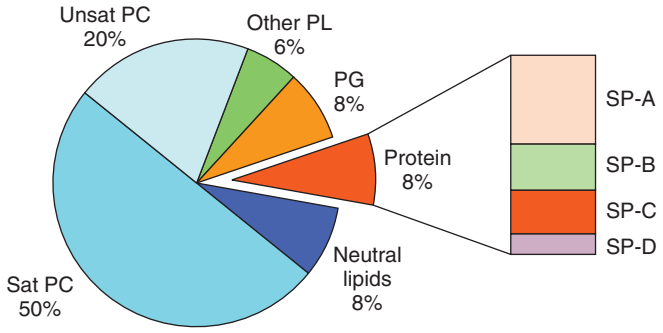

FIGURE 4-1. The composition of surfactant. Saturated phosphatidylcholines are the major components of alveolar surfactant. The proteins contribute about 8% to the weight of surfactant.

SURFACTANT METABOLISM AND SECRETION

The synthesis and secretion of surfactant by the type II cell is a complex sequence that results in the release of lamellar bodies to the alveolus by exocytosis.[20] Enzymes within the endoplasmic reticulum use glucose, phosphate, and fatty acids as substrates for phospholipid synthesis. The details of how the surfactant components condense with SP-B and SP-C to form the surfactant lipoprotein complex within lamellar bodies remain obscure. Ultrastructural abnormalities of type II cells in full-term infants with SP-B deficiency and ABCA3 deficiency indicate that these gene products are essential for lamellar body formation.[21] A basal rate of surfactant secretion occurs continuously, and surfactant secretion can be stimulated by β-agonists and purines, or by lung distention and hyperventilation.

The alveolar pool size of surfactant is about 4 mg/kg in the adult human.[22] The lung tissue of the adult human contains much more surfactant, and only about 7% of the surfactant lipids are in the secreted pool. The surfactant pool size per kilogram probably changes little with age after the newborn period. While no estimates exist for the full-term human, full-term animals have alveolar pool sizes of about 100 mg/kg, and this large pool decreases to adult values by about 1 week of age.[23] The alveolar surfactant pool size in the adult (and presumably young child) is small relative to other mammalian species (e.g., about 30 mg/kg in adult sheep), which may make the human lung more susceptible to surfactant deficiency with lung injury. Infants with respiratory distress syndrome (RDS) have alveolar surfactant pool sizes of less than 5 mg/kg.

The kinetics of surfactant metabolism have been extensively studied in adult, term, and preterm animal models.[24] In all species studied to date, including primates, the surfactant component synthesis to secretion interval is relatively long and the alveolar half-life of newly secreted surfactant is very long, on the order of 6 days in healthy newborn lambs.[25] The surfactant components are recycled back into type II cells, and recycling is more efficient in newborn than adult animals.[26] These observations have been validated by extensive studies in preterm and term humans using stable isotopes to label surfactant precursors or components.[27] A limitation of the studies is the need to have an endotracheal tube in place to allow repetitive sampling of lung fluid. Depending on the labeled precursor, the time from synthesis to peak secretion ranged from 2 to 3 days, and the half-life for clearance was 2 to 4 days in preterm infants. Similar values were measured for term infants. In general, preterm or term infants with lung disease have surfactant with smaller pool sizes, less synthesis and secretion, and shorter half-life values. These measurements include term infants with pneumonia, meconium aspiration syndrome, and congenital diaphragmatic hernia. There are no measurements of surfactant metabolism for older children. In one report in normal adults using sputum samples, peak labeling of surfactant phosphatidylcholine occurred about 2 days after the labeled precursor was given, and the subsequent half-life was about 7 days.[28] These studies demonstrate that replacement of endogenous surfactant pools is slow and alveolar pools turn over slowly.

ALVEOLAR LIFE CYCLE OF SURFACTANT

After secretion, surfactant goes through a series of form transitions in the airspace (Figure 4-2).[20] The lamellar bodies unravel to form the elegant structure called *tubular myelin*. This lipoprotein array has SP-A at the corners of the lattice and requires at least SP-A, SP-B, and the phospholipids for its unique structure.[29] Tubular myelin and other large surfactant lipoprotein structures are the reservoir in the fluid hypophase for the formation of the surface film within the alveolus and small airways. The hypophase is a very thin fluid layer covering the distal epithelium with a volume of about 0.5 mL/kg body weight that has a surfactant concentration of perhaps 10 mg/mL. New surfactant enters the surface film, and "used" surfactant leaves in the form of small vesicles. The surface-active tubular myelin contains SP-A, SP-B, and SP-C, while the biophysically inactive small vesicles that are recycled and catabolized contain very little protein. The total surfactant pool size is less than the amount of active surfactant because 30% to 50% of the alveolar phospholipids are in catabolic forms in the normal lung. Pulmonary edema and products of lung injury can accelerate form conversion and cause a depletion of the surface-active fraction of surfactant despite normal or high total surfactant pool sizes.[30] Surfactant is catabolized primarily by type II cells and alveolar macrophages. Granulocyte-macrophage colony-stimulating factor deficiency prevents alveolar macrophages from catabolizing surfactant and results in the clinical syndrome of alveolar proteinosis.[31] The important concept is that the alveolar pool of functional surfactant is maintained by dynamic metabolic processes that include secretion, reuptake, and resecretion balanced by catabolism.

SURFACTANT FUNCTION

Alveolar Stability

Alveoli are polygonal with flat surfaces and curvatures where the walls of adjacent alveoli intersect. Alveoli are interdependent in that their structure is determined by the shape and elasticity of neighboring alveolar walls.

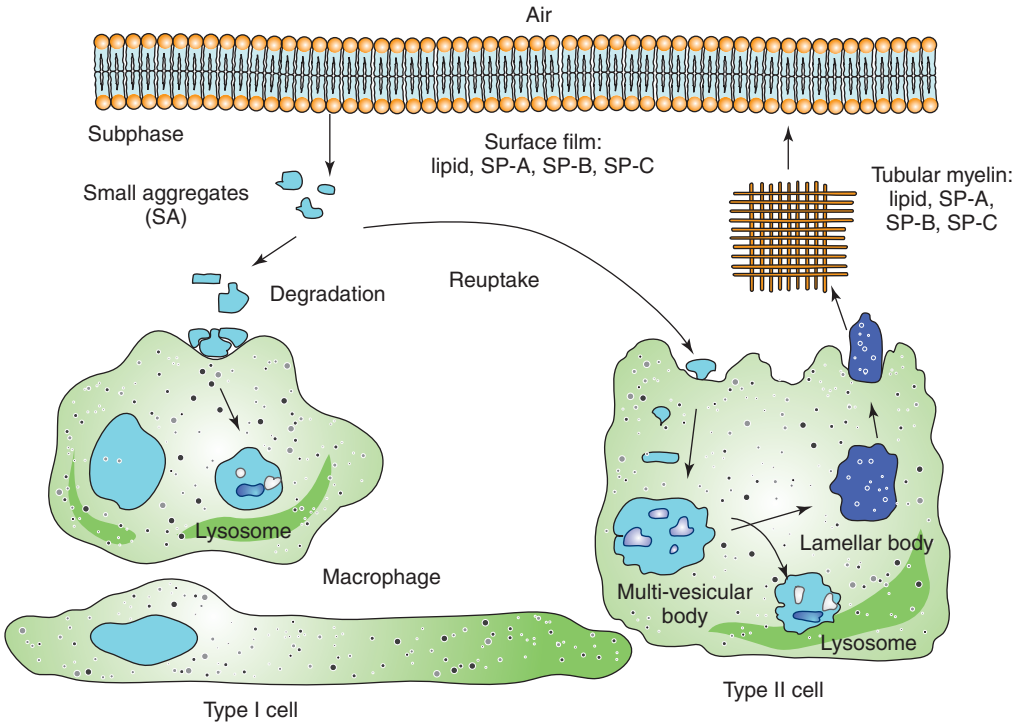

FIGURE 4-2. The alveolar life cycle of surfactant. Surfactant is secreted from lamellar bodies in type II cells. In the alveolar fluid lining layer, the surfactant transforms into tubular myelin and other surfactant protein–rich forms that facilitate surface adsorption. The lipids are catabolized as small vesicular forms by macrophages and type II cells and recycled by type II cells.

The forces acting on the pulmonary microstructure are chest wall elasticity, lung tissue elasticity, and surface tensions of the air-fluid interfaces of the small airways and alveoli. Although the surface tension of surfactant decreases with surface area compression and increases with surface area expansion, the surface area of an alveolus changes little with tidal breathing. The low surface tensions resulting from surfactant help to prevent alveolar collapse and keep interstitial fluid from flooding the alveoli. Surfactant also keeps small airways from filling with fluid and thus prevents the potentially ensuing luminal obstruction.[32] If alveoli collapse or fill with fluid, the shape of adjacent alveoli will change, which can result in distortion, overdistention, or collapse. When positive pressure is applied to a surfactant-deficient lung, the more normal alveoli will tend to overexpand and the alveoli with inadequate surfactant will collapse, generating a nonhomogeneously inflated lung.

Pressure-Volume Curves

The static effects of surfactant on a surfactant-deficient lung are evident from the pressure-volume curve of the preterm lung (Figure 4-3). Preterm surfactant-deficient rabbit lungs do not begin to inflate until pressures exceed 20 cm H_2O.[33] The pressure needed to open a lung unit is related to the radius of curvature and surface tension of the meniscus of fluid in the airspace leading to the 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. Surfactant decreases the opening pressure from greater than 20 to 15 cm H_2O, in this example, with preterm

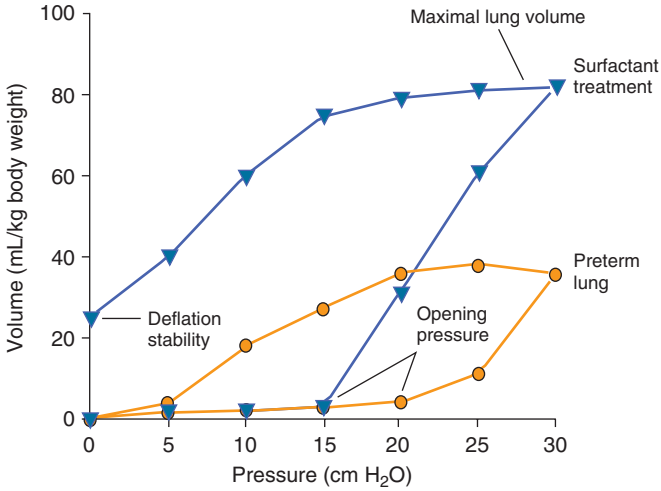

FIGURE 4-3. The effect of surfactant treatment on surfactant-deficient lungs. These idealized pressure-volume curves illustrate the effect of surfactant treatment with natural sheep surfactant on the opening pressure, the maximal lung volume, and the deflation stability of lungs from preterm rabbits. (Curves based on data from Rider ED, Jobe AH, Ikegami M, et al. Different ventilation strategies alter surfactant responses in preterm rabbits. *J Appl Physiol.* 1992;73:2089–2096.)

rabbit lungs. Because surfactant does not alter airway diameter, the decreased opening pressure results from surface adsorption of the surfactant to the fluid in the airways. The inflation is more uniform as more units open at lower pressures, resulting in 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 30 cm H_2O is increased over two times with

surfactant treatment. Surfactant also stabilizes the lung on deflation. The surfactant-deficient lung collapses at low transpulmonary pressures, whereas the surfactant-treated lung retains about 30% of the lung volume on deflation. This retained volume is similar to the total volume of the surfactant-deficient lung at 30 cm H_2O and demonstrates how surfactant treatments increase the functional residual capacity of the lung.

HOST DEFENSE FUNCTIONS OF SURFACTANT

SP-A and SP-D are pattern recognition molecules that bind a variety of polysaccharides, phospholipids, and glycolipids on the surface of bacterial, viral, and fungal pathogens.[4,5] SP-A and SP-D binding forms protein bridges between microbes that induce microbial aggregation and stimulate the recognition, uptake, and clearance of pathogens by host defense cells.[34,35] Although binding and aggregation of infectious microbes is a critical feature of SP-A and SP-D physiology, these proteins also have more complex roles in host defense.

SP-A and SP-D have been implicated in the stimulation and inhibition of several immune pathways. Both SP-A and SP-D bind CD14 and inhibit lipopolysaccharide-induced expression of pro-inflammatory cytokines through CD14 and toll-like receptor 4.[36–38] SP-A binds toll-like receptor 2 and inhibits pro-inflammatory cytokine release in response to peptidoglycan.[39] Gardai and colleagues proposed a model by which SP-A and SP-D might stimulate or inhibit inflammation through the competing actions of signal regulating protein α (SIRPα) and calreticulin/CD91.[40] Their model suggests that in the unbound state, the CRDs of SP-A or SP-D inhibit macrophage activation by binding to SIRPα, which inhibits activation of NFκB. In contrast, if the CRDs of SP-A or SP-D are occupied by a microbial ligand, binding to SIRPα is inhibited and instead the collectins bind to the macrophage-activating receptor, calreticulin/CD91, which turns on NFκB and subsequently induces pro-inflammatory mediator release and alveolar macrophage activation. SP-A also may contribute to adaptive immune responses. SP-A inhibits the maturation of dendritic cells in response to potent T-cell stimulators and enhances the endocytic ability of dendritic cells. In addition, SP-A downregulates lymphocyte activity and proliferation.[41]

The hydrophobic surfactant proteins SP-B and SP-C may also have host defense functions. Although SP-B can inhibit bacterial growth *in vitro,* overexpression of SP-B or reduced expression of SP-B in the lungs of mice does not alter bacterial clearance, suggesting that SP-B is not involved in innate host defense.[42] However, elevated levels of SP-B in the lungs of endotoxin-exposed mice decrease pulmonary inflammation.[43] Thus, SP-B may contribute to modulation of inflammation in the injured lung. SP-C binds lipopolysaccharide and blocks the production of tumor necrosis factor-α by macrophages.[44] However, possible roles for SP-C in bacterial clearance or lung inflammation *in vivo* have not been evaluated.

SURFACTANT DEFICIENCY

The Preterm Infant with Respiratory Distress Syndrome

RDS in preterm infants is a condition of surfactant deficiency that initially does not include lung injury, unless antenatal infection complicates the lung disease.[45] The surfactant system is normally mature by about 35 weeks' gestation, but early appearance of surfactant and lung maturation is observed in infants delivered prematurely. Early maturation is thought to occur in response to fetal stress resulting in increased fetal cortisol levels, or by exposure of the fetal lung to inflammation as a result of chorioamnionitis.[46] Maternal treatments with corticosteroids are routinely given to decrease the risk of RDS if delivery before 32 to 34 weeks gestation is anticipated.[47] Induced lung maturation includes not only an induction of surfactant but also thinning of the mesenchyme, which increases lung gas volumes. Unless preterm infants have early lung maturation, they develop progressive respiratory distress from birth characterized by tachypnea, grunting, an increased work of breathing, and cyanosis. Infants who die from RDS have alveolar pool sizes of surfactant of less than 5 mg/kg. Although similar in amount to the surfactant recovered from healthy adult humans, surfactant from the preterm infant has decreased function, probably because it contains less of the surfactant proteins that are critical for biophysical function.[48] The surfactant from the preterm infant also is more susceptible to inactivation by edema fluid, and the preterm lung is easily injured if a stable functional residual capacity (FRC) is not maintained, or if the lung is overstretched.

THE INJURED MATURE LUNG

Acute respiratory distress syndrome (ARDS) describes an overwhelming inflammatory reaction within the pulmonary parenchyma leading to global lung dysfunction.[49] ARDS is defined by acute onset, an oxygenation index less than 200 mm Hg, bilateral infiltrates on chest x-ray, and a pulmonary capillary wedge pressure of less than 18 mm Hg or absence of clinical evidence for left-sided heart failure (see Chapter 39). The etiology of ARDS is multifactorial and can occur in association with lung injury secondary to trauma, sepsis, aspiration, pneumonia, massive blood transfusions, or near drowning to name some associations. It is a common disease, affecting roughly 15% to 20% of all patients ventilated in the adult intensive care unit (ICU) and 1% to 4.5% of patients in the pediatric ICU. ARDS has a mortality rate of 25% to 50%.

Impairment of surfactant with ARDS can result from inhibition, degradation, or decreased production.[30,50,51] The proteinaceous pulmonary edema characteristic of ARDS can inactivate surfactant by dilution and by competition for the interface. Plasma proteins known to inhibit surfactant function include serum albumin, globulin, fibrinogen, and C-reactive protein. In addition to proteins, phospholipases (along with their products), fatty acids, and lipids inhibit surface activity. Epithelial cell injury by inflammatory mediators can decrease surfactant

production and contribute to surfactant deficiency. Normally in the lung, about 50% of surfactant is present in the bioactive form that has a high SP-B and SP-C content. In ARDS, small vesicular forms increase and the pool of active surfactant is depleted.

The phospholipid content is decreased and the phospholipid composition is abnormal in bronchoalveolar lavage fluid (BALF) from patients with ARDS.[52] SP-A, SP-B, and SP-C are also decreased in BALF from patients with ARDS. The surfactant protein levels can remain low for at least 14 days after the onset of ARDS. Changes in surfactant composition including phospholipids, fatty acids, and proteins likely represent alveolar type II cell injury with altered metabolism, secretion, or recycling of components. SP-A and SP-B concentrations are also reduced in the lungs of patients at risk for ARDS, even before lung injury is clinically apparent. In contrast, SP-D levels in BALF were shown to remain normal, except in a subgroup of patients who later died. Decreased SP-D levels in BALF were 85.7% sensitive and 74% specific in predicting death with ARDS.[53]

GENETIC DEFICIENCIES OF SURFACTANT IN MICE AND HUMANS

Mice with targeted deletion of the *Sftpa* gene (*Sftpa*$^{-/-}$) survive normally without changes in surfactant composition, function, secretion, and reuptake; however, there is no tubular myelin.[54] Although seemingly normal at baseline, significant defects are detected in pulmonary host defense in SP-A–deficient mice when they were subjected to a microbial challenge. Clearance of group B *Streptococcus, Haemophilus influenzae,* respiratory syncytial virus (RSV), and *Pseudomonas aeruginosa* is delayed in *Sftpa*$^{-/-}$ mice and the recognition and uptake of bacteria by alveolar macrophages are deficient.[55–57] Oxygen radical production and killing of engulfed microorganisms by *Sftpa*$^{-/-}$ macrophages are markedly reduced, while markers of lung inflammation are increased following infection in *Sftpa*$^{-/-}$ mice.[58]

Despite the considerable innate immune defects that are associated with SP-A deficiency in animal models, we have yet to find a human susceptibility to pulmonary infection that is caused by an *Sftpa* mutation. However, polymorphisms (genetic variants) in the human genes for SP-A, which affect their function, have been identified, and humans with these polymorphisms have increased susceptibility to infections with RSV and Mycobacterium tuberculosis.[59] Analyses suggest that SP-A polymorphisms may also affect infection severity since young children with RSV infection who are homozygous or heterozygous for asparagine at the amino acid position 9 are more likely to need intensive care, mechanical ventilation, or longer hospitalization.[50] Although there are no clear associations between *Sftpa* mutation and pulmonary infection, a recent study reported an association between familial pulmonary fibrosis and two heterozygous mutations in the *Sftpa* gene that caused SP-A misfolding and trapping of SP-A in the endoplasmic reticulum.[60] The extent to which these and other genetic variants will serve as clinically useful predictors of risk will require more analysis.

Mice with deletion of the *Sfptd* gene (*Sftpd*$^{-/-}$) survive normally, but unlike SP-A–deficient mice that have relatively normal lungs at baseline, *Sftpd*$^{-/-}$ mice spontaneously develop pulmonary inflammation and airspace enlargement. In addition, *Sftpd*$^{-/-}$ mice accumulate increased numbers of apoptotic macrophages, and enlarged, foamy macrophages that release reactive oxygen species and metalloproteinases.[61,62] When *Sftpd*$^{-/-}$ mice are exposed to a microbial challenge, the uptake and clearance of viral pathogens including influenza A and RSV are deficient, whereas the clearance of group B *Streptococcus* and *Haemophilus influenzae* is unchanged.[63,64] However, oxygen radical release and production of the proinflammatory mediators are increased in *Sftpd*$^{-/-}$ mice when exposed to either viral or bacterial pathogens indicating that SP-D plays an anti-inflammatory role in the lung, independent of the clearance of pathogens.[58,63,64] SP-D deficiency has not been described in humans, but polymorphisms at amino acid position 11 are associated with increased risk of RSV infection.[65]

Gene-targeted mice lacking SP-B and infants with hereditary SP-B deficiency demonstrate the critical role of SP-B in surfactant function, homeostasis, and lung function.[66] Targeted disruption of the mouse SP-B gene causes respiratory failure at birth. Despite normal lung structure, the mice fail to inflate their lungs postnatally. Type II cells of SP-B–deficient mice have large multivesicular bodies but no lamellar bodies, and the proteolytic processing of pro-SP-C (the preprocessed form of SP-C) is disrupted.[4] Infants with SP-B deficiency die from respiratory distress in the early neonatal period with the same pathologic findings.[67] Mutations leading to partial SP-B function have been associated with chronic lung disease in infants. Because SP-B is required for both intracellular and extracellular aspects of surfactant homeostasis, SP-B deficiency has not been treated successfully with surfactant replacement therapy and survival is dependent on lung transplantation. It is important to note that mice and infants without the adenosine triphosphate–binding cassette transporter A3 (ABCA3) have type II cells without lamellar bodies and the same lethal respiratory failure phenotype as observed in SP-B deficiency.[68]

SP-C–deficient mice survive and have normal surfactant composition and amounts. However, surfactant isolated from SP-C–deficient mice forms less stable bubbles, demonstrating a role for SP-C in developing and maintaining lipid films.[69] SP-C mutations recently were identified in patients with familial and sporadic interstitial lung disease.[70] In these patients, *Sftpc* mutations alter the ability of the protein to fold correctly and result in the retention of SP-C in the endoplasmic reticulum and the subsequent development of endoplasmic reticulum stress, which, in turn, leads to pulmonary cell injury and death. Histological features of lung disease in these individuals include lungs with a thickened interstitium, infiltration with inflammatory cells and macrophages, fibrosis, and abnormalities of the respiratory epithelium.

SURFACTANT TREATMENT OF SURFACTANT DEFICIENCY

Respiratory Distress Syndrome

The respiratory morbidities of preterm infants with RDS have decreased strikingly in recent years because of the combined effects of antenatal corticosteroid treatments on lung maturation and more gentle approaches to mechanical ventilation.[71] The original randomized trials of surfactant for RDS evaluated treatments given after the disease was established, generally after 6 hours of age.[72] Other trials evaluated treatment of all high-risk infants soon after birth to prevent RDS. Subsequent trials demonstrated that treatments of the highest-risk infants (generally infants with birth weights less than 1 kg) as soon after birth as convenient, and before significant mechanical ventilation, will minimize lung injury. However, many very low birth weight infants can be transitioned to air breathing successfully using continuous positive airway pressure (CPAP), and the decision to treat with surfactant can be made after the initial stabilization at birth.[73,74] An advantage of allowing the infant to breathe spontaneously with CPAP used to recruit and maintain FRC is that hyperventilation and overdistention of the delicate preterm lung can be avoided. Larger infants who develop RDS are generally treated with oxygen and nasal CPAP until the inspired oxygen concentration approaches 40%. They then are treated with surfactant. Preterm infants will respond to surfactant treatments even if the treatment is delayed for several days.

Full-term infants with severe meconium aspiration or pneumonia also will respond to surfactant treatments with improved oxygenation.[75] Surfactant also can improve lung function in infants with the group B streptococcal sepsis/pneumonia syndrome.[76] Current practice is to treat most infants with severe respiratory failure with surfactant because there are no contraindications.

The surfactants that are commercially available for clinical use in infants are made from organic solvent extracts of animal lungs or alveolar lavages of animal lungs. While there are differences in composition, the clinical results do not demonstrate any compelling differences in clinical responses. All of the commercial surfactants lack SP-A, contain SP-C, and have variable amounts of SP-B. Surfactants that contain synthetic peptides or surfactant proteins are being developed for clinical use.

Acute Respiratory Distress Syndrome

ARDS is a significant therapeutic challenge for intensivists despite recent advances in the understanding of its pathophysiology and new treatment modalities. Surfactant content and composition are altered in ARDS, resulting in decreased surface activity, atelectasis, and decreased lung compliance.[51] The injury is generally not uniform throughout the lung, resulting in overinflation of more normal lung and atelectasis and filling of alveoli with fluid in other lung regions. The injured lung makes less surfactant, surfactant is inhibited by the highly proteinaceous edema and inflammatory fluid, and the fluid-filled alveoli are difficult to recruit to improve ventilation. Multiple animal models of ARDS respond very positively to surfactant treatments when combined with lung recruitment ventilation strategies. Unfortunately, multiple large randomized controlled trials using different surfactants have not shown clinical benefit in humans.[77,78] Recent trials have evaluated surfactant treatment of selective causes for ARDS, but again with no overall benefit.[79]

The experience in adult patients with ARDS differs strikingly with the clinical responses of preterm infants with RDS. Somewhere in between are the clinical responses of term infants with meconium aspiration and pneumonia who have modest but consistent clinical improvements that can decrease ECMO use and save lives.[80] Several small trials and clinical experiences have suggested that older infants and children with diseases such as acute RSV pneumonia respond to surfactant treatment. A trial by Willson and colleagues[81] demonstrated that, for a range of children from 1 to 21 years of age with various causes of ventilator dependent ARDS, surfactant treatments improved oxygenation and decreased mortality. Future studies of surfactant intervention for ARDS should be refined to better define which populations benefit from surfactant treatment. Future studies also can explore the potential for surfactant components to enhance host defense in diseases such as ARDS.

Suggested Reading

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6 BIOLOGY AND ASSESSMENT OF AIRWAY INFLAMMATION

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INTRODUCTION

Inflammation is classically characterized by four cardinal signs: *calor* and *rubor* (due to vasodilatation), *tumor* (due to plasma exudation and edema), and *dolor* (due to sensitization and activation of sensory nerves. Inflammation is also characterized by infiltration with inflammatory cells, and these will differ depending on the type of inflammatory process. It is vital to recognize that inflammation is an important response that defends the body against invasion from microorganisms and the effects of external toxins. Failure of the components of the inflammatory response (e.g., neutrophil dysfunction, also known as Job's syndrome) has catastrophic consequences. Allergic inflammation is characterized by the fact that it is driven by exposure to allergens through IgE-dependent mechanisms, resulting in a characteristic pattern of inflammation.[1] The inflammatory response seen in allergic diseases is characterized by an infiltration with eosinophils and resembles the inflammatory process mounted in response to worm and other parasitic infections. The inflammatory response not only provides an acute defense against injury, but it is also involved in the healing and restoration of normal function after tissue damage from infection and toxins. In allergic disease, the inflammatory response is activated inappropriately and is harmful rather than beneficial. For some reason, allergens such as house dust mite and pollen proteins, activate eosinophilic inflammation, possibly as a result of their protease activity. Normally such an inflammatory response would kill the invading parasite (or the parasite would overwhelm the host) and the process would therefore be self-limiting, but in allergic disease the inciting stimulus persists and the normally acute inflammatory response turns into chronic inflammation, which may have structural consequences in the airways and skin. Cystic fibrosis (CF) bronchiectasis and persistent bacterial bronchitis are characterized by a neutrophilic pattern of inflammation, driven in part by chronic bacterial infection; the pathophysiology is covered in more detail in Chapters 26, 30, and 52. In this chapter, we place the most emphasis on allergic inflammation, as this underlies the most common noninfectious respiratory diseases of children.

ACUTE INFLAMMATION

Acute inflammation in the respiratory tract is an immediate defense reaction to inhaled allergens, pathogens, or noxious agents. Inhalation of an allergen (e.g., house dust mites) activates surface mast cells by an IgE-dependent mechanism. This releases multiple bronchoconstrictor mediators, resulting in rapid contraction of airway smooth muscle and wheezing. These mediators also result in plasma exudation and swelling of the airways and recruitment of inflammatory cells from the circulation—particularly eosinophils, neutrophils (transiently), and T-lymphocytes, mainly of the T helper 2 (Th2) type. This accounts for the late response that occurs 4 to 6 hours after allergen exposure and resolves within 24 hours, which should be regarded as an acute inflammatory reaction. The acute inflammatory response in the respiratory tract is usually accompanied by increased mucus secretion, which is a part of the defense system that protects the delicate mucosal surface of the airways. In CF, mucus secretion is a highly significant part of the airway pathology, in part mediated by the inflammatory response.

Chronic Inflammation

The normal consequence of an acute inflammatory process is complete resolution; for example, acute lobar pneumonia due to pneumococcal infection is characterized by a massive influx of neutrophils, with complete resolution and restoration of normal lung structure (unless the patient dies in the acute phase of the infection). Many inflammatory conditions of the respiratory tract are chronic and may persist for many years. This inflammation may persist even in the absence of causal mechanisms. This is well illustrated in patients with occupational asthma who continue to have asthma despite complete avoidance of sensitizing agents, and in adult patients with chronic obstructive pulmonary disease who have continued inflammation, even after stopping smoking for many years. The resolution of inflammation was previously thought to be a passive process, but it is now realized that there are important active control mechanisms. There are a number of potential mechanisms that are important in the normal resolution of inflammation. These include Interleukin-10 (IL-10),[2,3] CD200,[4,5] Annexin,[6] lung Kruppel-like factor (LKLF),[7] lipid mediators such as Resolvin E1 (RvE1) and Lipoxin A_4 (LXA_4), interferon (IFN)-γ, the IL-23 axis,[8,9] and Protectin D1 (PD1).[10] These mediators and regulators are discussed in more detail in the following paragraphs. The molecular and cellular mechanisms for the persistence of inflammation in the absence of its original causal mechanisms are not understood, but presumably involve some type of long-lived immunologic memory that drives the inflammatory process. Structural cells, such as airway epithelial cells that make up the airway wall, may also drive

the chronic inflammatory process. This is an important area of research, as understanding these mechanisms might lead to potentially curative therapies.

Structural Changes and Repair

The acute inflammatory response is usually followed by a repair process that restores the tissue to normal. This may involve proliferation of damaged cells (e.g., airway epithelial cells) and fibrosis to heal any breach in the mucosal surface. These repair processes may also become chronic in response to continued inflammation, resulting in structural changes in the airways that are referred to as *remodeling*.[11] However, it should be noted that the relationship between airway inflammation and remodeling is controversial; the conventional view—that inflammation leads to remodeling—has been challenged by human and animal work, which suggests that they may be parallel processes.[12–14] These structural changes in asthma and CF may result in irreversible narrowing of the airways, with a fixed obstruction to air flow. In asthma, several structural changes are found in the airway wall, including fibrosis, an increased amount of airway smooth muscle, and an increased number of blood vessels (angiogenesis). There is much debate about the importance of airway remodeling in asthma as it is not seen in all patients. It may contribute to airway hyperresponsiveness (AHR) in asthma, but it may also have some beneficial effects in limiting airway closure.[15]

INFLAMMATORY CELLS

Many types of inflammatory cells are involved in airway inflammation, although the precise roles of each cell type and the interrelationship among cells is not yet clear (Figure 6-1). The inflammatory mechanisms in early wheeze, especially episodic (viral) wheeze, are little studied, but probably differ from those seen in multiple trigger wheeze (asthma). The evidence in episodic (viral) wheeze shows that the pattern is neutrophilic.[16–19] In children with asthma, the same kind of inflammation is seen in bronchial biopsies as in adults, which indicates that similar inflammatory mechanisms are likely.[13,20–24] Of note, the inflammatory pattern seen at bronchoscopy is the same in children with multiple trigger (asthmatic) wheeze, independent of their atopic status.[25] No single inflammatory cell accounts for the complex pathophysiology of asthma, although some cells predominate in allergic inflammation. The pattern of inflammation in CF is different, and it results in different pathophysiologic consequences and different responses to therapy. It should be noted also that inflammation may vary in different compartments

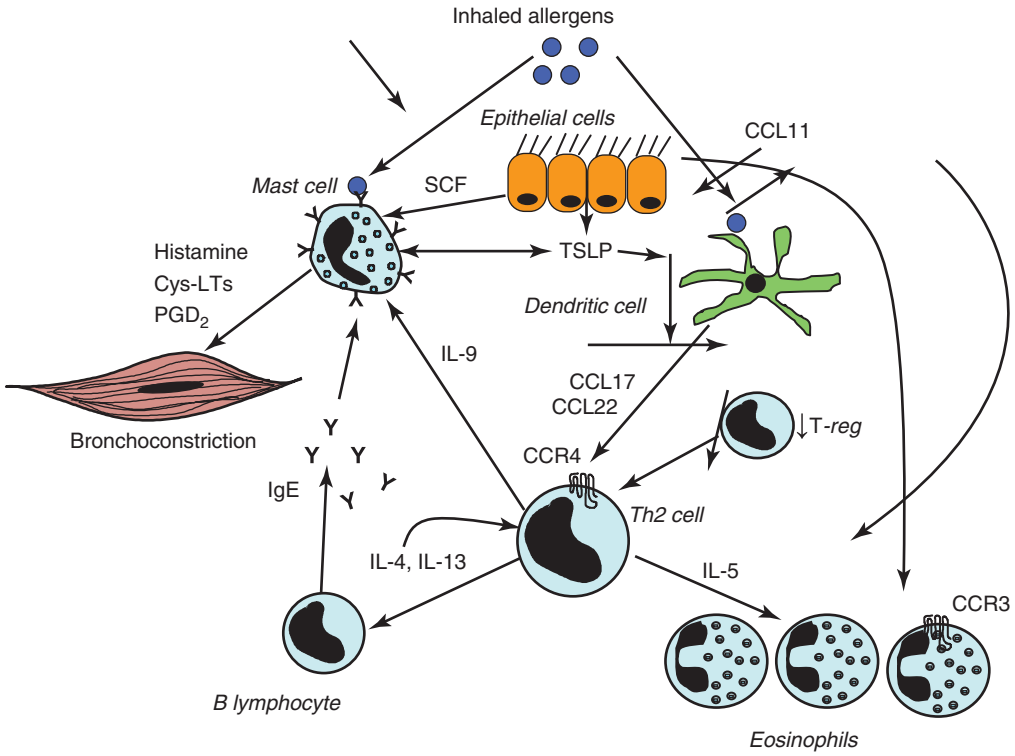

FIGURE 6-1. Inflammation in asthma. Inhaled allergens activate sensitized mast cells by cross-linking surface-bound IgE molecules to release several bronchoconstrictor mediators, including cysteinyl-leukotrienes (*cys*-LT) and prostaglandin D_2 (PGD_2). Epithelial cells release stem-cell factor (SCF), which is important for maintaining mucosal mast cells at the airway surface. Allergens are processed by myeloid dendritic cells, which are conditioned by thymic stromal lymphopoietin (TSLP) secreted by epithelial cells and mast cells to release the chemokines CC-chemokine ligand 17 (CCL17) and CCL22, which act on CC-chemokine receptor 4 (CCR4) to attract T helper 2 (Th2) cells. Th2 cells have a central role in orchestrating the inflammatory response in allergy through the release of interleukin-4 (IL-4) and IL-13 (which stimulate B cells to synthesize IgE), IL-5 (which is necessary for eosinophilic inflammation) and IL-9 (which stimulates mast-cell proliferation). Epithelial cells release CCL11, which recruits eosinophils via CCR3. Patients with asthma may have a defect in regulatory T cells (T-reg), which may favor further Th2-cell proliferation.

of the lung. In adults with asthma, transbronchial biopsy has shown evidence of very distal inflammation in the absence of proximal airway inflammation.[26–28] There is a dissociation between airway mucosal and airway luminal inflammatory patterns in asthma.[29] In CF, whereas the predominant inflammatory cell in the airway lumen is the neutrophil (as shown by sputum and bronchoalveolar lavage cytology), T-lymphocytes predominate in the proximal airway wall.[30]

Mast Cells

Mast cells are important in initiating the acute bronchoconstrictor responses to allergens and probably to other indirect stimuli such as exercise and hyperventilation (via osmolality or thermal changes) and fog. Treatment of asthmatic patients with prednisone results in a decrease in the number of tryptase-positive mast cells. Furthermore, mast cell tryptase appears to play a role in airway remodeling, as this mast cell product stimulates human lung fibroblast proliferation. Mast cells also secrete cytokines, including IL-4 and eotaxin, which may be involved in maintaining the allergic inflammatory response, and tumor necrosis factor-α (TNF-α).[31] Mast cells are found in increased numbers in airway smooth muscle of asthmatic patients, and this appears to correlate with AHR, suggesting that mast cell mediators mediate AHR.[32]

However, there are questions about the role of mast cells in more chronic allergic inflammatory events, and it seems more probable that other cells, such as macrophages, eosinophils, and T-lymphocytes, are more important in the chronic inflammatory process and in AHR. Classically, mast cells are activated by allergens through an IgE-dependent mechanism. The importance of IgE in the pathophysiology of asthma has been highlighted by recent clinical studies with humanized anti-IgE antibodies, which inhibit IgE-mediated effects. Anti-IgE therapy is effective in patients, including children, with severe asthma who are not well controlled with high doses of corticosteroids, and it is particularly effective in reducing exacerbations.[33] The role of IgE in the treatment of severe asthma is discussed in Chapter 48.

Macrophages

Macrophages, which are derived from blood monocytes, traffic into the airways in inflammatory diseases under the direction of specific chemokines. In the airways, these monocytes differentiate into macrophages, which have the capacity to secrete many inflammatory proteins, chemotactic factors, lipid mediators, and proteinases. In asthma, they may be activated by allergen via low-affinity IgE receptors ($Fc_{\varepsilon}RII$). The enormous immunologic repertoire of macrophages allows these cells to produce more than 100 different products, including a large variety of cytokines that may orchestrate the inflammatory response. Macrophages have the capacity to initiate a particular type of inflammatory response via the release of a certain pattern of cytokines. Macrophages may both increase and decrease inflammation, depending on the stimulus. Alveolar macrophages normally have a *suppressive* effect on lymphocyte function, but this may be impaired in asthma after allergen exposure. In patients with asthma, there is a reduced secretion of IL-10 (an anti-inflammatory protein secreted by macrophages) in alveolar macrophages. Macrophages may therefore play an important anti-inflammatory role by preventing the development of allergic inflammation. There may be subtypes of macrophages that perform different inflammatory, anti-inflammatory, or phagocytic roles in airway disease, but at present it is difficult to differentiate these subtypes. There is evidence that alveolar macrophages show reduced phagocytosis of apoptotic cells and carbon particles in severe asthma so that inflammation does not resolve.[34,35]

Dendritic Cells

Dendritic cells are specialized macrophage-like cells that have a unique ability to induce a T-lymphocyte–mediated immune response and therefore play a critical role in the development of asthma.[36] There are three major lineages of dendritic cells: myeloid DCs (mDCs),[37] plasmacytoid DCs (pDCs),[38] and Langerhans cells (LCs).[39] Dendritic cells in the respiratory tract form a network that is localized to the epithelium, and they act as very effective antigen-presenting cells. It is likely that dendritic cells play an important role in the initiation of allergen-induced responses in asthma. Dendritic cells take up allergens, process them to peptides, and migrate to local lymph nodes where they present the allergenic peptides to uncommitted T-lymphocytes. With the aid of co-stimulatory molecules (e.g., B7.1, B7.2, and CD40), they program the production of allergen-specific T cells. Animal studies have demonstrated that myeloid dendritic cells are critical to the development of T helper type 2 (Th2) cells and eosinophilia.

Eosinophils

Eosinophilic infiltration is a characteristic feature of allergic inflammation. Allergen inhalation results in a marked increase in eosinophils in bronchoalveolar lavage fluid at the time of the late reaction, and there is a correlation between eosinophil counts in peripheral blood or bronchial lavage and AHR. Eosinophils are linked to the development of AHR through the release of basic proteins and oxygen-derived free radicals.[40] Several mechanisms are involved in *recruitment* of eosinophils into the airways. Eosinophils are derived from bone marrow precursors, and the signal for increased eosinophil production is presumably derived from the inflamed airway. Eosinophil recruitment initially involves adhesion of eosinophils to vascular endothelial cells in the airway circulation, their migration into the submucosa, and their subsequent activation. The role of individual adhesion molecules, cytokines, and mediators in orchestrating these responses has been extensively investigated. Adhesion of eosinophils involves the expression of specific glycoprotein molecules on the surface of eosinophils (integrins) and expression

of such molecules as intercellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells. The adhesion molecule very late antigen-4 (VLA4) expressed on eosinophils, which interacts with VCAM-1 and IL-4, increases its expression on endothelial cells. GM-CSF and IL-5 may be important for the survival of eosinophils in the airways and for "priming" eosinophils to exhibit enhanced responsiveness.

There are several mediators involved in the migration of eosinophils from the circulation to the surface of the airway. The most potent and selective agents appear to be chemokines (e.g., CCL5, CC11, CCL13, CCL24, and CCL26) that are expressed by epithelial cells. There appears to be a co-operative interaction between IL-5 and chemokines, so that both are necessary for the eosinophilic response in the airway. Once recruited to the airway, eosinophils require the presence of various growth factors, of which GM-CSF and IL-5 appear to be the most important. In the absence of these growth factors, eosinophils may undergo programmed cell death (apoptosis).

After humanized monoclonal antibody to IL-5 is administered to asthmatic patients, there is a profound and prolonged reduction in circulating eosinophils, and eosinophils recruited into the airway following allergen challenge.[41] However, there is no effect on the response to inhaled allergen and no reduction in AHR. Clinical studies with anti-IL-5–blocking antibody showed a similar profound reduction in circulating eosinophils, but no improvement in clinical measures of asthma control. A subsequent study attributed this to a failure to reduce mucosal eosinophilia.[42] Recent studies with highly selected patients with persistent sputum eosinophilia despite high doses of inhaled corticosteroids have shown a reduction in exacerbations.[43,44] These two studies underscore the importance of understanding the differing inflammatory process in subgroups of patients with asthma, rather than applying the same strategies to all patients.

Neutrophils

Neutrophils are the predominant inflammatory cells in patients with CF, and they appear to be involved in severe asthma, when increased numbers of neutrophils are found in the sputum and in bronchial biopsies (Figure 6-2).[45–47] Putative causes for airway neutrophilia are corticosteroid therapy, which inhibits neutrophil apoptosis, chronic infection with atypical organisms such as Chlamydia or Mycoplasma, exposure to passive smoking, and gastroesophageal reflux and aspiration. It is not certain whether these neutrophils play a pathophysiologic role. However, they may generate oxidative stress that could play an important role in the pathophysiology of severe asthma. There is an increase in sputum neutrophils in patients following loss of asthma control.[48]

T-Lymphocytes

T-lymphocytes play a very important role in coordinating the inflammatory response in asthma through the release of specific patterns of cytokines, resulting in the recruitment and survival of eosinophils and in the maintenance of mast cells in the airways.[49] T-lymphocytes are coded to express a distinctive pattern of cytokines, which are similar to that described in the murine T helper 2 (Th2) type of T-lymphocytes, which characteristically express IL-4, IL-5, IL-9, and IL-13 (Figure 6-3). This programming of T-lymphocytes is presumably due to antigen-presenting cells, such as dendritic cells, which may migrate from the epithelium to regional lymph nodes or which interact with lymphocytes resident in the airway mucosa. The naïve immune system is skewed to express the Th2 phenotype; data now indicate that children with atopy are more likely to retain this skewed phenotype than normal children. There is some evidence that early infections or exposure to endotoxins might promote Th1-mediated responses to predominate and that a lack of infection or a clean environment in childhood may favor Th2 cell expression and thus atopic diseases.[50] Indeed, the balance between Th1 cells and Th2 cells is thought to be determined by locally released cytokines, such as IL-12, which tip the balance in favor of Th1 cells, or IL-4 or IL-13, which favor the emergence of Th2 cells. Regulatory T cells (Tregs) suppress the immune response through the secretion of inhibitory cytokines (e.g., IL-10 and TGF-β) and play an important role in immune regulation with suppression of Th1 responses, and there is some evidence that Treg function may be defective in asthmatic patients.[51]

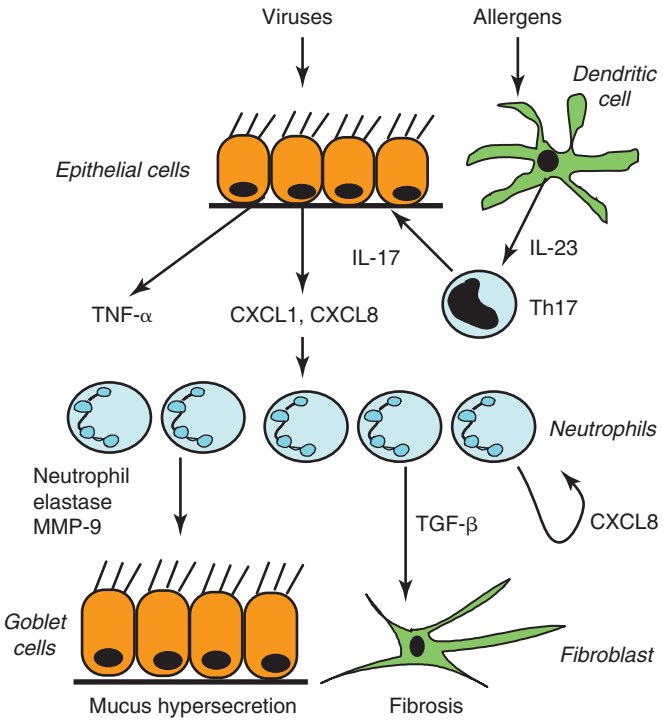

FIGURE 6-2. Neutrophilic inflammation in asthma. Viruses, such as rhinovirus, stimulate the release of CXCL8 and CXCL1 from airway epithelial cells. Allergens activate dendritic cells to release IL-23, which recruits helper T-cells that secrete IL-17 (Th17 cells) to release tumor necrosis factor-α (TNF-α), which amplifies inflammation, and CXCL1 and CXCL8, which recruit neutrophils into the airways. Neutrophils release more CXCL8 and also transforming growth factor-β (TGF-β), which activates fibroblasts to cause fibrosis, and neutrophil elastase and matrix metalloproteinase-9 (MMP-9), which stimulate mucus hypersecretion from goblet cells.

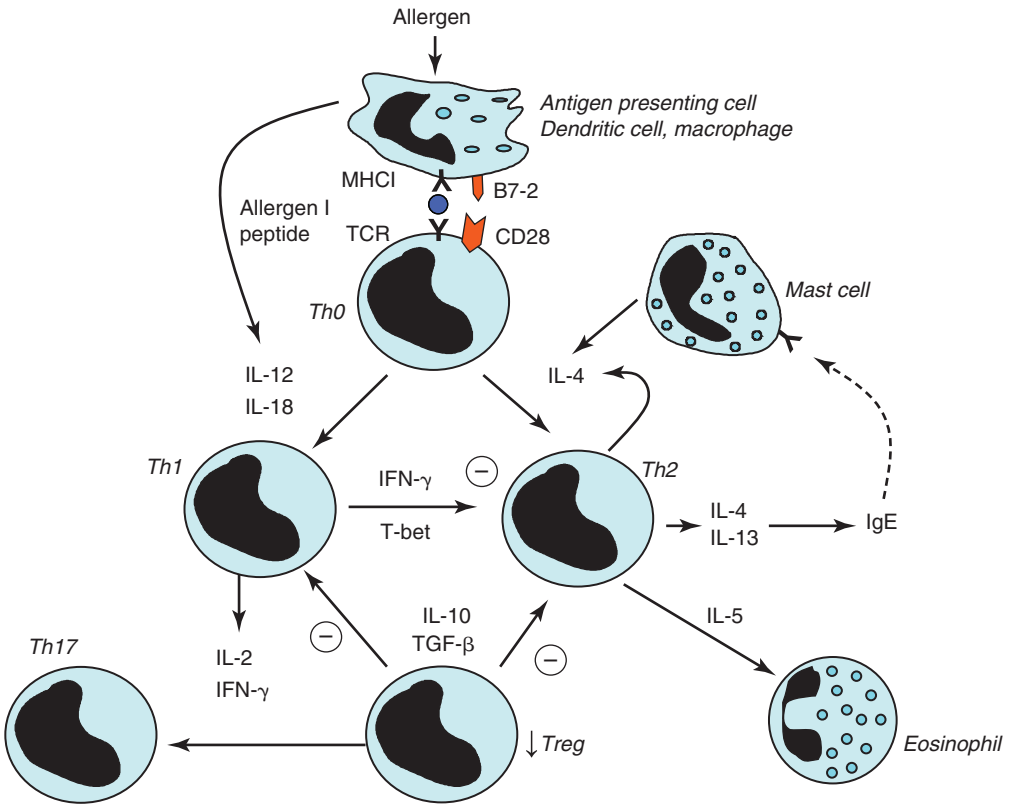

FIGURE 6-3. T lymphocytes in asthma. Asthmatic inflammation is characterized by a preponderance of T helper 2 (Th2) lymphocytes over T helper 1 (Th1) cells. Regulatory T cells (Treg) have an inhibitory effect, whereas T helper 17 (Th17) cells have a pro-inflammatory effect. *MHCI,* Class 1 major histocompatibility complex; *IL,* interleukin; *IFN-γ,* interferon gamma; *TGF-β,* transforming growth factor beta; *IgE,* immunoglobulin E; *Tho,* uncommitted T cell.

Th17 cells are CD4+ cells that predominantly release IL-17 and IL-22 and may be involved in neutrophilic inflammation in severe asthma and CF.[52] In contrast to Th1 and Th2 cells, Th17 cells are corticosteroid-resistant.

B-Lymphocytes

In allergic diseases B-lymphocytes secrete IgE, and the factors regulating IgE secretion are now much better understood.[53] IL-4 is crucial in switching B cells to IgE production, and CD40 on T cells is an important accessory molecule that signals through interaction with CD40-ligand on B cells. There is increasing evidence for local production of IgE, even in patients with intrinsic asthma.[54,55]

A subset of CD4+ T cells termed *invariant natural killer T* (*i*NKT) cells secrete IL-4 and IL-13, but their role in asthma is currently uncertain as there appears to be a discrepancy between the data from murine models of asthma and humans.[56]

Basophils

The role of basophils in asthma is uncertain, as these cells have previously been difficult to detect by immunocytochemistry. Using a basophil-specific marker, a small increase in basophils has been documented in the airways of asthmatic patients, with an increased number after allergen challenge. However, these cells are far outnumbered by eosinophils (approximately 10:1 ratio), and their functional role is unknown.[57] There is also an increase in the numbers of basophils, as well as mast cells, in induced sputum after allergen challenge.

Platelets

There is some evidence for the involvement of platelets in the pathophysiology of allergic diseases, since platelet activation may be observed and there is evidence for platelets in bronchial biopsies of asthmatic patients. After allergen challenge, there is a significant decrease in circulating platelets, and circulating platelets from patients with asthma show evidence of increased activation and release the chemokine CCL5.

STRUCTURAL CELLS AS SOURCES OF MEDIATORS

Structural cells of the airways, including epithelial cells, endothelial cells, fibroblasts, and even airway smooth muscle cells, may be an important source of inflammatory mediators, such as cytokines and lipid mediators in asthma and CF. Indeed, because structural cells far outnumber inflammatory cells in the airway, they may become the major source of mediators driving chronic airway inflammation. Epithelial cells may have a key role in translating inhaled environmental signals into an airway inflammatory response and are probably the major target cell for inhaled corticosteroids in asthma (Figure 6-4). Epithelial cells may also play an important role in CF in driving the neutrophilic inflammatory response through the release of CXCL1 and CXCL8. Through the release

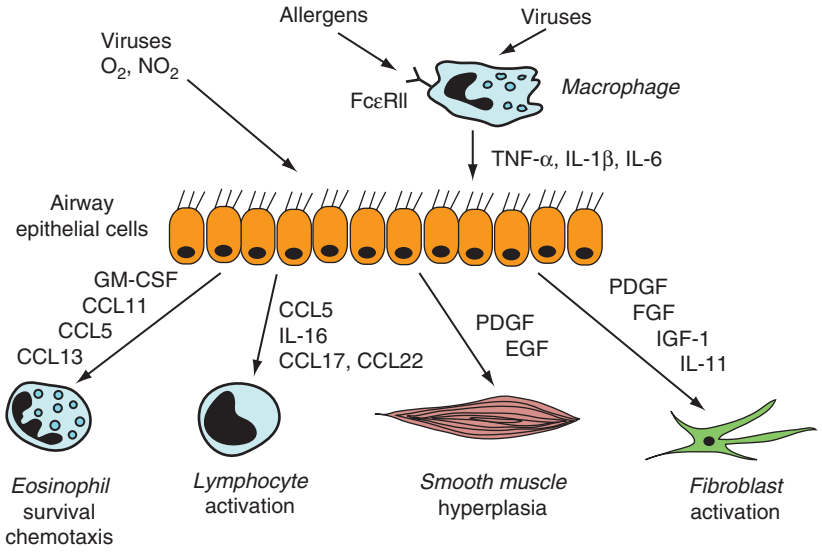

FIGURE 6-4. Airway epithelial cells in asthma. Airway epithelial cells may play an active role in asthmatic inflammation through the release of many inflammatory mediators, cytokines, chemokines, and growth factors. *TNF-α,* tumor necrosis factor alpha; *IL,* interleukin; *CCL,* C-C chemokine; *PDGF,* platelet-derived growth factor; *EGF,* epidermal growth factor; *FGF,* fibroblast growth factor; *IGF,* insulin-like growth factor.

of growth factors, airway epithelial cells may also be important in driving the structural changes that occur in chronic airway inflammation.[58] Epithelial cell integrity may also be an important factor in denying allergens exposure to the immune system; an increasing number of asthma susceptibility genes are expressed in the airway epithelium.[59–61]

INFLAMMATORY MEDIATORS

Many different mediators have been implicated in asthma, and they may have a variety of effects on the airway, which accounts for all of the pathological features of asthma[62] (Figure 6-5). Although less is known about the mediators of CF,[63] it is becoming clear that they differ from those implicated in asthma. Because each mediator has many effects, the role of individual mediators in the pathophysiology of airway inflammatory disease is not yet clear. The multiplicity and redundancy of effects of mediators make it unlikely that preventing the synthesis or action of a *single* mediator will have a major impact in the therapy of these diseases. However, some mediators may play a more important role if they are upstream in the inflammatory process. The effects of single mediators can only be evaluated through the use of specific receptor antagonists or mediator synthesis inhibitors.

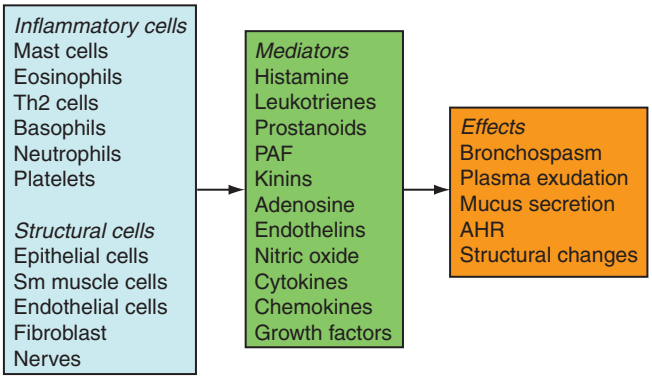

FIGURE 6-5. Multiple cells, mediators and effects. Many cells and mediators are involved in asthma and lead to several effects on the airways. *Th2,* T helper 2 cells; *Sm,* smooth; *PAF,* platelet-activating factor; *AHR,* airway hyperresponsiveness.

Lipid Mediators

The cysteinyl-leukotrienes, LTC_4, LTD_4, and LTE_4, are potent constrictors of human airways and may also increase AHR. Leukotriene antagonists have some bronchodilator and anti-inflammatory effects but are much less effective than inhaled corticosteroids in the management of childhood asthma.[64] Platelet-activating factor (PAF) is a potent inflammatory mediator that mimics many of the features of asthma, including eosinophil recruitment and activation and induction of AHR; yet even potent PAF antagonists, such as modipafant, do not control asthma symptoms, at least in chronic asthma. Prostaglandins (PG) have potent effects on airway function, and there is increased expression of the inducible form of cyclo-oxygenase (COX-2) in asthmatic airways; however inhibition of their synthesis with COX inhibitors, such as aspirin or ibuprofen, does not have an effect in most patients. Prostaglandin D_2 is a bronchoconstrictor prostaglandin produced predominantly by mast cells; it also activates a novel chemoattractant receptor termed *chemoattractant receptor of Th2 cells* (CRTh2) or *DP2-receptor,* which is expressed on Th2 cells and eosinophils and mediates chemotaxis of these cell types; it may provide a link between mast cell activation and allergic inflammation. Several oral CRTh2/DP_2 antagonists are now in clinical development.[65]

Cytokines

Cytokines are increasingly recognized to be important in chronic inflammation and to play a critical role in orchestrating the type of inflammatory response. They are the target for the development of new asthma therapies[66] (Figure 6-6). Many inflammatory cells (macrophages, mast cells, eosinophils, and lymphocytes) and airway