chapter 4

GENE EXPRESSION IN ALVEOLAR DEVELOPMENT

Jacques R. Bourbon

RESPIRATORY epithelium, strictly defined as the epithelium of distal lung that lines walls of respiratory bronchioles and alveoli, is composed of two cell types known as *alveolar type I* and *II cells* or *type I* and *II pneumocytes*. The former is a flat, attenuated, terminally differentiated cell through which air–blood gas exchanges occur. The latter is a cuboidal cell that elaborates and releases all the phospholipid and protein components of pulmonary surfactant (87, 113); it also serves as a progenitor cell for itself and for the type I cell that does not divide (64). Although it belongs to the bronchiolar epithelium at the junction of conducting and respiratory portions of the lung, the nonciliated bronchiolar, or Clara, cell shares features with the type II pneumocyte since, in addition to secretory products proper to this cell type, it also releases some surfactant protein components (87). It also represents the progenitor for itself and for the basal and ciliated cells in the renewal process of the epithelium (64). These cell types, however, represent only a small part of all epithelial lung cell phenotypes. In total, pulmonary epithelium comprises at least 11 differentiated cell types that, to a large extent, determine lung function. To understand phenotypic differentiation in the distal parts of the lung, we need to investigate (*1*) how lung-specific identity is determined, and (2), how distal cell lineages are specified relative to those of more proximal parts of the lung, the epithelial cells of conducting airways.

Alveolar and Clara cells differentiate relatively late in gestation, once the bronchial and bronchiolar tree is completed (30). All epithelial cells of the lung derive from a ventral outgrowth of the embryonic endodermal tube. During the earliest phases of lung development, i.e., the embryonic and glandular periods, when

C. Gaultier et al. (eds.), *Lung Development*

branching of the bronchial tree becomes established by dichotomous growing of the anlage (see Chap. 1), epithelial cells remain morphologically undifferentiated and apparently homogeneous. At a definite developmental stage that takes place at the end of the glandular period, the proximal and distal areas of growing tubules become sharply demarcated. Whereas proximal tubules, considered to be the prospective bronchial system (primordial or bronchial tubules), keep the pseudostratified columnar appearance of the primordial epithelium, cells of distal tubules, considered to be the prospective respiratory system (acinar tubules), become cuboidal (170). Functional maturation of the various cell types begins before the completion of the branching process of the bronchiolar and respiratory systems. The precise time when a given cell type or cell type precursor is present is nevertheless in dispute, depending on the nature of the criterion used for investigation: morphological aspects, immunological reactivity, or in situ hybridization.

There is considerable information on diffusible factors capable of modulating (enhancing or decreasing) the expression of lung cell markers. The sequence of molecular events driving pattern formation in the developing lung, i.e., the temporal cascade of gene activation and repression that leads to differential expression characteristic of the various cell phenotypes and to their architectural arrangement, however, remains largely unknown. On the basis of currently available data, only conjectural assumptions can be made with regard to the ultimate mechanisms of cell determination, i.e., to the master genes that specify a pulmonary identity or to the possible morphogens that instruct lung cells to assume proximal- or distal-specific functions. Recently, however, a rapidly increasing number of studies have produced new insights into the molecular mechanisms that confer the ability to express lung-specific genes to alveolar and Clara cells. This chapter reviews the data dealing with distal lung development in this fast evolving field of research. Control mechanisms of growth, morphogenesis, and cell-specific expression of markers are considered, with particular emphasis given to the latter.

ONTOGENY OF DISTAL LUNG MARKERS AND COMMITMENT OF LUNG EPITHELIAL CELLS

Markers of Alveolar Cells and Clara Cells

Alveolar Type II Cell Markers

The alveolar type II cell is the major source of pulmonary surfactant, the proteolipid complex that lines lung alveoli and plays a variety of roles crucial for respiratory function. It presents remarkable and unique biophysical and biological features, including surface tension–lowering capacity, water repellency, anti-edematous activity, and antimicrobial properties. Its major components are lipids, principally phospholipids, that account for as much as 90% of its mass and of which dipalmitoylphosphatidylcholine is the most abundant molecule. Another phospholipid, phos-

phatidylglycerol, appears to be surfactant-specific, as it is virtually absent from animal cells other than alveolar type II cells. The high rate of phospholipid production by type II cells is reflected in the high level of activity of the enzymes involved in their biosynthetic pathway, compared with other cell types. Among the 10% protein components of surfactant, four characteristic proteins, SP-A, -B, -C, and -D, have been described (87). SP-A and SP-D are amphiphilic proteins that belong to the C-type collectin family and are closely related to two seric proteins, mannose-binding protein and conglutinin, respectively. They are active in alveoli as an octadecamer and a dodecamer. Both SP-A and SP-D exhibit a non-phospholipid-linked property of opsonizing microorganisms, which likely facilitates phagocytosis by alveolar macrophages. Furthermore, SP-A, but not SP-D, interacts with surfactant phospholipids and SP-B. The simultaneous presence of SP-A and SP-B is required for the transformation of the content of lamellar bodies, the intracellular storage form of surfactant, into tubular myelin, a three-dimensional square lattice that appears to be a precursor form of the active surfactant film. SP-B and SP-C are much smaller, highly hydrophobic peptides that are closely associated with phospholipids. Their presence appears to be essential for rapid spreading and absorption of surfactant onto the aqueous hypophase lining the alveolar wall. These four proteins are excellent markers of distal lung epithelium. It should be stressed, however, that only SP-C is specific to type II cells, since Clara cells also express SP-A, SP-B, and SP-D genes (91). Their genes, including large upstream promoter regions, have been cloned in several species, allowing one to study transactivation mechanisms of their expression. Attempts to characterize type II cell apical membrane markers have also been made. Characterization and cDNA cloning of an SP-A binding protein considered a type II cell–specific SP-A receptor (165) have been reported.

A major problem in neonatology, respiratory distress syndrome (RDS) of the newborn, is mainly a consequence of pulmonary surfactant deficit; thus thorough knowledge of lung maturation is a prerequisite to efficient prevention of RDS. Prevention of its sequelae, bronchopulmonary dysplasia (BPD), a condition that involves necrotizing bronchiolitis, thickening of septal walls, and fibrosis, also calls for a better understanding of mechanisms governing cell growth, differentiation, and epithelial repair.

Alveolar Type I Cell Markers

The phenotypic transition that type II cells undergo during their transdifferentiation into type I cells includes not only the loss of surfactant component expression but also the acquisition of new membrane markers of the second cell type. This was first illustrated by changes in lectin-binding properties. Lectin ligands, however, have not all been characterized. Whereas *Maclura pomifera* lectin binds to apical surface proteins of type II cells, *Ricinus communis* (reviewed in ref. 113) and *Bauhinia purpurea* (93) lectins specifically label type I cells. Only recently have more specific markers of type I cells begun to be characterized. Antibodies have been raised against apical surface proteins that appear to be type I cell–specific (62). These tools appear to be useful for studying the type II cell–type I cell transition in vitro (55). Similarly,

a novel gene encoding for a type I cell protein designated T1α has been described. Interestingly, T1α, which appears to be a transmembrane protein also expressed in the brain and in ciliary epithelia, has been reported to be developmentally regulated (185).

Clara Cell Secretory Protein

A secreted, covalently bound homodimeric protein called the *Clara cell secretory protein* [CCSP; (80)], otherwise designated 10 kDa Clara cell specific protein [CC10; (159)], CC17, RLL (for rat lung lavage), and polychlorinated biphenyl-binding protein, has been described in lung lavage or tissue of various species and localized to vesicles within the Clara cell (12, 159). Its functions are not precisely established, but it displays phospholipase A_2 and proteinase-inhibitory activity (80). It may also act as an immunomodulatory and anti-inflammatory protein similar to its uterine counterpart, uteroglobin (111). Its gene has been cloned in human (186) and rat (147). Along with SP-C, it represents a useful marker for differentiating alveolar type II and Clara cells.

Ontogeny of Surfactant Proteins and CC10 in the Developing Lung

In human pregnancy (term at 40 weeks), the first lamellar bodies appear at about 19–20 weeks. Surfactant amount regularly increases until term. In the rat and rabbit, two species widely used for investigations on lung development, the first lamellar bodies are seen at gestational days 19 and 26 for gestational length of 22 and 31 days, respectively (30). In various species, significant amounts of surfactant-associated proteins have been detected along with the increase in surfactant phospholipids, although their mRNAs could be detected somewhat earlier (30, 148), and the course of phospholipid and protein storage parallels that of development of biophysical properties (65). It has been shown, however, that a low level of expression of surfactant proteins is present much earlier and is not limited to cells destined to become alveolar type II cells. These proteins therefore represent precocious markers of developing pulmonary epithelia, before differentiation of actual type II cells. Thus, SP-A and its mRNA have been evidenced in human tracheal and bronchial epithelium as early as the 13th gestational week (96)—i.e., at least 6 weeks before formation of the first lamellar bodies. SP-A expression in proximal portions ceases when mucociliary differentiation takes place and becomes limited to Clara cells (96). Similarly, in the rat, the mRNAs of SP-A, SP-B, and SP-C have been detected by reverse-transcriptase polymerase chain reaction (RT-PCR) on gestational day 12, the day after the formation of the lung anlage, and were present in all tubules, including the trachea, during the 3 following days (176). In the mouse fetus, immunoreactive pro-SP-B and pro-SP-C have been found in large proximal airways, including main and lobar bronchi, during early lung development, and become restricted to bronchiolar and alveolar epithelial cells in late gestation (195).

CC10 mRNA was first detected in rat lung on gestational day 16 by in situ hy-

bridization and was found to be restricted to bronchial tubules lined with tall columnar cells in which expression appeared to be uniform (167). By gestational day 19, it became limited to secretory epithelial cells lining the bronchi and terminal bronchioles. Ciliated cells and cells lining the prealveolar saccules lacked hybridization signal (167). CC10 transcript increased in abundance during late fetal life and reached adult levels 2 weeks postpartum in this species (82). Surprisingly, in the adult rat, alveolar type II cells appear to express CC10 mRNA (167), which is in contrast with the Clara cell-specific expression of the protein itself, suggesting post-transcriptional control of the message. This overlap could be a peculiarity of the rat, however, since the murine type II cells do not appear to contain CC10 transcripts (179).

An important observation was made through immunocytochemical investigations in the mouse fetus (188): lung epithelial progenitor cells simultaneously express immunohistochemical markers characteristic of different mature cell lineages. Thus, in an early phase spanning from gestational days 13 to 15 (term is 20 days), all epithelial cells of distal airways co-express CC10, SP-A, and calcitonin gene–related peptide (CGRP), a neuropeptide marker of neuroendocrine cells of the bronchiolar system. These markers subsequently seggregate when cell types differentiate from each other. The emergence of staining of the differentiated cell types occurs between days 16 and 18. These temporal expression patterns were recapitulated in serumless organ culture. Consistently, in situ hybridization study revealed SP-C expression throughout the distal epithelium from days 11 to 16 (179). According to Wuenschell et al. (188), the expression of all these gene products in a progenitor cell population may reflect some aspect of an underlying mechanism of developmental gene regulation, rather than early commitment to specific lineages.

Ontogeny of Surfactant in Culture Models

It has been long established that the entire pulmonary anlage can pursue its development in vitro even in the absence of serum (reviewed in ref. 30) This includes branching of the complete bronchial tree and differentiation of ciliated cells in bronchi and bronchioli. Type II cells differentiate at the tips, although actual alveolar sacs do not develop. Type II cell maturation was also observed in cultured fetal lung explants from various species in numerous studies. Starting in the 1960s, a number of experiments (reviewed in ref. 30) using the dissociation-reassociation approach of various epithelia with either homologous or heterologous mesenchyme showed that, even if the presence of mesenchyme was an absolute requirement for bronchial growth and branching, and if the branching pattern was dictated by mesenchyme according to the organ from which it originated, mesenchymes were not able to phenotypically transform epithelial cells that seemed to be already determined. Thus, lung mesenchymal cells appeared to control the degree of differentiation of lung epithelium, but were not able to induce expression of lung epithelial markers in nonpulmonary epithelia.

Although lung mesenchyme does not appear to induce the pulmonary identity of the epithelium, regionalization of the latter appears to be a consequence of region-

alization of the mesenchyme. Thus, grafting distal lung mesenchyme onto explanted fetal rat tracheal epithelium induced budding, branching, and alveolar type II cell differentiation in the epithelium. Type II cell differentiation was attested to by the presence of SP-C mRNA (normal tracheal epithelium at the considered stage expressed only SP-A and SP-B mRNAs), lamellar bodies, SP-B and SP-C proteins, and tubular myelin figures, all normally absent in normal tracheal epithelial cells (152). It should be pointed out that the ability of tracheal epithelium to respond to induction by distal mesenchyme was limited in time; day 13–14 tracheal cells were responsive to mesenchyme of the same stage, but day 16 tracheal cells failed to respond. Only distal mesenchyme from the presumptive acinar areas was inductory; mesenchyme of the main bronchus induced at most a barely detectable outpocketting of tracheal epithelium (152). By the same token, distal lung epithelium recombined with tracheal mesenchyme did not branch, but instead formed cysts and differentiated into ciliated and mucous secretory cells (156). Importantly, no lamellar bodies and no SP-C expression were detected with this combination (156). In addition, in another study (38), it was suggested that, depending on the distance of mesenchymal fibroblasts from the epithelium, these fibroblasts could produce different mediators that would promote either epithelial growth or differentiation, the latter being favored by fibroblasts in close proximity to the epithelium and more distant fibroblasts favoring epithelial growth. The phenotypic plasticity of epithelial cells and the role of their local environment was also recently illustrated by results from experiments in which tumor cells from a cell line (MLE 15) were implanted in nude mice. This cell line is derived from lung adenocarcinoma formed in a mouse rendered transgenic for the simian virus 40 early region driven by the SP-C promoter. Whereas cells of this line expressed SP-C but not CC10 in vitro, they expressed both markers in tumor nodules formed in vivo. Both markers were not co-expressed in the same cell, however; SP-C mRNA was detected at the periphery of the tumors in close association with surrounding stromal tissue, and CC10 mRNA was detected in central tumor regions (184). This connects with the existence of direct contact between alveolar type II and stromal cells through focal interruptions of the basement membrane in the normal lung, as this contact is thought to facilitate instruction transfer from the mesenchyme (30). Such contact is not present in bronchiolar cells.

Although adult type II cells cultured on plastic rapidly loose their markers, these markers, including expression of surfactant proteins, are retained when cells are grown on basement membrane matrix material prepared from the murine Engelbreth-Holm-Swarm (EHS) tumor (94, 153). This substratum was therefore used for studying type II cell differentiation in vitro. A first methodological approach consisted of complete enzymic dispersion of cells and removal of fibroblasts by differential adhesion before seeding epithelial cells onto the basement membrane matrix where they formed pseudo-alveolar structures (45, 72). A second method consisted of separating the epithelial rudiment of embryonic rat lung from its surrounding mesenchyme by mild enzyme treatment; successful in vitro development of the entire epithelial tube was obtained through substitution of mesenchymal influence by

EHS basement membrane matrix and a growth factor–enriched medium (60, 130). Branching was observed in the presence of serum and various growth factors in one study using the second model (60), and was in another study obtained in a defined medium in the presence of acidic fibroblast growth factor (FGF-1) (130). In both the dispersed-cell model and the entire rudiment model, full differentiation of type II cells was obtained within a few days, including appearance and accumulation of typical lamellar bodies (45, 60, 72), formation of tubular myelin figures (45), appearance of SP-C mRNA (60, 72), onset of SP-A and SP-B synthesis, a sharp rise in the rate of precursor incorporation in surfactant lipids, and responsiveness to fibroblast-conditioned medium (71, 72). Figure 4.1 illustrates this differentiation capacity of fetal rat lung epithelial cells. With dissociated cells, an increase in the steady state of mRNAs encoding for various enzymes involved in phospholipid synthetic pathway that reproduced normal developmental changes was also observed (71). Scarce ciliated cells also differentiated in this model (71, 72). It should be underscored that the differentiation process was achieved in chemically defined medium devoid of hormones in the first model (45, 71, 72), but not in the second one (60). No evaluation of epithelial differentiation was done in the study of whole rudiment growth in defined medium (130); however, a more recent investigation with the same model indicated poor expression of type II cell markers (40).

Pluripotency of Fetal Lung Epithelial Cells and the Role of Mesenchyme

Taken together, the early expression of surfactant protein genes in undifferentiated lung epithelium, including those areas destined to differentiate into proximal structures that no longer express these markers, and the co-expression in a same progenitor cell of markers subsequently restricted to distinct cell lineages lead to the following conclusions: (*1*) cell commitment to a pulmonary identity is extremely precocious and (2) all the information necessary to generate all lung epithelial cell phenotypes is present in the primordial lung bud, including control of the biphasic pattern (common, then specific) of gene expression. The ability of undifferentiated epithelial cells to pursue in vitro the differentiation program of alveolar cells in an autonomous way (at least beyond a definite stage of development) shows that even if an initial mesenchymal induction takes place, it does not need to be maintained. This pulmonary identity may already be imprinted in the initial bud in a potential form, possibly as a consequence of the combined expression of homeotic genes that dictate commitment along the anterior–posterior axis. Regionalization in the capacity of mesenchyme to modulate or reverse epithelial gene expression indicates, however, that the final orientation of a progenitor cell toward a given phenotype among the possible pulmonary cell fates is submitted to mesenchymal control. Presumably, paracrine and/or matricial information compartmentalized along the anterior–posterior axis of the lung orients gene expression in pluripotent epithelial cells. This control is likely to be exerted at the level of differential expression of transcriptional factors, leading in turn to expression of specific epithelial cell markers.

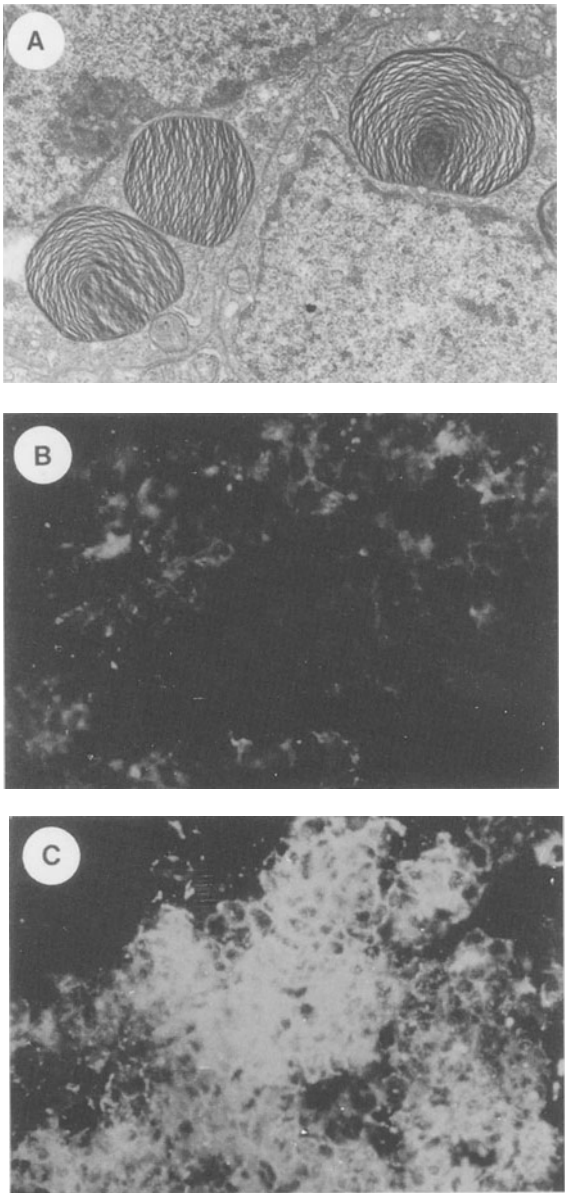

Fig. 4.1 In vitro differentiation of isolated epithelial cells from fetal rat lung. Cells were taken on gestational day 17 and cultured on basement membrane matrix in defined medium. (*A*) Transmission electron microscopy aspect of lamellar bodies formed during a 6-day culture. [Original picture from C. Fraslon.] SP-B immunoreactivity (immunofluorescent-coupled anti SP-B antibody) occurred after 2 days (*B*) and 6 days (*C*) of culture. Note the increase in SP-B content during culture ; immunoreactive SP-B was not detected at culture initiation. [Original pictures from B. Chailley-Heu.]

ROLE OF TRANSCRIPTION FACTORS IN LUNG DEVELOPMENT AND THE CONTROL OF SPECIFIC PROTEIN EXPRESSION

Control of gene expression resides in a combinatorial interaction between gene promoter regions and various nuclear protein ligands known as *transcription factors.* These *trans*-acting factors present characteristic DNA-binding motifs that interact with specific DNA sequences or *cis*-acting elements. Some transcription factors are ubiquitous and are needed for transcription of a number of genes. Tissue, area, or cell-type specificity in expression pattern of other transactivating factors, along with chromatin accessibility to these factors, accounts for restricted expression of cell markers. Analysis of the 5′-flanking regions of marker genes and of the cell-specific nuclear factors that control their transcription may therefore enlighten our understanding of cell differentiation mechanisms. Only these specific transcription factors will be considered here. Transcription factors from three different families, namely thyroid transcription factor 1, hepatocyte nuclear factors-3/forkhead factors, and CCAAT/enhancer binding proteins, have been recently recognized as playing crucial roles in distal lung-specific gene expression and lung developmental processes.

Role of Thyroid Transcription Factor-1

Thyroid transcription factor-1 (TTF-1), otherwise designated *thyroid-specific enhancer-binding protein* (T/EBP) or *Nkx-2.1,* is a homeodomain-containing DNA-binding protein (78) initially described as a transcription factor for thyroid-specific genes (47). It is homologous to the *Drosophila* transcription factor NK-2 and belongs to the NKx class of nuclear factors (53). Homeodomain transcription factors are characterized by a conserved 60 amino acid DNA-binding domain designated the *homeodomain,* which is characterized by a helix-turn-helix motif and is encoded by a 180 nucleotide domain referred to as the *homeobox* (75).

During fetal development, TTF-1 mRNA is present in cell nuclei of the brain, thyroid, and pulmonary epithelium, but is absent from the foregut epithelium prior to the onset of thyroid and lung morphogenesis (53, 105). In the lung and thyroid, TTF-1 is expressed in cells of the primitive lung bud epithelium as well as in subsets of epithelial cells that subsequently differentiate. Interestingly, a gradient establishes along the anterior–posterior axis of these structures, with expression restricted to the distal epithelial tubules and absent from the most proximal segments (105). TTF-1 mRNA is absent from mesenchymal cells (105). It is present in subsets of bronchiolar and alveolar cells in the mature lung (78, 84) and is particularly abundantly expressed in postnatal lung alveolar type II cells (84). Recently, systematic immunohistochemical studies have established more precisely the spatial–temporal changes in TTF-1 protein localization in the developing mouse (195) and human (162) fetal lung. In mouse lung (195), TTF-1 was detected on gestational day 10 in nuclei of main bronchial epithelial cells. From days 12 through 16, it was expressed at high levels in all airways including the trachea, and was particularly prominent in

newly formed distal airway tubules. In late gestation, labeling was more intense in type II cells than in cells of conducting airways. In the adult mouse lung, however, type II cells and bronchial or bronchiolar epithelial cells appeared equally stained, which appears to contradict previous mRNA analysis (105). In fetal human lung (162), early detection (11 weeks) was found in undifferentiated columnar epithelial cells. Subsequently, labeling became most prominent in cells at the tip of the developing buds. Close to term, TTF-1 disappeared from proximal columnar epithelium to become restricted to alveolar type II cells, Clara cells, and cells lining bronchioloalveolar portals. It is striking that in both species, TTF-1 distribution overlaps that of SP-A, SP-B, SP-C, and CC10, and decline of TTF-1 from proximal territories coincides with the disappearance of transiently expressed markers such as SP-A or pro-SP-B/C. The persistence of TTF-1 in large airways throughout life in the mouse suggests, however, that other transcription factors could be involved in this regulation. Interestingly, TTF-1 is absent from alveolar type I cells, which implies an arrest of its production during the type II–to–type I transition (162). It should be emphasized that TTF-1 was found in the same study (162) to be deficient in atelectatic areas of the lung in RDS and in collapsed airways in BPD, but was prominent in regenerating tips of terminal airways in the latter condition. Elucidation of mechanisms that regulate TTF-1 gene expression may therefore be important for the treatment of acute and chronic lung disease in infants.

Inhibition of TTF-1 translation through the use of an antisense oligonucleotide in an in vitro model of embryonic mouse lung morphogenesis led to abnormal phenotype, including reduced branching and unorganized hyperplastic epithelium which kept tall columnar immature cells (124). Consistently, inactivation of the TTF-1 gene through homologous recombination in the mouse permitted development until birth but, among other major abnormalities, led to arrested lung development. Fetuses homozygous for the disrupted gene displayed lack of lobar bronchi branching, rudimentary bronchial tree, and absence of bronchioli, alveoli, and lung parenchyma, which were replaced by dilated saccules limited by a columnar epithelium of bronchial type (97). Altogether, these observations point out TTF-1 as having an essential role in the control of lung organogenesis, particularly with regard to epithelial branching growth and development of distal structures, including the ontogenesis of cuboidal cells that serve as progenitors to alveolar epithelial cells.

With regard to expression of lung-specific protein genes, TTF-1 was first demonstrated to control SP-B gene expression. An isolated fragment of the promoter region of the SP-B gene has been shown to contain several sites protected against S1 nuclease by nuclear ligand proteins and to allow strong expression of a reporter gene in a tumoral cell line that expresses SP-B in vitro (28). Two *cis*-acting TTF-1 binding sites have been identified in this DNA sequence, relatively near the start site of transcription (27, 34). A distinct enhancer also mediating TTF-1 transactivation was later found further upstream in the 5′-flanking region of the gene (190). Point mutations in these sites markedly decreased the activity of expression constructs in transfected lung cells in vitro, whereas co-transfection of a reporter gene under the control of the SP-B promoter along with an expression vector of TTF-1 cDNA considerably increased expression of the former (27, 190). TTF-1 was subse-

quently demonstrated to regulate SP-A (35, 168) and SP-C (95) gene expression. Four distinct TTF-1 responsive fragments were identified in the SP-A promoter and five were found in the SP-C promoter. The same site-specific mutagenesis and co-transfection approaches led to the same conclusions as for SP-B. Three binding sites in the SP-A gene and two in the SP-C gene were shown to be required for TTF-1 activation (35, 95). In SP-A, -B, and -C promoters TTF-1 binds with varying affinity to the different sites, and cooperative binding has been observed at the clustered sites.

TTF-1 has been found to similarly regulate CC10 expression (139, 171). No less than seven TTF-1 binding sites have been identified—five in the distal part of the promoter and two in the proximal part. Deletion and mutational analyses have identified a site located between –282 and –272 base pairs (bp) of the transcription initiation point as the major regulator of CC10 expression. A reporter gene fused to a CC10 promoter fragment containing a mutation in this binding site presented a markedly reduced expression in transgenic mice (139). Co-transfection assays have also demonstrated specific activation by TTF-1 at the level of the most proximal TTF-1 site (171). DNase-I footprint analysis enabled identification of a binding site for a second transactivating factor of the NKx family in the CC10 promoter, namely cardiac muscle–specific homeobox protein (CSX) (139).

Role of Hepatocyte Nuclear Factor 3/Forkhead Factor Family

The hepatocyte nuclear factor 3/forkhead (HNF-3/fkh) factors belong to a wide group of nuclear factors initially discovered in the liver, but subsequently found to be expressed in a number of other tissues. The HNF-3α, β, and γ isoforms were first identified as factors controlling expression of the liver transthyretin and α1-antitrypsin genes (52). Their genes constitute the mammalian counterparts (103) of the homeotic gene *forkhead* in *Drosophila*, thus named because its mutation results in an embryo with a duplicated anterior pole (177). Forkhead factors have been identified in all phyla. This family of DNA-binding proteins is characterized by a conserved 110 amino acid DNA-recognition domain named the *forkhead domain*. This domain presents a characteristic α-helical structure with two wing-like loops resembling the shape of a butterfly, leading to the term the *winged-helix motif* (48). To date, the family includes about 40 identified mammalian members involved in differentiation of various cell lineages. In addition to HNF-3/fkh factors, a number of related factors designated *hepatocyte nuclear factor/forkhead homologues* (HFH) have been identified in rodents (50). The human forkhead-related factors FREAC-1 and -2 expressed in lung are homologous to the murine HFH-8 (136). The human counterpart to the murine HNF-3α has recently been cloned from a pulmonary adenocarcinoma cell line (20).

The adult lung strongly expresses transcripts of HNF-3α and β, HFH-4, and HFH-8, and more weakly expresses HFH-1, HFH-2, HFH-5, and HFH-6 (52, 103). HNF-3γ is not expressed. In situ hybridization experiments have revealed regionalization of expression. For instance, whereas HFH-1 expression is diffuse throughout the organ, HNF-3α and HFH-4 are expressed in Clara cells, but not in type II cells, which by contrast express HFH-8 (50, 52, 81, 103). HFH-8 is also expressed in the pulmonary endothelium and connective fibroblasts of the alveolar sac in the adult

lung and in the mesenchyme in the developing lung (135). During development, the expression pattern of HNF-3 family members suggests that they play roles in regional differentiation of the intestinal endoderm and its derivatives, including the lung (8, 127). While HNF-3α expression is sustained in the trachea, HNF-3β, which is expressed in the rat lung bud, disappears from trachea but continues to be expressed, along with HNF-3α, in the growing bronchial tree (21). In the fetal mouse lung, HNF-3β protein, is present at early stages throughout the pulmonary epithelium, including in the trachea. It becomes restricted to epithelial cells of conducting airways and to type II cells in late gestation, and shows a decreased level of expression compared with earlier stages (195). This decrease may provide signals influencing acinar cell differentiation. Similar changes in the temporal–spatial pattern of HNF-3β expression have been found in the developing human lung (163). Interestingly, in infants with BPD, HNF-3β was abundantly expressed in regenerating epithelial cells (163). In the adult lung, HNF-3β mRNA has been found to be expressed in mesenchyme in only one study (49), but HNF-3β protein has been detected in nuclei of epithelial bronchial, bronchiolar, and alveolar (type II) cells in another study (195). Targeted disruption of the HNF-3β gene has been attempted, but because of the early requirement of the gene for node, notochord, and gut tube development, the homozygous genotype is lethal prior to lung budding and the model has not been useful in determining more precisely the role of this gene in lung development (7, 178). Strong expression of HFH-4 has been found in epithelium of bronchiolar tubules but not in more distal tubules destined to generate the alveolar epithelium (81). Thus, the limit of HFH-4 expression coincides precisely with the demarcation between proximal and distal epithelia, which suggests a direct role for HFH-4 in regulating the initial differentiation of airway and alveolar epithelial cells.

A recently described forkhead gene, named *LUN*, is strongly expressed in the lung and more weakly in the small intestine (125). Its forkhead domain is identical to that of HFH-8. This factor has been shown to act as a positive transactivator for the SP-B promoter, which suggests that it may play a role in the determination and maintenance of some cell types in the lung (125).

Transcription factors of the HNF-3/fkh family appear to specify the endodermal lineage and therefore the tissues derived from this embryonic sheet, such as the liver, intestine, thyroid, and lung (8, 127). Organs of endodermal origin posterior to the lung, however, express HNF-3γ, but not TTF-1, which by contrast is expressed in more anterior segments of the body, such as thyroid and brain. Thyroid also expresses Pax-8, which is absent from the lung. A lung-cell identity would therefore be determined by the simultaneous expression of TTF-1, HNF-3α, and HNF-3β in the absence of Pax-8, a pattern found only in lung epithelial cells (27).

A characteristic HNF-3/fkh protein binding site has been shown to be present in the SP-B promoter with a canonic palindromic sequence, TGTTTGT, which is also found in liver-specific gene regulatory regions (27). Point mutation of this binding site eliminated HNF-3 binding and resulted in significantly decreased activity of the transfected SP-B promoter (27). Co-transfection experiments with either HNF-3α or HFH-8 expression vectors and gene constructs that included a fragment of the SP-B promoter have shown that both transcription factors are able to activate reporter

gene expression to a similar extent (49). Since SP-B is expressed both in Clara and type II cells, but HNF-3α is restricted to the former and HFH-8 to the latter, it has been proposed that SP-B expression is controlled at the same regulatory sequence by HNF-3α in Clara cells and by HFH-8 in type II cells (49). Although HFH-8 expression during lung development has not been characterized, this suggests a role for this factor in cell differentiation of the developing distal epithelium.

Binding sites for HNF-3 proteins have also been demonstrated in the CC10 promoter (19, 147). In co-transfection experiments with CC10 promoter elements and HNF-3α and β expression vectors in HeLa cells, HNF-3α and HNF-3β have been shown to have opposite effects on CC10 expression: HNF-3α stimulated activity whereas HNF-3β inhibited activity (146). This finding may result, however, from a specificity of HeLa cells, since in another study (21), similar co-transfection in the Clara cell–like NCI-H441 adenocarcinoma cell line led to cooperative action between both factors. More recently, a synergistic activation of the rabbit CC10 promoter was reported for HNF-3α and the ubiquitous transcription factor Sp1 and shown to be absolutely dependent on the integrity of two Sp binding sites (31). Surprisingly, it was shown in the same study that contrary to the rat promoter, the human and rabbit CC10 promoters are not regulated by TTF-1 (31). Figure 4.2 summarizes the

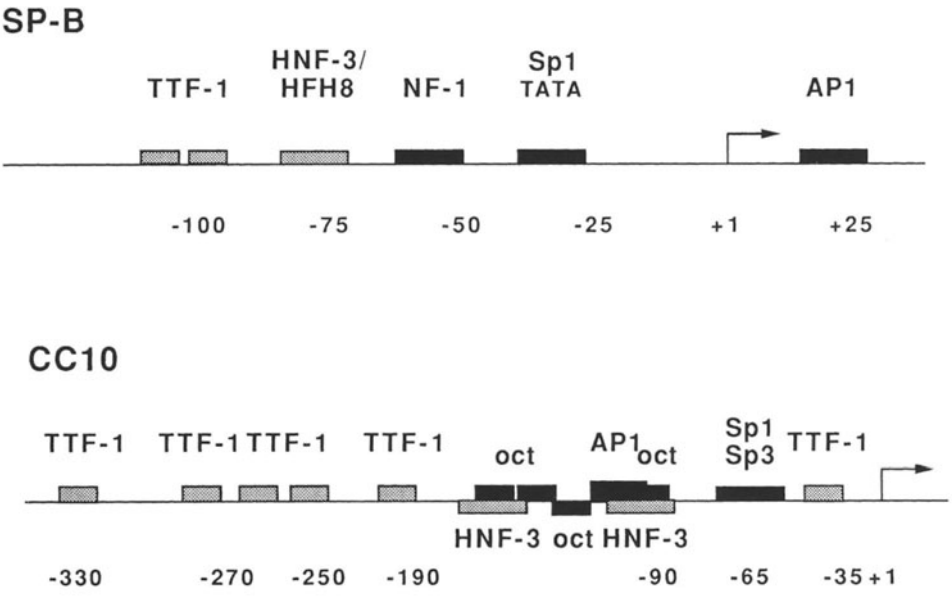

Fig. 4.2 Schematic illustration of the gene promoter region of surfactant protein B (*SP-B*) and of Clara cell protein 10 kDa (*CC10*). *+1* indicates the transcription start site. Ubiquitous transcription factor–binding sites are represented by *black rectangles*. Tissue-specific transcription factor–binding sites are represented by *shaded rectangles*. Drawn from data by Bohinski et al. (28) and Clevidence et al. (49) for SP-B, and from data by Ray et al. (139), Sawaya et al. (147), and Toonen et al. (171) for CC10.

organization of the SP-B and the CC10 gene promoter with localization of ubiquitous and specific transcription factor–binding sites.

In addition to direct effects on lung cell marker genes, HNF-3/fkh factors also transcriptionally control the expression of TTF-1 since, on the one hand, HNF-3β expression precedes that of TTF-1 in lung cells and, on the other hand, HNF-3β and, to a lesser extent, HNF-3α enhance the expression of a TTF-1-reporter construct in MLE cells (85). A hierarchical relationship therefore appears to exist between winged-helix transcription factors and TTF-1.

Role of CCAAT/Enhancer Binding Proteins and Peroxisome Proliferator-Activated Receptor γ

The CCAAT/enhancer binding proteins (C/EBP) are a family of the leucine zipper class of transcription factors that bind to DNA as homo- or heterodimers. The prototype member of the family, C/EBPα, may be involved in the control of type II cell functions. Expression of C/EBPα is restricted to certain tissues and cell types, including the liver, lung, white and brown adipose tissue, and placenta (59). Several genes of enzymes involved in lipid metabolism have been shown to be targets of this transcription factor (59). In the lung, C/EBPα mRNA levels have been found to be substantially enriched in alveolar type II cells, and C/EBPα protein is present in type II cell nuclei (106). Interestingly, C/EBPα is rapidly lost when type II cells are removed from the lung, but similar to surfactant proteins, it is re-expressed when cells are cultured on basement membrane matrix (106). Consistent with a putative role in type II cell differentiation is the fact that C/EBPα mRNA level increases in the developing rat lung between gestational days 18 and 20 (106). Three putative C/EBP-binding sites have been identified in the SP-A gene, one in the 5′ untranscribed region and the others in the first and last introns (160).

Invalidation of the C/EBPα gene in the mouse was reported to result in defects in liver and lung structure and in failure to accumulate lipids in adipocytes (67). The lung of nullizygous mice exhibited disturbed alveolar architecture, with immature appearance and type II cell hyperproliferation. Although no alteration was found in the expression patterns of SP-C, TTF-1, or CC10, these mice displayed respiratory problems and generally died within the first 10 h after birth. Surfactant abnormalities, including an inadequate amount of other surfactant proteins or of surfactant phospholipids, have been assumed but not yet explored. The role of C/EBPα in the control of lipogenetic enzyme expression, especially of fatty acid synthase and acetyl-CoA carboxylase, argues for a role in the synthesis of lipid precursors of surfactant phospholipids by type II cells.

More recently, C/EBPδ messenger RNA has been found to be developmentally regulated in fetal rabbit lung (32). In human fetal lung explants, C/EBPδ expression was induced rapidly during organ culture and increased further by treatment with dexamethasone and cyclic AMP, which, along with its localization primarily in alveolar epithelial cells, suggests a role for this factor in the regulation of type II cell differentiation (32).

Finally, another transcription factor known to be involved in adipogenesis, the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), was reported to be expressed in rabbit lung type II cells, to occur coincidentally with SP-A expression during type II cell differentiation, and to be up-regulated by cyclic AMP (121). A role in type II cell differentiation and expression of lipogenic enzymes in this cell type has been postulated for PPARγ (121).

Promoters of Distal Lung-Marker Proteins and Cell Specificity of Expression

SP-A and SP-B Promoters

Analysis of the SP-A promoter function in tissue-specific expression has led to somewhat contradictory findings. It has been reported that a 215 bp fragment of the rat SP-A gene upstream of the transcription start site supports in vitro transcription in the presence of lung nuclear extract but not of liver nuclear extract (102). Consistent with this finding is the fact that the binding profile of nuclear protein extract from the liver or kidney to fragments of the same promoter differed from that of the lung (73, 99). Lung nuclear proteins were found to protect two palindromic sequences in the distal and proximal parts of the promoter (73). Some binding activity was found, however, in nuclear proteins from cardiac and skeletal muscle (73), and from the liver (168) using fragments of the promoter region of different sizes. The transfection of reporter genes coupled with a large portion of the 5′-flanking region of the SP-A gene that included these elements into cells of various origins (including primary cultures of type II cells and cell lines of pulmonary and extrapulmonary origin) led to tissue-specific expression in one study (35) but not in others (6, 160), even if expression was higher in cells of pulmonary origin. These data suggest that regulation of the SP-A gene is complex and that the appropriateness of the different cell systems used should be evaluated carefully. Stuempfle and co-workers (168) have suggested that the lack of in vitro tissue-specific expression of the rat SP-A gene is due to one or more possible factors. Lung-specific DNA–protein complexes identified in various studies may require interaction with other DNA–protein complex(es) to confer tissue specificity to the SP-A gene. A silencer element may be present further upstream or downstream of the region covered by these constructs. The lack of chromatin structure that characterizes transient transcription assays could play a role in the nonspecific transcription. Finally, regulation of the SP-A gene may not be readily discerned via a cell-free transcriptional assay. In addition, it should be pointed out that thus far there has been no report of in vivo transgenic experiments with the SP-A promoter.

In contrast, study of the human SP-B promoter (174) has shown unambiguously that a fragment from −403 to −35 bp was sufficient to confer expression specificity to the Clara cell–like H441 cell line, compared with other cell lines. Moreover, transcriptional control elements were found both 5′ and 3′ of the transcription start site. Promoter activity was strongly and dose-dependently inhibited by phorbol ester, an in-

hibitor of surfactant protein synthesis. The phorbol-ester response localization does not coincide with a consensus AP1 site (binding site of the Jun-Fos heterodimer) identified in the promoter. It therefore appears possible that novel regulatory sequence binding proteins other than Jun and Fos are involved in this inhibition (174).

Differential Control of SP-C and CC10 Expression

Since SP-C and CC10 are differential markers of alveolar type II cells and bronchiolar Clara cells, respectively, analysis of their promoter sequences and respective regulation processes by transcription factors may produce clues to the compartimentalized expression of regulatory factors that account for the demarcation between bronchiolar and alveolar epithelia. Upon transfection, a small fragment located at –165 to +57 bp of the transcriptional starting point of CC10 has been shown to support expression of a reporter gene exclusively in the Clara cell–like H441 cell line and to elicit expression of a reporter gene in transgenic mice in Clara cells only, suggesting that a Clara cell–specific transcriptional element resides on this fragment (166). Regulation by the ubiquitous transcription factors AP-1 and octamer and by HNF-3/fkh family factors has been accounted for by binding sites in this fragment (147). However, there is no information about specific ligands responsible for differential expression. The presence of the –132 to –76 fragment of the CC10 promoter placed in its normal position, i.e., upstream of its homologous –75 to +37 fragment, is sufficient to allow HNF-3α and HNF-3β to activate reporter gene expression in H441 cells, whereas no reporter gene expression was found when the same fragment was placed upstream of the tyrosine kinase promoter (21). Regulatory elements that control Clara cell–specific expression of CC10 should therefore be investigated in the promoter part extending upstream to the –75 position.

In contrast with CC10 studies, transgenic experiments in mice with chimeric genes that include the 5′-flanking sequence derived from the human SP-C gene have led to lung epithelium–specific but not type II cell–specific expression of the transgene. Indeed, the latter was found to be expressed both in bronchiolar and alveolar cells (76, 179). Expression of the transgene was developmentally regulated, but it did not coincide strictly with either temporal or spatial expression of the SP-C gene. The transgene product was detected early in the development of the primordial lung bud, before the appearance of endogenous SP-C mRNA. Its quantity increased with progressing development, but it disappeared from trachea and main bronchi while its expression was maintained in secondary bronchi and bronchioles. Differentiation of bronchial–bronchiolar epithelium was consistent with the disappearance of SP-C expression and onset of CC10 expression, whereas that of the SP-C chimeric transgene persisted (179). The features of transgene expression nonetheless led to a strategy based on the use of the SP-C promoter for targeting the expression of various transgenes in the developing lung. This approach, however, did not allow the investigators to identify mechanisms responsible for specific SP-C expression in alveolar cells. Because the human SP-C gene was used in transgenic experiments, subtle differences may exist in the *cis*-active elements that would account for the lack of cell-specific expression in the mouse. Alternatively, the authors of this study have proposed that

possible differences in the expression of the endogenous and chimeric genes may be related to peculiarities of the human gene being expressed in the mouse. The lack of cell-specific expression may also be explained by a positional effect. In a more recent study (57), microinjection in fertilized eggs of a reporter gene coupled with a smaller human SP-C fragment was reported to lead to expression of the transgene only in alveolar type II cells. Further investigations are therefore required to explore the molecular mechanisms that drive type II cell–specific expression of SP-C.

PUTATIVE MECHANISMS OF ANTERIOR–POSTERIOR SPECIFICATION

In contrast to the rapidly growing body of knowledge about nuclear factors that readily control transcription of distal lung markers at the level of *cis*-acting elements, much less is known about the mechanisms taking place upstream in the cascade of developmental events and that determine cell fate along the anterior–posterior axis of the lung. Currently, putative control factors can only be inferred from indirect arguments such as correlative expression with cell markers or findings from gain- or loss-of-function experiments. Although the list is not limited, possible control factors include the N-*myc* proto-oncogene, homeotic genes, and the retinoic acid–retinoic acid receptor system.

Role of N-myc

Transcription factors of the Myc protein family mediate specific responses to signals that regulate cell growth and differentiation during development. Expression of the N-*myc* proto-oncogene appears to be restricted by tissue and stage specificity, in contrast to c-*myc*, which is ubiquitous. During the course of murine lung development, N-*myc* expression has been shown to be greatest during the early phase of branching, and then to decline (83, 129). By in situ hybridization, this expression has been localized to the bronchiolar epithelium, with higher levels occurring in the tips of the developing airways. During the same stages of lung development, *c-myc* expression was found to be restricted to the mesenchyme (83).

Creation of a null mutation in the N-*myc* gene by homologous recombination resulted in death of the homozygous mutant mouse embryos soon after the surge of the lung bud (145, 164). It resulted in the failure of many epithelial structures to develop, including the lung, which showed no sign of branching beyond the main stem bronchi. Culturing explanted lungs from homozygous mutant embryos in a medium containing fetal bovine serum allowed some recovery of bronchial morphogenesis to occur (145), indicating that to some extent, serum components can overcome the lack of N-*myc* expression. The null mutation indicates the probable major importance of N-*myc* in lung development, but it is not useful for evaluating the more specific role of N-*myc* in the development of distal regions. More interesting in this respect are the consequences of an insertional mutation in the N-*myc* locus that did

not completely eliminate, but resulted in the formation of, truncated N-*myc* transcripts (126). This "leaky" mutation thus allowed for a 75% reduction in N-*myc* expression through alternative splicing. Homozygous mice developed until term but died at birth of respiratory failure. With the exception of a reduced spleen size, abnormalities were limited to the lung. Whereas the number and distribution of the more proximal bronchi were normal, distal airways and air spaces failed to develop. Along with the highest rate of N-*myc* expression in distal developing airways, this finding is indicative of a major role for N-*myc* in the developmental control of distal lung epithelium. N-*myc* may not be involved in the differentiation of particular cell types, but it may represent an important element in the control of distal lung growth and morphogenesis.

Role of Homeotic Genes

The homeotic *Hox* (mouse) and *HOX* (human) genes are homeodomain-containing genes that share a high degree of homology with the *Drosophila HOM-C* complex. They are expressed during embryonic development and have been shown to regulate pattern formation in specifying anterior–posterior positional information. In vertebrates, 38 *Hox* genes are arranged in four chromosomal clusters designated *a* through *d*. Corresponding genes in the different clusters are called *paralogs* and are numbered 1 through 13. A colinear relation exists between a gene's position in the cluster and its anterior boundary of expression. The combination of expression of different paralogs in various organs forms a *Hox* code that appears to specify regional identity (reviewed in refs. 39 and 92).

Early expression, regionalization, and spatial–temporal developmental regulation of *Hox* genes in the lung argue for an important role for these genes in lung morphogenesis and pattern formation. Twenty *Hox* genes from the four clusters have been found to be expressed in the developing lung—some in both fetal and postnatal lung, others either in fetal or in neonatal lung; no expression of genes located 5′ to paralog group 9 has been detected (reviewed in ref. 39). *Hox* genes appear to be expressed in mesenchyme only (reviewed in ref. 92). Steady-state mRNA levels of *Hoxa-5*, *Hoxb-5*, *Hoxb-6*, and *Hoxb-8* are high at early stages and decrease with advancing age in fetal mouse or rat lung (24). By contrast, *Hoxa-2* mRNA levels remain unchanged throughout development (39). Active branching morphogenesis appears to be associated with high levels of expression of several *Hox* genes (reviewed in ref. 39). Thus, *Hoxb-3*, *b-4*, and *b-5* are highly expressed in the mouse embryonic foregut where the lung buds form (25). Background levels of *Hoxb-6* expression are seen in trachea and proximal lung, while high levels of expression are associated with the distal epithelial forming tubules in the branching lung; these levels become undetectable, however, at late gestation when acinar areas develop (41). The expression of some paralogs is consistent with the principle of co-linearity, and it has been postulated that specific combinations of *Hox* genes may function to specify the developmental fate of distinct regions of the lung through involvement in epithelial–mesenchymal interactions (26). Thus, once the bronchial tree has

formed, *Hoxb-3* and *b-4* are expressed in the mesenchyme of the trachea, main-stem bronchi, and distal lung, whereas *Hoxb-2* and *b-5* are expressed only in the mesenchyme of the distal lung buds (26). *Hox* genes in other clusters also appear to be involved in this determination. Elements in the 5′ upstream region of the *Hoxa-4* gene directed a transgene expression with prominent localization in lower lobes in the mouse fetal lung (13). Regulatory elements may therefore control the expression of *H*ox genes in specific regions of the lung.

Limited information has been gained from mutation disruption of *Hox* gene expression or transgenic overexpression, most likely because of redundancy in the sites of expression. It has nevertheless been reported that *Hoxa-3* mutants die at birth of respiratory failure and have smaller trachea and bronchi with disorganized epithelial cells (46, 110). These results, together with defects in differentiation of endodermal cells in the thyroid, may indicate a role for *Hoxa-3* in specification of endodermally derived cells, including lung epithelial cells. *Hoxa-5* has more recently been shown to be essential for normal organogenesis and function of the respiratory tract (9). In homozygous newborn mutants for this gene, improper tracheal and lung morphogenesis lead to tracheal occlusion and respiratory distress associated with a marked decrease in the production of surfactant proteins and with a reduced TTF-1, HNF-3β, and N-myc gene expression in the pulmonary epithelium (9). Thus, *Hox* gene products appear to control TTF-1 expression, an assumption also supported by the report that the Hoxb-3 protein is an upstream regulator of TTF-1 (77). This hypothesis, however, appears to conflict with the expression of *Hox* genes in mesenchyme and of TTF-1 in epithelium.

Despite the sparse and preliminary character of current data on *Hox* genes , an involvement of *Hox* gene expression in anterior–posterior specification in the developing lung is strongly suggested. Further investigations are needed to determine whether and how a particular *Hox* gene complement specifies the differences between proximal and distal identity of lung mesenchyme—particularly to what degree they affect the expression of factors that may in turn control the expression of epithelial cell markers.

Role of Retinoic Acid and Retinoic Acid Receptors

Retinoic acid (RA) is clearly necessary for the maintenance of mature tracheal-bronchial epithelium. By contrast, its effects on the developing alveolar epithelium have been a matter of debate. Whereas high concentration and/or long-term exposure of in vitro models led to a dramatically altered branching pattern, including total suppression of distal tubules and extension of proximal-like tubules toward the pleural surface (42), and to a decreased amount of surfactant proteins or of their mRNAs (42, 70, 119), low concentration and short-term exposure increased surfactant protein mRNAs (25). It should be stressed that stimulating effects were observed with concentrations in the range of the dissociation constant for in vitro nuclear binding of RA in the lung (10^{-9} M in the rat). Moreover, exogenous retinol and RA enhanced surfactant phospholipid synthesis in vivo and in vitro (69, 70). Several

lines of evidence indicate that retinoids are also involved in the control of alveolar morphogenesis. Retinoic acid increased transcription of the elastin gene and elastin synthesis in cultured neonatal rat lung fibroblasts (107), when elastin deposition is a central element in the process of alveolar septation. Furthermore, in vivo postnatal treatment with RA in the rat increased the number of alveoli and prevented the glucocorticoid-induced inhibition of septation (115).

Retinoic acid exerts its effects through binding to a number of different receptors designated RARs α, β, and γ, RXRs α, β, and γ, and their subtypes. These are members of the steroid-thyroid-retinoid nuclear receptor superfamily and form heterodimeric combinations among themselves and other nuclear receptors to transactivate gene expression through site-specific DNA binding. While all-*trans* RA binds specifically to RARs, 9-*cis* RA can bind to both RARs and RXRs. Differential expression of RA receptors has been reported in the different pulmonary structures by in situ hybridization (63). RAR-α and RXR-α/β are ubiquitously expressed in the lung. RAR-β2 mRNA is present in the foregut endodermal precursor of the lung bud. As branching proceeds, it becomes restricted to the trachea and the most proximal bronchi and is not detected in distal lung epithelium. RAR-γ expression is limited to tracheal and bronchial mesenchyme. Whereas the null mutation of a single receptor subtype generally lead to little developmental alteration, double null mutations in RARs α and β result in a variety of abnormalities in many organs, including various lung developmental defects from agenesis to simple growth retardation (118). These abnormalities, however, show little correlation with spatial distribution of RARs in the lung (RXRs are ubiquitous) and provide no information about a role for these receptors in regional specification. Other knockout experiments (reviewed in ref. 39) have not led to any aberrant lung phenotype. Conversely, when the gene of the receptor subtype RAR-β4 was overexpressed in transgenic mice, alveolar hyperplasia and an excess of alveolar type II cells were observed (17), which suggests a role for RA in the control of type II cell differentiation and the involvement of RAR-β4 in the process. A putative RXR responsive element has been found in the third intron of the rat SP-A gene (25), and the SP-B gene has been demonstrated to be transactivated by RAR/RXR heterodimers in the presence of 9-*cis* RA in the H441 adenocarcinoma cell line (189). In the latter study, the RA response element mediating RA stimulation of the human SP-B promoter was identified.

Since the capacity of RA to activate expression of homeotic genes is a fundamental characteristic feature in vertebrate development, the hypothesis that RA's effects on the lung may be mediated through changes in *Hox* gene expression must be considered. Retinoic acid markedly increased *Hoxa-5*, *Hoxb-5*, and *Hoxb-6* mRNA levels in lung explants (24), and that of *Hoxa-5* in lung fibroblasts (18). Retinoic acid also prevented the decrease in *Hoxb-6* and *Sonic hedgehog* expression that spontaneously occurs in cultured whole embryonic lung and reproduces the changes occurring during the course of in vivo development (41). It has been proposed (41) that retinoic acid acts to maintain high levels of expression of pattern-related genes in a fashion characteristic of the immature lung, thus promoting continued formation of proximal lung structures and preventing formation of typical distal lung structures

of the mature lung. It should be pointed out, however, that this conclusion was drawn from the results of embryonic lung culture experiments in the presence of high RA concentrations (up to 10^{-5} M).

On the whole, RA appears to be an important element in the control of lung development, including alveolar development and expression of alveolar markers, but its functions are not understood in detail. Current data are still insufficient to determine whether RA is a morphogen along the anterior–posterior axis of the developing lung, as has been shown in other organs. Further investigations are necessary to determine more precisely the local tissue level of retinoids and of cellular binding proteins that sequester retinoids, the distribution of the various RA receptors, and the developmental changes of these parameters. Figure 4.3 summarizes the putative

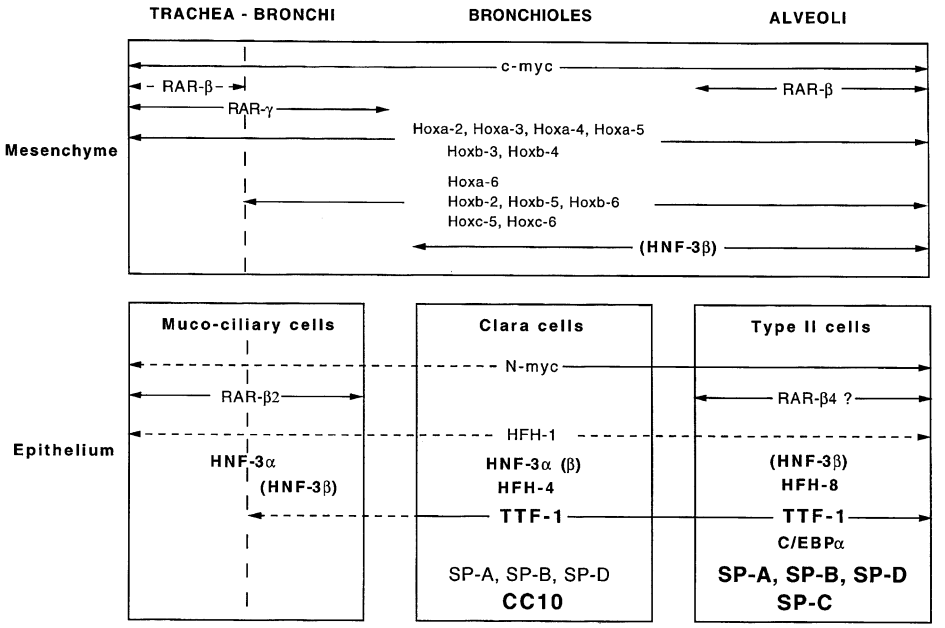

Fig. 4.3 Transcription factors potentially involved in anterior–posterior determination of epithelial cell fate in the developing lung and in Clara cell and alveolar type II cell differentiation. Ubiquitously expressed transcription factors are not shown. Factors are presented vertically in a putative hierarchical order, i.e., retinoic acid receptors (RARs) may control *Hox* gene expression, which may in turn determine expression of cell-specific transcription factors that would finally control cell-specific expression of markers (SP-A, -B, -C, -D, and CC10). For SP-A, -B, -D, *bold characters* indicate that the type II cell is the major source. *Dashed lines* indicate a diffuse weak expression, or, in the instance of TTF-1, contradictory data. *Parentheses* indicate that HNF-3β has been detected in one study but not in another one. Potential mesenchymal influences upon transcription factor expression by epithelium are not included. C/EBPα, CCAAT/enhancer binding protein α; HFH, hepatocyte nuclear factor 3/forkhead homologs; HNF, hepatocyte nuclear factor; TTF, thyroid transcription factor.

factors of anterior–posterior specification and specific transactivating factors of distal lung markers with their possible hierarchical interrelationships.

ROLE OF EXTRACELLULAR MATRIX COMPONENTS

Extracellular matrix is an essential element in the process of branching morphogenesis (see Chap. 1), but it also appears to participate in the control of cell phenotype expression. As stated above, type II cells cultured on tissue culture plastic rapidly flatten and loose their markers. Changes in lectin-binding pattern suggest that they acquire type I cell features (55, 61). Attempts have therefore been made to develop in vitro models supporting type II cell marker expression. The matrix extracted from the murine EHS tumor, which presents features similar to those of the *lamina densa* of basement membrane (98), has emerged as the most favorable substratum for adult (155) and fetal (22) type II cells. The mechanism by which this matrix functions is difficult to interpret, however, because its composition is complex, it includes not only typical basement membrane components but also various growth factors (98). Current evidence suggests that integrins, the cell receptors to matrix components, transmit signals via both the cytoskeleton and second messengers, and that these signals may in turn elicit specific gene expression (2, 89). It appears that formation of close, hollow, alveolar-like structures by type II cells on the EHS matrix and the fact that cells keep a cuboidal shape are crucial factors for sustained expression of their markers (22, 155). The role of cell shape has been illustrated in an investigation that consisted of seeding type II cells onto a collagen gel fixed on a culture dish, then detaching the gel to allow it to float and shrink (154). The cells spread out on fixed collagen, as when cultured directly on plastic, and lost their markers, but when their collagen substratum was detached, they retrieved their cuboidal shape and differentiation markers, including lamellar bodies and surfactant proteins. Further investigation (54) has shown that cells acquire membrane markers of type I cells on attached gels and that this is reversed when the gel is detached. The potential to express type II cell markers therefore appears to be retained in type I cells, and the type II–to–type I cell conversion represents a reversible transdifferentiation process. Since interactions with multiple matrix components are not involved in these elegant experiments, it appears that geometric constraint, which is likely to induce major changes in the cytoskeleton, plays a major role in the control of cell-specific feature expression.

Attempts have been made to explore the role of individual matrix components in growth and differentiation of type II cells. Whereas fibronectin appears to enhance DNA synthesis in isolated type II cells (137), laminin appears to play a role in the preservation of type II cell morphology and function, as cells flattened on an anti-laminin serum-treated EHS matrix (138). Epithelial cells adhere more readily to laminin than fibroblasts, possibly because of higher expression of α_6 integrin subunit and specific expression of α_3 integrin subunit (36, 187). Laminin may play a key role in alveolar morphogenesis, particularly its carboxy-terminal fragment desig-

nated *E8,* as an anti-E8 antibody prevented alveolar-like type II cell assembly on EHS matrix (116). A peptide sequence SINNNR in this domain was demonstrated to mediate cation-dependent adhesion of type II cells to the matrix (116). It should be pointed out, however, that type II cells seeded onto purified laminin or fibronectin spread, lose their features, and develop type I cell markers with kinetics similar to those on uncoated plastic (55). This suggests that association with other matrix components is needed for the differentiation and morphogenetic responses to laminin. Collagens have been found to have no effect on DNA or on surfactant phosphatidylcholine synthesis by isolated type II cells (3). Changes in lung glycosaminoglycans that occur in the course of development appear to be temporally related with epithelial differentiation and alveolarization (reviewed in ref. 30). Caniggia et al. (37) have shown that while fibroblasts in close proximity to the epithelium produce and secrete mainly hyaluronan, more distant fibroblasts shift from producing principally heparan sulfate and chondroitin sulfate during the pseudoglandular stage to producing hyaluronan during the canalicular stage of lung development. Because this shift to hyaluronan production occurs simultaneously with the thinning of the alveolar septal wall, the authors suggest that this developmentally regulated glycosaminoglycan production may facilitate epithelial–fibroblast interaction, thus influencing fetal lung growth and cell differentiation.

Little is known about the mechanisms that drive type I cell differentiation in vivo. Type I cells overlie a fused basement membrane that is shared with the vascular endothelium. It had been observed in the developing human lung (104) that cuboidal cells start decreasing in height at focal points where blood vessels contact with the epithelium. Type I cell differentiation is therefore probably induced by endothelial cells. This assumption had not received experimental support until Adamson and Young (4) studied rat lung type II cell behavior on a matrix secreted by a pulmonary vascular endothelial cell line. They observed that after an initial burst of growth, cell proliferation dropped while cells acquired type I cell features, as judged by morphology and *Bauhinia purpurea* lectin binding. These temporal changes reproduce the in vivo process of re-epithelialization that follows type I cell necrosis and denudation of the capillary endothelial basement membrane. Although the components of endothelial basement membrane controlling this process are not yet defined, it is clear that different microdomains must exist in the alveolar basement membrane beneath type I and II cells and that basement membrane produced by endothelial cells provides information first for epithelial proliferation, then for differentiation of type I from type II cells.

ENDOCRINE AND PARACRINE MODULATORS OF GENE EXPRESSION

It is beyond the scope of this chapter to extensively review the role of diffusible factors in the control of alveolar cell development. Only those recent studies relevant to the present purpose are considered here.

Glucocorticoids

Glucocorticoid hormones play crucial roles in architectural maturation of the pulmonary gas-exchange region (reviewed in ref. 114) and in biochemical lung maturation. For architectural maturation, glucocorticoids inhibit epithelial cell growth and alveolar septation and accelerate thinning of the alveolar walls. In neonatal rats, exogenous glucocorticoids induce a precocious termination of the lung developmental process. The clinical relevance of this finding pertains to the use of glucocorticoid hormones in the fetus for accelerating biochemical lung maturation and in the neonate as part of the treatment of RDS and BPD, since the lungs of infants with BPD fail to septate (112). For biochemical maturation, glucocorticoids were historically the first modulators of lung maturation to be recognized. Their key role in the control of lung maturation is well established, and they are known to stimulate in vivo the synthesis of all surfactant components, which has led to their use in the prevention of RDS. Their actions, however, are complex and not always clearly understood at the molecular level.

Insight into the requirement of glucocorticoids for lung development has recently been gained from the gene inactivation approach. One strategy consisted of creating a mammalian model of corticotropin-releasing hormone (CRH) deficiency by targeted mutation in mouse embryonic stem cells (128). This resulted in a markedly atrophic appearance of the zona fasciculata of the adrenal gland, a reduced basal blood level of corticosterone, and impaired corticosterone response to stress. Homozygous CRH-deficient mice born to heterozygous mothers developed normally and exhibited normal viability and fertility. Mating between homozygous animals, however, yielded progeny that died within the first 12 h of life, despite a normal appearance at birth and a normal length of pregnancy. Corticosterone supplementation to pregnant homozygous females resulted in the production of viable litters, demonstrating that maternal corticosteroids in heterozygous pregnant mice are sufficient to compensate for the fetal deficit. Histologic examination of newborn lungs revealed lung dysplasia in the offspring of homozygous mating, including marked hypercellularity, thickened alveolar septae, and paucity of air spaces. SP-B mRNA was reduced to 44% of the wild-type value. Corticosterone treatment completely reversed lung abnormalities. Thus, the study revealed a fetal glucocorticoid requirement for lung maturation, but postnatally, despite marked glucocorticoid deficiency, mice exhibited normal growth, fertility, and longevity, which suggests that the major role of glucocorticoids is during fetal rather postnatal life. In another study (51), the glucocorticoid receptor (GR) gene was disrupted. Similar to the CRH deficiency, homozygous GR-deficient mice developed normally in utero, but died within a few hours after birth because of respiratory failure. Lung development appeared to be impaired from gestational day 15.5, i.e., it arrested in a pseudoglandular state. SP-A, SP-B, and SP-C gene expression did not seem to be markedly impaired, but protein content was not explored. Other abnormalities included blocked adrenergic chromaffin cell development and reduced capacity to activate genes for key gluconeogenic enzymes.

The molecular mechanism of action of glucocorticoids in the control of surfactant synthesis remains imperfectly understood. With regard to the phospholipid moiety of surfactant, an indirect action through stimulated production by the lung fibroblast of a peptide mediator that in turn stimulates phospholipid metabolic pathway was postulated long ago; the putative mediator has not yet been characterized, however (see Chap. 11). Medium conditioned by fibroblasts in the presence of cortisol enhanced the expression of fatty acid–synthesizing enzymes at a pretranslational level in isolated type II cells (11), but this observation was made with type II cells cultured on plastic. In fetal lung explants, dexamethasone did not increase the mRNA level of the rate-limiting enzyme of phosphatidylcholine synthesis, CTP:phosphocholine cytidylyltransferase (68), which supports the concept that the increased activity of this enzyme by glucocorticoids is due to increased fatty acid synthesis.

The actual impact of glucocorticoids on surfactant proteins remains unclear, despite many investigations in this area. Whereas stimulatory effects of glucocorticoids have invariably been observed for SP-B and SP-C gene expression in vivo, in lung explant culture, or in pulmonary adenocarcinoma cell line, SP-A gene expression has been found to be either increased or decreased, or submitted to a biphasic control, i.e., enhanced at low glucocorticoid concentrations and diminished at higher concentrations. Differences among the species, the use of in vivo or in vitro models, the hormonal dose, or length of exposure may account for these conflicting data. Adrenalectomy in the rat showed that glucocorticoids play a minor role in SP gene expression, at least in the adult, and have rather limited translational or post-translational effects (66).

The role of glucocorticoids in the control of SP gene transcription is unclear. Although putative glucocorticoid regulatory elements (GRE) have been evidenced in the 5′-flanking sequence of the SP-A and SP-B genes (102, 180, 181) their role in the regulation of transcription has not been established. No GRE consensus sequence was found in the entire structural SP-A gene in the rabbit (44), despite enhanced transcription of the SP-A gene at all glucocorticoid doses in fetal lung explants of this species (23). When a reporter gene construct including a large portion of the 5′-flanking region of the SP-A gene was transfected in type II cells or in two lung adenocarcinoma cell lines, not only did glucocorticoids fail to increase its transcription but the stimulation of its transcription by cyclic AMP (see below) was unexpectedly antagonized by dexamethasone in a dose-dependent manner (6). Three possible glucocorticoid receptor–binding sites with modest homology to the consensus GRE that appeared to overlap the cAMP-response element (CRE) may account for this inhibition of expression (6). By contrast, evidence for direct stimulation of SP-D expression at the transcriptional level has been demonstrated in transfection experiments with constructs including a large 5′-flanking sequence of the human SP-D gene (142).

Glucocorticoid effects on the developing lung appear to involve interaction with other regulatory factors. Another possible mechanism of inhibited SP-A transcription in lung explants might be through diminished prostaglandin secretion (1). Glu-

cocorticoids also appear to interact with transforming growth factor (TGF) β expression in the developing lung, but conflicting data have been reported. Glucocorticoids were reported by some investigators to down-regulate TGF-β2 and TGF-β3 transcripts in mouse lung (86, 117), whereas others found an induction of TGF-β3 by glucocorticoids in fetal rat lung fibroblasts (175). These contradictory findings, along with the complexity of TGF-β effects on the developing lung (see below), make it difficult to draw conclusions about glucocorticoid-TGF-β interactions. Finally, lung proteins designated A2, B, and D that bind the SP-B promoter at a NF 1 site have been recently characterized. Their cDNAs have been cloned and sequenced; all three proteins are novel (108, 109). Co-transfection of a reporter construct containing 212 bp from the SP-B promoter together with a protein A2 or B expression vector in a lung adenocarcinoma cell line resulted in slightly increased reporter gene expression that was further enhanced by glucocorticoids in the instance of protein B (109). The protein D carboxy-terminal end contains a modified leucine zipper-like DNA-binding motif. Co-transfection with a reporter construct containing 212 bp of the SP-B promoter together with a protein D expression vector led to strongly enhanced reporter gene expression in the presence of dexamethasone only (108). At least for SP-B regulation, glucocorticoid responsiveness appears to involve additional gene transactivating factors that are distinct from the glucocorticoid receptor.

As a whole, glucocorticoids appear to accelerate surfactant accumulation in fetal lung through complex mechanisms that involve cell-specific transcription factors and balanced control of other modulating factors. Moreover, in view of the precocious and marked delay of overall lung development reported in GRH or GR knockout models, it is likely that glucocorticoids play multiple roles and elicit expression of a number of genes other than those of the specific markers of distal lung. The control of some genes may be a prerequisite for expression of the others, thus illustrating the concept of glucocorticoid permissive effect. Clarification of these mechanisms calls for further investigations, especially into the cascade of successive gene activations/inactivations.

Cyclic Adenosine 3′,5′ Monophosphate (cAMP) and cAMP Increasing Agents

Cyclic AMP has been shown to be a potent positive regulator of SP-A gene expression. It markedly increases the transcription rate of the SP-A gene in fetal lung explant culture. Two enhancers have been identified in the 5′-flanking region of the rabbit SP-A gene, whose mutagenesis resulted in a marked reduction in basal and cAMP-stimulated fusion gene expression in transfected type II cells (73). A putative c-AMP response element (CRE) that differs from the consensus CRE by one nucleotide only has been identified in this promoter by the same group and is presumed to mediate transactivation by cAMP of a reporter gene in transfected type II cells grown on extracellular matrix prepared from Madin-Darby canine kidney cells (6). Cyclic AMP did not enhance expression of the reporter gene in type II–related cell lines that do not express SP-A in vitro, despite a relatively high basal rate of

transcription in these cells (6). Type II cell–specific transactivator(s) of the CRE or, alternatively, some component of the cAMP response pathway is absent from these cell lines. More recently, mutagenetic study of the SP-A CRE led to the conclusion that this site does not bind classical CRE ligands of the CRE-binding protein/activating transcription factor family, but rather an orphan member of the steroid receptor family (120).

The finding that terbutaline, a β2-adrenergic agonist, increased the accumulation of SP-A in human fetal lung explants (131), suggests a role of catecholamines in the regulation of SP-A expression through β-adrenergic receptors and cAMP. Prostaglandin E_2 synthesis may also promote increased cAMP formation with consequent type II cell differentiation and SP-A gene expression (1).

In comparison, cAMP appears to have more modestly enhancing effects on SP-B and SP-C mRNA levels in rabbit or human lung. Cyclic AMP is nevertheless a stimulating factor of SP-B gene transcription in organ cultures of fetal rabbit lung. Evidence has recently shown that the stimulation of SP-B expression by cAMP is mediated by protein kinase A (PKA)-dependent activation of TTF-1 through the phosphorylation of a site at its NH_2 terminus (191). Consistent with this finding is the fact that a DNA homology search revealed no CRE consensus element within the concerned region of the SP-B promoter, suggesting that the stimulatory effects of PKA were not mediated by direct activation of CRE elements in the SP-B gene (191).

Epidermal Growth Factor and Transforming Growth Factor α

Epidermal growth factor (EGF) has long been recognized as an important control factor of both lung branching growth (see Chap. 1) and lung maturation (reviewed in ref. 30). Location of EGF and TGF-α mRNAs principally in mesenchyme of fetal human lung and of their common receptor (EGF-R) in epithelia suggests their probable role as paracrine mediators of mesenchymal–epithelial interactions (141). Since a high level of TGF-α has been found in fetal lung during the late canalicular and saccular stages when distal lung epithelial cells differentiate and mature (100), this factor may be involved primarily in the corresponding control mechanisms.

Both TGF-α and EGF stimulated type II cell proliferation, and this effect was antagonized by TGF-β (143). Epidermal growth factor also enhanced phosphatidylcholine synthesis in isolated type II cells, particularly when maintained on a basement membrane matrix (69, 43). Since EGF also increased SP-A mRNA and protein in human fetal lung in culture in a dose-dependent fashion (183), EGF and TGF-α both appear to be proliferative and differentiation factors for the alveolar epithelium. Despite this fact, no attempts to further analyze their effects on gene expression have been made.

The EGF-R gene has been invalidated in the mouse (122). Homozygous EGF-R-deficient mice died within the first 8 postnatal days. They exhibited growth retardation and a number of abnormalities, including marked lung abnormalities and breathing problems. Their lungs were condensed, with collapsed alveoli and dilated

terminal bronchioles closer to the pleura. Alveolar septa were thicker and more cellular, which is typical of lung immaturity. SP-A and proSP-C immunoreactivity was reduced. This picture resembles that of human neonatal RDS. These findings demonstrate the involvement of EGF-R and therefore of EGF and/or TGF-α in lung development, especially in the morphogenesis and cell maturation of distal areas.

Keratinocyte Growth Factor (Fibroblast Growth Factor 7) and Hepatocyte Growth Factor/Scatter Factor

Both these heparin-binding growth factors have been reported to be present in lung-fibroblast conditioned medium and to synergistically mediate the proliferation-stimulating activity of this medium upon isolated type II cells (133). Keratinocyte growth factor (KGF) has also been shown to be a potent stimulus of type II cell multiplication in vivo through intratracheal administration (173). Again, the restriction of KGF gene expression in mesenchymal tissues and of KGF receptor [KGFR, a fibroblast growth factor receptor (FGFR)-2 splicing variant also designated FGFR2/IIIb] in epithelia (132), as well as the expression of hepatocyte growth factor/scatter factor (HGF/SF) in mesenchymal tissues and of the HGF/SF receptor (c-*met*) in epithelia (161) suggest their implication as mediators of mesenchymal–epithelial interactions in the developing lung. The KGF and KGFR mRNAs have indeed been found to be expressed early in the course of embryonic rat lung development, during the pseudoglandular stage (15, 157), and have been proposed to be involved in the control of early branching morphogenesis (see Chap 1).

With regard to marker expression, it has been shown that exogenous KGF is a potent enhancer of SP-A and SP-B mRNA steady-state levels in isolated type II cells from the adult rat lung (169). In cultured whole embryonic rat lung rudiments, KGF was found to elicit formation of cystic structures filled with fluid and limited by an epithelium that displayed characteristic features of alveolar type II cells, including lamellar bodies and expression of SP-C mRNA (157). This time, the SP-A mRNA was not enhanced, although there was strong stimulation of SP-C mRNA expression. More recently, using cultures of mesenchyme-free embryonic mouse lung epithelium on EHS matrix, it was consistently shown that KGF induced cystic structure formation and a precocious differentiation of type II cells with advanced SP-A and SP-B expression, but SP-C expression seemed to be reduced (40). In type II cells isolated from fetal rat lung during the late gestational period, when surfactant storage takes place, and cultured on the EHS matrix, KGF not only enhanced expression of all three SP-A, SP-B, and SP-C proteins but also stimulated phosphatidylcholine synthesis and storage through the increase of fatty acid synthase gene expression and activity, and the increase of choline phosphate cytidylyltransferase activity (43). Compared with other growth factors, KGF appeared to be the most potent stimulating factor (43). Differences in the models may account for these partially discrepant findings. Nevertheless, taken together, these investigations suggest that KGF is a stimulus of distal lung development and that it plays a role in the differentiation of type II cells. Acidic FGF (FGF-1), which also binds the KGFR, mimicked the effects of

KGF, although at a higher concentration, in three studies (43, 157, 169), but failed to induce type II cell differentiation in the fourth one (40). The HGF/SF neither influenced expression of SP mRNAs (169) nor stimulated phospholipid synthesis (43) nor affected lung morphogenesis in vitro (157), but it was shown to induce formation by LX-1 lung carcinoma cells of pseudo-alveolar structures lined with thin type I–like cells (33). A possible involvement of HGF/SF in the type II–to–type I cell conversion can therefore not be ruled out.

Several genetic manipulations have been made in the mouse to explore the role of KGF and KGFR in lung development. The key role of KGF transduction signal for lung development has been illustrated by lung-targeted expression of a dominant-negative FGFR2 placed under the control of the SP-C promoter for targeting the transgene expression specifically to the lung epithelium (134). Newborn mice expressing the transgene were completely normal, except that instead of having normally developed lungs, they had two unbranched, undifferentiated epithelial tubes that extended from the bifurcation of the trachea down to the diaphragm (see Figure 12.4c). No expression of the endogenous SP-C, chosen as a molecular marker of epithelial differentiation, was detected. Surprisingly, invalidation of the KGF gene (79) led to no obvious abnormality, which suggests that alternative ligands to KGFR, for instance, FGF-1 (40) or FGF-10 (15), play the major role in the KGFR-mediated events of lung morphogenesis. Misexpression of KGF in the lung epithelium of mice rendered transgenic for a human KGF gene fused to the SP-C promoter (158) led to embryonic lethality and to profound disturbance in fetal lung branching, with cystic lungs composed of exaggerated large airways, a deficit in small branching airways, and numerous dilated saccules lined by an immature, columnar epithelium that was unable to produce surfactant proteins B and C despite abundant expression of their genes. Morphological findings are consistent with those obtained with exogenous KGF in embryonic lung cultures (157). The absence of SP-B and SP-C may appear surprising, but the unregulated expression of KGF in epithelial cells that normally do not elaborate it may have profoundly disturbed a regulatory mechanism that implies interaction between distinct cell types in the normal lung. Moreover, the KGF mRNA level has been shown to increase steadily in rat lung during late saccular and early alveolar stages (58, 157). In the transgenic model, the precocious presence of high levels of KGF in the lung bud may therefore have impaired lung development at a stage when the bronchiolar tree is not completed.

More in-depth investigations are necessary to determine the underlying mechanisms that control gene expression, but taken together, these data indicate that KGF and related factors of the FGF family that are acting through the KGFR are important regulatory mediators in lung development. Taking into account the branching effects of FGF-1 (40) and FGF-10 (15), these factors may play a major role in branching morphogenesis, while KGF may control alveolar cell differentiation. Like EGF and TGF-α, KGF is both a mitogenic and differentiation factor of the alveolar epithelium. In this respect, it could become useful for the prevention of RDS through acceleration of lung maturation in utero and for the treatment of epithelial lung injury, including BPD.

Transforming Growth Factor β and Related Substances

Factors of the TGF β family have multiple roles in lung development. Three different TGF-β isoforms are present in mammals, and each has different effects on lung development. TGF-β1 appears to regulate early morphogenesis and extracellular matrix deposition in the developing lung (see Chap. 1). In distal parts of the lung, it inhibits histodifferentiation and the synthesis of SP-A and phospholipids (38, 72, 172, 183). The TGF-β-related Müllerian inhibiting substance, which is responsible for the Müllerian duct regression in the male, has also been reported to inhibit surfactant synthesis and has been proposed to be involved in the delay of lung maturation in males (reviewed in ref. 30). Conversely, TGF-β has been reported to enhance expression of laminin and of α and β subunits of its integrin cell receptors in alveolar epithelial cells, an effect potentially linked to their differentiation process (101). TGF-β1, which inhibited branching morphogenesis in a dose-dependent and reversible manner, also inhibited N-*myc* expression in cultured mouse lung bud (150). It appears, therefore, that reduced levels of N-*myc* resulting from TGF-β1 treatment may have impaired epithelial cell proliferation in lung buds and consequently have affected branching. Expression in the mouse of a TGF-β1 transgene targeted to respiratory epithelial cells by the SP-C promoter led to an arrest of lung development in the pseudoglandular stage (194). Contrary to branching morphogenesis of the bronchial tree, which was only slightly delayed, dilation of terminal buds, thinning of mesenchyme, and epithelial cell differentiation were markedly impaired, including failure to express marker proteins such as CC10 and pro-SP-C also occurred. In addition, the distribution of smooth muscle actin was abnormal, which may account at least in part for the limited expansion of distal leading edges of terminal buds. Consistent with the probable implication of N-*myc* in distal lung morphogenesis is the finding that reduced N-*myc* expression could be involved in these abnormalities. These findings are in keeping with the concept that alveolar epithelial differentiation implies that the inhibition exerted by a TGF-β-like activity produced at early developmental stages by lung mesenchyme is released (172).

Little is known about the role of TGF-β2 in lung development. TGF-β2 knockout mice are born cyanotic, exhibit respiratory distress, and die within minutes (144). Histological examination of the lung did not reveal morphological defects. Collapsed conducting airways were observed, but surfactant was unexplored (144). The role of TGF-β3 is better documented and appears to be crucial. The TGF-β3 null mutant mice exhibited grossly abnormal lung development, including pseudoglandular histology, alveolar hypoplasia, mesenchymal thickening and hypercellularity, and decreased pro-SP-C immunoreactivity (90). This study indicates that this TGF-β isoform is necessary for normal lung development and that, in contrast to TGF-β1, TGF-β3 favors alveolar cytodifferentiation. Transforming growth factor β3 has also been shown to control tropoelastin expression by the fetal lung fibroblast, which is consistent with a possible involvement in alveolarization process (192).

Studies on localization of TGF-β isoforms in the developing lung do not provide much insight into their functions. Through in situ hybridization, TGF-β1 mRNA

has been found to be present in both mesenchyme and epithelium (74, 149). While in situ hybridization detected TGF-β2 expression in epithelial cells of the growing tips of developing bronchioles in the human and mouse (74, 123, 149), the immunoreactive peptide was detected in isolated fibroblasts but not in isolated epithelial cells from the developing rat lung (56). TGF-β3 expression appears to be restricted to the columnar epithelial cells of future airways and to be absent in the epithelium of growing end buds or in differentiated alveolar epithelium (123). Since mesenchymal cells express the TGF-β3 gene (123, 149) and contain the immunoreactive peptide (56), this absence from distal lung epithelial cells is consistent with a possible mediation of a mesenchymal–epithelial interaction related to the role of TGF-β3 in alveolar cytodifferentiation.

The TGF-β receptors belong to a superfamily of transmembrane serine-threonine kinases. Three different-sized classes termed types I, II, and III receptors have been identified, although the precise role of each of those in TGF-β signal transduction is not clearly established. Consistent with the involvement of TGF-β in the development of alveolar cells is the finding that the TGF-β receptor type II displays maximal expression in fetal rat lung around birth and is expressed along a proximal-distal gradient. Whereas the cuboidal epithelium of the presumptive alveolar tubes shows intense expression of the receptor, only a low level of expression is detectable in the columnar epithelium of the large proximal conducting airways (193).

Bone morphogenetic protein-4 (BMP-4), a TGF-β-related substance, also appears to play a role in lung morphogenesis and cytodifferentiation (16). The *Bmp-4* gene and the *Sonic hedgehog* (*Shh*) gene, the product of which appears to play a role in *Bmp-4* regulation, have been shown to be expressed at high levels in the distal tips of the growing mouse lung tubules, in contrast with the HNF-3β gene, which is expressed uniformly throughout the epithelium. Moreover, BMP-4 is also present in the mesenchyme adjacent to the most distal epithelium. Its expression declines during late gestation. Overexpression of *Bmp-4* in mice transgenic for a SP-C promoter-*Bmp-4* construct led to abnormal lung development. Transgenic lungs were about half the size of normal lungs and had fewer, greatly distended, epithelial terminal buds separated by abundant mesenchyme. Epithelial cell multiplication was reduced and mesenchymal cell death was enhanced. Whereas the pattern of CC10 expression appeared unchanged, the number of SP-C-expressing cells was greatly diminished. It should be stressed that contrary to normal *Bmp-4* expression, which is limited to the tips of distal lung tubules, the transgene was expressed throughout the distal epithelium. Differences from SP-C- driven TGF-β1 overexpression (194) should be pointed out: whereas TGF-β1 overexpression led to arrested development at the pseudoglandular stage, with a reduced expression of both SP-C and CC-10 but no lung size reduction, *Bmp-4* overexpression led to decreased lung growth and specific reduction in expression of alveolar markers. The factor BMP-4 therefore appears to be associated with growth of the lung epithelial tree, and a down-regulation of its expression may be a prerequisite for alveolar cell differentiation. It is questionable whether BMP-4 is the mediator of the TGF-β-like activity evidenced in previous investigations (172). In a more recent study (14), it was shown that *Shh* overexpres-

sion targeted to the mouse lung affected lung growth, with increased mesenchymal and epithelial proliferation and absence of typical alveoli. Type II cells, however, displayed normal ultrastructure, and there was no change in the expression of *Bmp-4*, the KGF gene, or of the specific lung markers CC10 and SP-C. By contrast, the expression of *Ptc*, a murine homologue of the *Drosophila* segment polarity gene *Patched*, was up-regulated in the transgenic mice (14).

Tumor Necrosis Factor α and Interleukins

Although the proinflammatory cytokines, i.e., tumor necrosis factor α and various interleukins, may not have major function in lung developmental processes, they are worth brief mention because of their implication in acute respiratory distress syndrome (ARDS), neonatal RDS, and BPD. Tumor necrosis factor α has indeed been reported to be a potent inhibitor of SP-A, SP-B, and SP-C gene transcription and synthesis (10, 182), which would represent an aggravating factor in RDS and ARDS. A potential role of elevated IL-1 (140) and IL-8 (88) in neonatal RDS has been proposed in the development of BPD, while IL-6 has been assumed to be implicated in excessive alveolar fibroblast proliferation in interstitial lung diseases (151). Therapeutic approaches to these conditions should therefore aim at blockade of the cytokine-mediated inflammatory cascade.

Platelet-Derived Growth Factors

Platelet-derived growth factors (PDGFs) are dimers of two different peptide chains (AA or BB or AB) that interact with two related receptor tyrosine kinases designated PDGF receptor α (PDGFRα) and PDGFRβ. Only PDGFRα binds PDGF-A chains. PDGF-AA is involved in the mesenchymal-epithelial interactions that direct early lung branching (see Chap. 1). The recent production of PDGF-A null mice has provided evidence for the key inductory role that this mediator also plays later in development in the process of alveologenesis (29). The null allele has been shown to be homozygous lethal with two restriction points—one prenatally before embryonic day 10 and one postnatally. In the homozygous mutant mice that survived after birth, fetal and early postnatal lung development appeared to be normal, including the formation of prealveolar saccules, but postnatal alveolarization failed to take place. Emphysematous appearance and large areas of collapsed lung tissue (atelectasis) extended progressively, thus leading to death. The defect in alveologenesis appeared to result from the lack of septal smooth-muscle cells (alveolar myofibroblasts), with subsequent absence of elastin fiber deposition. In addition, PDGFRα-positive cells having the location of putative alveolar myofibroblast progenitors were specifically absent in PDGF-A null mutants. The ontogeny of alveolar myofibroblasts therefore appears to be a prerequisite for alveolar septal formation, most likely because these cells are the source of elastin, and the specification of alveolar myofibroblasts is clearly dependent upon the presence of PDGF-A. Because epithelial cells appear to be the source of PDGF-A, myofibroblast differentiation is likely to be

under paracrine control exerted by epithelial cells in close apposition to the mesenchymal cells from which myofibroblasts derive. That differentiation of myofibroblasts is driven through positive direct induction of PDGFRα by PDGF-A appears to be a likely assumption. These remarkable findings illustrate that alveolar development implicates reciprocal action between the epithelium and mesenchyme, an idea that was formerly proposed for lung cell growth (5), and not exclusively one-way mesenchymal influence upon epithelial cell differentiation.

CONCLUSION

The control of gene expression in pulmonary epithelial cells has recently become a field of intensive research. Even if the question of how the fate of lung primordial cells is determined across the developmental process remains largely unanswered, a number of pieces of the puzzle have already been gathered. It appears that when the lung anlage emerges, its epithelial pulmonary identity is already imprinted, likely through positional information. Branching growth and differentiation of the various epithelial cell types are still imperfectly understood processes, but the crucial role of mesenchyme in their control, through actions of diffusible mediators or extracellular matrix components, is firmly established. Transcription factors determining lung-specific marker expression have been identified. Markers of the distal epithelium are early and basically expressed genes, and cell differentiation along an anterior–posterior axis appears as either a repression or reinforcement of their expression in proximal and distal areas, respectively. The same genetic and epigenetic factors control both morphogenesis and lung-specific gene expression. A complex, subtle balance between various mediators with developmentally adjusted changes appears to control morphogenetic and cell maturational events. Multiple approaches are necessary to explore mechanisms more fully. Tumoral or transformed cell lines expressing a particular cell phenotype will certainly be useful. Transgenic models and null mutations have yielded and will continue to produce important new insights (see Chap. 13). It should be pointed out, however, that gene inactivation has led to somewhat disappointing findings in some instances because it sometimes induced little change (e.g., KGF gene), or conversely, the developmental consequences were so dramatic and precocious that no information was provided for the understanding of organ formation (e.g., HNF-3β gene). In this respect, the use of dominant-negative transgenes or conditional gene-expression/inactivation systems, such as Cre-LoxP, would be especially informative. Other strategies, such as differential display RT-PCR using oligo-dT primers and random 5′ oligonucleotides, or investigations of mammalian homologues of genes important for the development of invertebrates (for instance the *Drosophila*'s tubulogenesis driver *trachealess*) may help to identify novel gene expression in the lung. All these approaches, along with investigations of exogenous factors capable of accelerating or facilitating normal processes, should provide the molecular bases for new treatments of various lung diseases.

REFERENCES

1. Acarregui, M.J., J.M. Snyder, M.D. Mitchell, and C.R. Mendelson. Prostaglandins regulate surfactant protein A (SP-A) gene expression in human fetal lung in vitro. *Endocrinology* 90: 1105–1113, 1990.
2. Adams, J.C. and F.M. Watt. Regulation of development and differentiation by the extracellular matrix. *Development* 117: 1183–1198, 1993.
3. Adamson, I.Y.R., G.M. King, and L. Young. Influence of extracellular matrix and collagen components on alveolar type 2 cell morphology and function. *In Vitro Cell. Dev. Biol.* 25: 494–502, 1989.
4. Adamson, I.Y.R. and L. Young. Alveolar type II cell growth on a pulmonary endothelial extracellular matrix. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L1017–L1022, 1996.
5. Adamson, I.Y.R., L. Young, and G.M. King. Reciprocal epithelial:fibroblast interactions in the control of fetal and adult rat lung cells in culture. *Exp. Lung Res.* 17: 821–835, 1991.
6. Alcorn, J.L., E. Gao, Q. Chen, M.E. Smith, R.D. Gerard, and C.R. Mendelson. Genomic elements involved in transcriptional regulation of the rabbit surfactant protein-A gene. *Mol. Endocrinol.* 7: 1072–1085, 1993.
7. Ang, S.L. and J. Rossant. HNF-3β is essential for node and notochord formation in mouse development. *Cell* 78: 561–574, 1994.
8. Ang, S.L., A. Wierda, D. Wong, K.A. Stevens, S. Cascio, J. Rossant, and K.S. Zaret. The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of the HNF3/*forkhead* proteins. *Development* 119: 1301–1315, 1993.
9. Aubin, J., M. Lemieux, M. Trembly, J. Berard, and L. Jeannotte. Early postnatal lethality in Hoxa-5 mutant mice is attributable to respiratory tract defects. *Dev. Biol.* 192: 432–445, 1997.
10. Bachurski, C.J., G.S. Pryhuber, S. Glasser, S.E. Kelly, and J.A. Whitsett.Tumor necrosis factor-α inhibits surfactant protein C gene transcription. *J. Biol. Chem.* 270: 19402–19407, 1995.
11. Batenburg, J.J. and R.H. Elfring. Pre-translational regulation by glucocorticoid of fatty acid and phosphatidylcholine synthesis in type II cells from fetal rat lung. *FEBS Lett.* 307: 164–168, 1992.
12. Bedetti, C.D., J. Singh, G. Singh, S.L. Katyal, and M.L. Wong-Chong. Ultrastructural localization of rat Clara cell 10kD secretory protein by the immunogold technique using polyclonal and monoclonal antibodies. *J. Histochem. Cytochem.* 35: 789–794, 1987.
13. Behringer, R.R., D.A. Crotty, V.M. Tennyson, R.L. Brinster, R.D. Palmiter, and D.J. Wolgemuth. Sequences 5′ of the homeobox of the hox-1,4 gene direct tissue specific expression of *lacZ* during mouse development. *Development* 117: 823–833, 1993.
14. Bellusci, S., Y. Furuta, M.G. Rush, R. Henderson, G. Winnier, and B.L.M. Hogan. Involvement of Sonic hedgehog (*Shh*) in mouse embryonic lung growth and morphogenesis. *Development* 124: 53–63, 1997.
15. Bellusci S., J. Grindley, H. Emoto, N. Itoh, and B.L.M. Hogan. Fibroblast growth factor 10 (FGF 10) and branching morphogenesis in the embryonic mouse lung. *Development* 124: 4867–4878, 1997.
16. Bellusci S., R. Henderson, G. Winnier, T. Oikawa, and B.L.M. Hogan. Evidence from normal expression and targeted overexpression that Bone Morphogenetic Protein-4 (BMP-4) plays a role in mouse embryonic lung morphogenesis. *Development* 122: 1693–1702, 1996.

17. Berard, J., L. Gaboury, M. Landers, Y. de Repentigny, B. Houle, R. Kothary, and W.E.C. Bradley. Hyperplasia and tumors in lung, breast and other tissues in mice carrying a *RAR-β4*-like transgene. *EMBO J.* 13: 5570–5580,1994.
18. Bernacki, S.H., C. Nervi, T.M. Volberg, and A.M. Jetten. Homeobox 1.3 expression: induction by retinoic acid in bronchial fibroblasts. *Am. J. Respir.Cell Mol. Biol.* 7: 3–9, 1992.
19. Bingle, C.D. and J.D. Gitlin. Identification of hepatocyte nuclear factor-3 binding sites in the Clara cell secretory protein gene. *Biochem. J.* 295: 227–232, 1993.
20. Bingle, C.D. and S. Gowan. Molecular cloning of the *forkhead* transcription factor HNF-3α from a human pulmonary adenocarcinoma cell line. *Biochim. Biophys. Acta* 1307: 17–20, 1996.
21. Bingle, C.D., B.P. Hackett, M. Moxley, W. Longmore, and J.D. Gitlin. Role of HNF-3α and HNF-3β in Clara cell secretory protein gene expression in the bronchiolar epithelium. *Biochem. J.* 308: 197–202, 1995.
22. Blau, H., D.E. Guzowski, Z.A. Siddiqi, E.M. Scarpelli, and R.S. Bienkowski. Fetal type 2 pneumocytes form alveolar like structures and maintain long term differentiation on extracellular matrix. *J. Cell. Physiol.* 136: 203–214,1988.
23. Boggaram, V. and C. Mendelson. Transcriptional regulation of the gene encoding the major surfactant protein (SP-A) in rabbit fetal lung. *J. Biol. Chem.* 263: 19060–19065, 1988.
24. Bogue, C.F., I. Gross, H. Vasavada, D.W. Dynia, C.M. Wilson, and H.C. Jacobs. Identification of *Hox* genes in newborn lung and effects of gestational age and retinoic acid on their expression. *Am. J. Physiol.* 266 (*Lung Cell. Mol. Physiol.* 10): L448–L454, 1994.
25. Bogue, C.F., H.C. Jacobs, D.W. Dynia, C.M. Wilson, and I. Gross. Retinoic acid increases surfactant protein mRNA in fetal rat lung in culture. *Am. J. Physiol.* 271 (*Lung Cell. Mol. Physiol.* 15): L862–L868, 1996.
26. Bogue, C.F., L.J. Lou, and H.C. Jacobs. Expression of *Hoxb* genes in the developing mouse foregut and lung. *Am. J. Respir. Cell Mol. Biol.* 15: 163–171, 1996.
27. Bohinski, R.J., R. Di Lauro, and J.A. Whitsett. The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol. Cell. Biol.* 14: 5671–5681, 1994.
28. Bohinski, R.J., J.A. Huffman, J.A. Whitsett, and D.L. Lattier. *Cis*-active elements controlling lung cell-specific expression of human pulmonary surfactant protein B gene. *J. Biol. Chem.* 268: 11160–11166, 1993.
29. Boström, H., K. Willets, M. Pekny, P. Levéen, P. Lindhal, H. Hedstrand, M. Pekna, M. Hellström, S. Gebre-Medhin, M. Schalling, M. Nilsson, S. Kurland, J. Törnell, J.K. Heath, and C. Betscholtz. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* 85: 863–873, 1996.
30. Bourbon, J.R. and C. Fraslon. Developmental aspects of the alveolar epithelium and the pulmonary surfactant system. In: *Pulmonary Surfactant: Biochemical, Functional, Regulatory and Clinical Concepts*, edited by J.R. Bourbon. Boca Raton, FL: CRC Press, 1991, p. 257–324.
31. Braun, H. and G. Suske. Combinatorial action of HNF3 and Sp family transcription factors in the activation of the rabbit uteroglobin/CC10 promoter. *J. Biol. Chem.* 273: 9821–9828, 1998.
32. Breed, D.R., L.R. Margraf, J.L. Alcorn, and C. Mendelson. Transcription factor C/EBPδ in fetal lung: developmental regulation and effects of cyclic adenosine 3′,5′-monophosphate and glucocorticoids. *Endocrinology* 138: 5527–5534, 1997.

33. Brinkman, V., H. Foroutan, M. Sachs, K.M. Weidner, and W. Birchmeier. Hepatocyte growth factor/scatter factor induces a variety of tissue specific morphogenic programs in epithelial cells. *J. Cell Biol.* 131: 1573–1586,1995.
34. Bruno, M.D., R.J. Bohinski, J.E. Carter, K.A. Foss, and J.A. Whitsett. Structure and function of the mouse surfactant protein B gene. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L381–L389, 1995.
35. Bruno, M.D., R.J. Bohinski, K.M. Huelsman, J.A. Whitsett, and T.R. Korfhagen. Lung cell-specific expression of the murine surfactant protein A(SP-A) gene is mediated by interactions between the SP-A promoter and thyroid transcription factor-1. *J. Biol. Chem.* 270: 6531–6536, 1995.
36. Caniggia, I., J. Liu, R. Han, J. Wang, A.K. Tanswell, G. Laurie, and M. Post. Identification of receptors binding fibronectin and laminin on fetal rat lung cells. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L459–L468, 1996.
37. Caniggia, I., K. Tanswell, and M. Post. Temporal and spatial differences in glycosaminoglycan synthesis by fetal lung fibroblasts. *Exp. Cell Res.* 202: 252–258, 1992.
38. Caniggia, I., I. Tseu, R.N. Han, B.T. Smith, A. K. Tanswell, and M. Post. Spatial and temporal differences in fibroblast behavior in fetal rat lung. *Am. J. Physiol.* 261 (*Lung Cell. Mol. Physiol.* 5): L424–L433, 1991.
39. Cardoso, W.V. Transcription factors and pattern formation in the developing lung. *Am. J. Physiol.* 269 (*Lung Cell. Mol. Physiol.* 13): L429–L442, 1995.
40. Cardoso, W.V., A. Itoh, H. Nogawa, I. Mason, and J.S. Brody. FGF-1 and FGF-7 induce distinct patterns of growth and differentiation in embryonic lung epithelium. *Dev. Dyn.* 298: 398–405, 1997.
41. Cardoso, W.V., S.A. Mitsialis, J.S. Brody, and M.C. Williams. Retinoic acid alters the expression of pattern-related genes in the developing lung. *Dev. Dyn.* 207: 47–59, 1996.
42. Cardoso, W.V., M.C. Williams, S.A. Mitsialis, M. Joyce-Brady, A.K. Rishi, and J.S. Brody. Retinoic acid induces changes in the pattern of airway branching and alters epithelial cell differentiation in the developing lung in vitro. *Am. J. Respir. Cell Mol. Biol.* 12: 464–476, 1995.
43. Chelly, N., O.-B. Mouhieddine-Gueddiche, A.-M. Barlier-Mur, B. Chailley-Heu, and J.R. Bourbon. Keratinocyte growth factor enhances maturation of fetal rat lung type II cells. *Am. J. Respir. Cell Mol. Biol.* 20: in press, 1999.
44. Chen, Q., V. Boggaram, and C.R. Mendelson. Rabbit lung surfactant protein A (SP-A) gene: identification of a lung-specific DNAse I hypersensitive site in the 5′-flanking region. *Am. J. Physiol.* 262 (*Lung Cell. Mol. Physiol.* 6): L662–L671, 1992.
45. Chinoy, M.R., M.T. Antonio-Santiago, and E.M. Scarpelli. Maturation of undifferentiated lung epithelial cells into type II cells in vitro: a temporal process that parallels cell differentiation in vivo. *Anat. Rec.* 240: 545–554,1994.
46. Chisaka, O. and M.R. Capecchi. Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene *hox-1,5*. *Nature* 350: 473–479, 1991.
47. Civitareale, D., R. Lonigro, A.J. Sinclair, and R. Di Lauro. A thyroid-specific nuclear protein essential for tissue expression of the thyroglobulin promoter. *EMBO J.* 8: 2537–2542, 1989.
48. Clark, K.L., E.D. Halay, E. Lai, and K.S. Burley. Co-crystal structure of the HNF-3/fork head DNA recognition motif resembles histone H5. *Nature* 364: 412–420, 1993.
49. Clevidence, D.E., D.G. Overdier, R.S. Peterson, A. Porcella, H. Ye, K.E. Paulson, and

R.H. Costa. Members of the HNF-3/forkhead family of transcription factors exhibit distinct cellular expression patterns in lung and regulate the surfactant protein B promoter. *Dev. Biol.* 166: 195–209,1994.

50. Clevidence, D.E., D.G. Overdier, W. Tao, X. Qian, L. Pani, E. Lai, and R.H. Costa. Identification of nine tissue-specific transcription factors of the hepatocyte nuclear factor 3/forkhead DNA-binding domain family. *Proc. Natl. Acad. Sci. USA* 90: 3948–3952, 1993.
51. Cole, T.J., J.A. Blendy, A.P. Monaghan, K. Krieglstein, W. Schmid, A. Aguzzi, G. Fantuzzi, E. Hummler, K. Unsicker, and G. Schütz. Targeted disruption of the glucocorticoid receptor gene blocks chromaffin cell development and severely retards lung maturation. *Genes Dev.* 9: 1608–1621, 1995.
52. Costa, R.H., D.R. Grayson, and J.E. Darnell, Jr. Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and α1-antitrypsin. *Mol. Cell. Biol.* 9: 1415–1425, 1989.
53. Damante, G. and R. Di Lauro. Thyroid specific gene expression. *Biochim. Biophys. Acta* 1218: 255–266, 1994.
54. Danto, S.I., J.M. Shannon, Z. Borok, S.M. Zabski, and D.E. Crandall Reversible transdifferentiation of alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 12: 497–502, 1995.
55. Danto, S.I., S.M. Zabski, and E.D. Crandall. Reactivity of alveolar epithelial cells in primary culture with type I cell monoclonal antibodies. *Am. J. Respir. Cell Mol. Biol.* 6: 296–306, 1992.
56. De Bortoli, C., B. Chailley-Heu, and J.R. Bourbon. Production of transforming growth factor (TGF) β by fetal lung cells. *Biol. Cell* 84: 215–218, 1995.
57. Degryse, E., M.M. De Santi, M. Dietrich, D. Ali Hadj, J.-F. Spetz, D. Villeval, and G. Lungarella. A human SP-C promoter fragment targets a1-proteinase inhibitor gene expression to lung alveolar type II cells in transgenic mice. *Transgenic Res.* 5: 139–143, 1996.
58. Dekowski, S.A., M. Pajeau, and R.J. Panos. Keratinocyte growth factor and keratinocyte growth factor mRNA content in late gestation fetal and neonatal rat lung. *Pediatr. Res.* 39: 56A, 1996.
59. De Simone, V. and R. Cortese. Transcription factors and liver-specific genes. *Biochim. Byophys. Acta* 1132: 119–126, 1992.
60. Deterding, R.R. and J.M. Shannon. Proliferation and differentiation of fetal rat pulmonary epithelium in the absence of mesenchyme. *J. Clin. Invest.* 95: 2963–2972, 1995.
61. Dobbs, L.G., M.C. Williams, and A.E. Brandt. Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. *Biochim. Biophys. Acta* 846: 155–166, 1985.
62. Dobbs, L.G., M.C. Williams, and R. Gonzalez. Monoclonal antibodies to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. *Biochim. Biophys. Acta* 970: 146–156,1988.
63. Dollé, P., E. Ruberte, P. Leroy, G. Morriss-Kay, and P. Chambon. Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* 110: 1133–1151, 1990.
64. Evans, M.J. and S.G. Shami. Lung cell kinetics. In: *Lung Cell Biology*, Vol. 41, edited by D. Massaro. New York: Marcel Dekker, 1989, p. 1–36.
65. Farrell, P.M., J.R. Bourbon, R.H. Notter, L. Marin, L. Nogee, and J.A. Whitsett. Relationships among surfactant fraction lipids, proteins and biophysical properties in the developing lung. *Biochim. Biophys. Acta* 1044: 84–90, 1990.

66. Fisher, J.H., F. McCormack, S.S. Park, T. Stelzner, J.M. Shannon, and T. Hofmann. In vivo regulation of surfactant by glucocorticoids. *Am. J. Respir. Cell Mol. Biol.* 5: 63–70, 1991.
67. Flodby, P., C. Barlow, H. Kylefjord, L. Ährlund-Richert, and K.G. Xanthopoulos. Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein α. *J. Biol. Chem.* 271: 24753–24780, 1996.
68. Fraslon, C. and J.J. Batenburg. Pre-translational regulation of lipid synthesizing enzymes and surfactant proteins in fetal rat lung in explant culture. *FEBS Lett.* 325: 285–290, 1993.
69. Fraslon, C. and J.R. Bourbon. Comparison of effects of epidermal and insulin-like growth factors, gastrin-releasing peptide and retinoic acid on fetal lung cell growth and maturation in vitro. *Biochim. Biophys. Acta* 1123: 65–75, 1992.
70. Fraslon, C. and J.R. Bourbon. Retinoids control surfactant phospholipid biosynthesis in fetal rat lung. *Am. J. Physiol.* 266 (*Lung Cell. Mol. Physiol.* 10): L705–L712, 1993.
71. Fraslon, C., B. Chailley-Heu, J.J. Batenburg, R. Elfring, and J.R. Bourbon. Ontogeny of surfactant proteins and lipid-synthesizng enzymes in cultured fetal lung epithelial cells. *Am. J. Physiol.* 267 (*Lung Cell. Mol. Physiol.* 11): L375–L383, 1994.
72. Fraslon, C., T. Lacaze-Masmonteil, V. Zupan, B. Chailley-Heu, and J.R. Bourbon. Fetal rat lung type II cell differentiation in serum-free isolated cell culture: modulation and inhibition. *Am. J. Physiol.* 264 (*Lung Cell. Mol. Physiol.* 8): L504–L516, 1993.
73. Gao, E., J.L. Alcorn, and C.R. Mendelson. Identification of enhancers in the 5′-flanking region of the rabbit surfactant protein A (SP-A) gene and characterization of their binding proteins. *J. Biol. Chem.* 268: 19697–19709, 1993.
74. Gatherer, D., P.T. Dijke, D.T. Baird, and R.J. Arkhurst. Expression of TGF-β isoforms during first trimester human embryogenesis. *Development* 110: 445–460, 1990.
75. Gehring, W.J., M. Affolter, and T. Bürglin. Homeodomain proteins. *Annu. Rev. Biochem.* 63: 487–526, 1994.
76. Glasser, S.W., T.R. Korfhagen, S.E. Wert, M.D. Bruno, K.M. McWilliams, D.K. Vorbroker, and J.A. Whitsett. Genetic element from human surfactant protein SP-C gene confers bronchiolar-alveolar cell specificity in transgenic mice. *Am. J. Physiol.* 261 *(Lung Cell. Mol. Physiol.* 5*)*: L349–L356, 1991.
77. Guazzi, S., R. Lonigro, L. Pinotello, E. Bonicelli, R. DiLauro, and F. Mavilio. The thyroid transcription factor-1 is a candidate target for regulation by Hox proteins. *EMBO J.* 13: 3339–3347, 1994.
78. Guazzi, S., M. Price, M. De Felice, G. Damante, M.-G. Mattei, and R. DiLauro. Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. *EMBO J.* 9: 3631–3639, 1990.
79. Guo, L., L. Degenstein, and E. Fuchs. Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* 10: 165–175,1996.
80. Gupta, R.P., S.E. Patton, A.M. Jetten, and G.E.R. Hook. Purification, characterization and proteinase activity of a Clara-cell secretory protein from the pulmonary extracellular lining of rabbits. *Biochem. J.* 248: 337–344, 1987.
81. Hackett, B.P., S.L. Brody, M. Liang, I.D. Zeitz, L.A. Bruns, and J.D. Gitlin. Primary structure of hepatocyte nuclear factor/forkhead factor homologue 4 and characterization of gene expression in the respiratory and reproductive epithelium. *Proc. Natl. Acad. Sci. USA* 92: 4249–4253, 1995.
82. Hackett, B.P., N. Shimizu, and J.D. Gitlin. Clara cell secretory protein gene expression in bronchiolar epithelium. *Am. J. Physiol.* 262 *(Lung Cell. Mol. Physiol. 6)*: L399–L404, 1992.
83. Hirning, U., P. Schmid, W.A. Schultz, G. Rettenberger, and H. Hameister. A comparative

analysis of N-*myc* and c-*myc* expression and cellular proliferation in mouse organogenesis. *Mech. Dev.* 33: 119–126, 1991.

84. Ikeda, K., J.C. Clark, J.R. Shaw-White, M.T. Stahlman, C. Bouteil, and J.A.Whitsett. Gene structure and expression of human TTF-1 in respiratory epithelial cells. *J. Biol. Chem.* 270: 8108–8114, 1995.
85. Ikeda, K., J.R. Shaw-White, S. Wert, and J.A. Whitsett. Hepatocyte nuclear factor 3 activates transcription of thyroid transcription factor 1 in respiratory epithelial cells. *Mol. Cell. Biol.* 16: 3626–3636, 1996.
86. Jaskoll, T., H.A. Choy, and M. Melnick. The glucocorticoid-glucocorticoid receptor signal transduction pathway, transforming growth factor-β, and embryonic mouse lung development in vivo. *Pediatr. Res.* 39: 749–759,1996.
87. Johansson, J., T. Curstedt, and B. Robertson. The proteins of the surfactant system. *Eur. Respir. J.* 7: 372–391, 1994.
88. Jones, C.A., R.G. Cayabyab, K.Y.C. Kwong, C. Stotts, B. Wong, H. Hamsdan, P. Minoo, and R. de Lemos. Undetectable interleukin (IL)-10 and persistent IL-8 expression early in hyaline membrane disease: a possible developmental basis for the predisposition to chronic lung inflammation in preterm newborns. *Pediatr. Res.* 39: 966–975, 1996.
89. Juliano, R.L. and S. Haskill. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120: 577–585, 1993.
90. Kaartinen, V., J.W. Voncken, C. Shuler, D. Warburton, D. Bu, N. Heisterkamp, and J. Groffen. Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial mesenchymal interaction. *Nat. Genet.* 11: 415–421, 1995.
91. Kalina, M., R.J. Mason, and J.M. Shannon. Surfactant protein C is expressed in alveolar type II cells but not in Clara cells of rat lung. *Am. J. Respir. Cell Mol. Biol.* 6: 594–600, 1992.
92. Kappen, C. *Hox* genes in the lung. *Am. J. Respir. Cell Mol. Biol.* 15: 156-162, 1996.
93. Kasper, M., D. Schuh, and M. Muller. *Bauhinia purpurea* lectin (BPA) binding of rat type I pneumocytes: alveolar epithelial alterations after radiation-induced lung injury. *Exp. Toxicol. Pathol.* 46: 361–367, 1994.
94. Kawada, H., J.M. Shannon, and R.J. Mason. Improved maintenance of adult rat type II cell differentiation in vitro: effect of serum-free hormonally defined medium and a reconstituted basement membrane. *Am. J. Respir. Cell Mol. Biol.* 3: 33–43, 1990.
95. Kelly, S.E., C.J. Bachurski, M.S. Burhans, and S.W. Glasser. Transcription of the lung-specific surfactant protein C gene is mediated by thyroid transcription factor 1. *J. Biol. Chem.* 271: 6881–6888, 1996.
96. Khoor, A., M.E. Gray, W.M. Hull, J.A. Whitsett, and M.T. Stahlman.Developmental expression of SP-A and SP-A mRNA in the proximal and distal respiratory epithelium in the human fetus and newborn. *J. Histochem. Cytochem.* 41: 1311–1319, 1993.
97. Kimura, S., Y. Hara, T. Pineau, P. Fernandez-Salguero, C.H. Fox, J.M.Ward, and F.J. Gonzalez. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* 10: 60–69, 1996.
98. Kleinman, H.K., M.L. McGarvey, J.R. Hassell, V.L. Star, F.B. Cannon,G.W. Laurie, and G.R. Martin. Basement membrane complexes with biological activity. *Biochem. USA* 28: 312–318, 1986.
99. Kouretas, D., A.M. Karinch, A. Rishi, K. Melchers, and J. Floros. Conservation analysis of rat and human SP-A gene identifies 5′ flanking sequences of rat SP-A that bind rat lung nuclear proteins. *Exp. Lung Res.* 19: 485–503, 1993.

100. Kubiak, J., M.M. Mitra, R. Steve, J.D. Hunt, P. Davies, and B.R. Pitt.Transforming growth factor-α gene expression in late-gestation fetal rat lung. *Pediatr. Res.* 31: 286–290, 1992.
101. Kumar, N.M., S.L. Sigurdson, D. Sheppard, and J.S. Lwebuga-Mukasa. Differential modulation of integrin receptor and extracellular matrix laminin by transforming growth factor-β1 in rat alveolar epithelial cells. *Exp. Cell Res.* 221: 385–394, 1995.
102. Lacaze-Masmonteil, T., C. Fraslon, J. Bourbon, M. Raymondjean, and A. Khan. Characterization of the rat pulmonary surfactant protein A promoter. *Eur. J. Biochem.* 206: 613–623, 1992.
103. Lai, E., V.R. Prezioso, W. Tao, W. Chen, and J.E. Darnell, Jr. Hepatocyte nuclear factor 3A belongs to a gene family in mammals that is homologous to the *Drosophila* homeotic gene *fork head. Genes Dev.* 5: 416–427, 1991.
104. Langston, C., K. Kida, M. Reed, and W.M. Thurlbeck. Human lung growth in late gestation and in the neonate. *Am. Rev. Respir. Dis.* 129: 607–613, 1984.
105. Lazzaro, D., M. Price, M. De Felice, and R. Di Lauro. The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* 113: 1093–1104, 1991.
106. Li, F., E. Rosenberg, C.I. Smith, K. Notarfrancesco, S.R. Reisher, H. Shuman, and S.I. Feinstein. Correlation of expression of transcription factor C/EBPα and surfactant protein genes in lung cells. *Am. J. Physiol.* 269 (*Lung Cell. Mol. Physiol.* 13): L241–L247, 1995.
107. Liu, R., C.S. Harvey, and S.E. McGowan. Retinoic acid increases elastin in neonatal rat lung fibroblast cultures. *Am. J. Physiol.* 265 (*Lung Cell. Mol. Physiol.* 9): L430–L437, 1993.
108. Luzi, P., M. Anceschi, and D.S. Strayer. Glucocorticoid responsiveness conferred by a cloned DNA binding protein. *Receptor* 5: 93–103, 1995.
109. Luzi, P. and D.S. Strayer. Binding proteins that amplify surfactant protein B gene expression: isolation and characterization. *Biochem. Biophys. Res.Commun.* 208: 153–160, 1995.
110. Manley, N.R. and M.R. Capecchi. The role of Hoxa-3 in the thymus and thyroid development. *Development* 121: 1989–2003, 1995.
111. Mantile, G., L. Miele, E. Cordella-Miele, G. Singh, S.L. Katyal, and A.B. Mukherjee. Human Clara cell 10-kDa protein is the counterpart of rabbit uteroglobin. *J. Biol. Chem.* 25: 20343–20351, 1993.
112. Margraf, L.R., J.R. Tomashefski, Jr, M.C. Bruce, and B.B. Dahms. Morphometric analysis of the lung in bronchopulmonary dysplasia. *Am. Rev. Respir. Dis.* 143: 391–400, 1991.
113. Marin, L. The type II pneumocyte. In: *Pulmonary Surfactant: Biochemical, Functional, Regulatory and Clinical Concepts*, edited by J.R. Bourbon, Boca Raton, FL: CRC Press, 1991, p.15–36.
114. Massaro, G.D. and D. Massaro. Formation of pulmonary alveoli and gas-exchange surface area: quantitation and regulation. *Annu. Rev. Physiol.* 58: 73–92, 1996.
115. Massaro, G.D. and D. Massaro. Postnatal treatment with retinoic acid increases the number of pulmonary alveoli in rats. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L305–L310, 1996.
116. Matter, M.L. and G.W. Laurie. A novel laminin E8 cell adhesion site required for lung alveolar formation in vitro. *J. Cell Biol.* 124: 1083–1090,1994.
117. Melnick, M., H.A. Choy, and T. Jaskoll. Glucocorticoids, tumor necrosis factor-α, and epidermal growth factor regulation of pulmonary morphogenesis: a multivariate in vitro analysis of their related actions. *Dev. Dyn.* 205: 365–378, 1996.

118. Mendelsohn, C., D. Lohnes, D. Decimo, T. Lufkin, M. LeMeur, P. Chambon, and M. Mark. Function of retinoic acid receptors (RARs) during development. II. Multiple abnormalities at various stages of organogenesis in *RAR* double mutants. *Development* 120: 2749–2771, 1994.
119. Metzler, M.D. and J.M. Snyder. Retinoic acid differentially regulates expression of surfactant-associated proteins in human lung. *Endocrinology* 133: 1990–1998, 1993.
120. Michael, L.F., J.L. Alcorn, E. Gao, and C.R. Mendelson. Characterization of the cyclic adenosine 3′-5′-monophosphate response element of the rabbit surfactant protein-A gene: evidence for transactivators distinct from CREB/ATF family members. *Mol. Endocrinol.* 10: 159–170, 1996.
121. Michael, L.F., M.A. Lazar, and C.R. Mendelson. Peroxisome proliferator-activated receptor γ1 expression is induced during cyclic adenosine monophosphate-stimulated differentiation of alveolar type II pneumonocytes. *Endocrinology* 138: 3695–3703, 1997.
122. Miettinen, P.J., J.E. Berger, J. Meneses, Y. Phung, R.A. Pedersen, Z. Werb, and R. Derynck. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* 376: 337–341, 1995.
123. Millan, F.A., F. Denhez, P. Kondaiah, and R.J. Arkhurst. Embryonic gene expression patterns of TGF-β1, β2 and β3 suggest different developmental functions in vivo. *Development* 111: 131–144, 1991.
124. Minoo, P., H. Hamdam, D. Bu, D. Warburton, P. Stepanik, and R. de Lemos. TTF-1 regulates lung epithelial morphogenesis. *Dev. Biol.* 172: 694–698,1995.
125. Miura, N., H. Kakinuma, M. Sato, N. Aiba, K. Terada, and N. Sugiyama. Mouse forkhead (winged helix) gene *LUN* encodes a transactivator that acts in the lung. *Genomics* 50: 346–356, 1998.
126. Moens, C.B., A.B. Auerbach, R.A. Conlon, A.L. Joyner, and J. Rossant. A targeted mutation reveals a role for N-*myc* in the embryonic mouse lung. *Genes Dev.* 6: 691–704, 1992.
127. Monaghan, A.P., K.H. Kaestner, E. Grau, and G. Schütz. Postimplantation expression patterns indicate a role for the mouse *fork head*/HNF-3 α, β and γ genes in determination of the definitive endoderm, chordamesoderm and neurectoderm. *Development* 119: 567–578, 1993.
128. Muglia, L., L. Jacobson, P. Dikkes, and J.A. Majzoub. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. *Nature* 373: 427–432, 1995.
129. Murgrauer, G., F.W. Alt, and P. Ekblom. N-*myc* proto-oncogene expression during organogenesis in the developing mouse as revealed by in situ hybridization. *J. Cell Biol.* 107: 1325–1335, 1988.
130. Nogawa, H. and T. Ito. Branching morphogenesis of embryonic mouse lung epithelium in mesenchyme-free culture. *Development* 121: 1015–1022,1995.
131. Odom, M.J., J. Snyder, and C. Mendelson. Adenosine 3′, 5′-monophosphate analogs and β-adrenergic agonists induce the synthesis of the major surfactant apoprotein in human fetal lung in vitro. *Endocrinology* 1212: 1155–1163, 1987.
132. Orr-Urtreger, A., M.T. Bedford, T. Burakova, E. Arman, Y. Zimmer, A. Yayon, D. Givol, and P. Lonai. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). *Dev. Biol.* 158:475–486, 1993.
133. Panos, R.J., J.S. Rubin, S.A. Aaronson, and R.J. Mason. Keratinocyte growth factor and hepatocyte growth factor/scatter factor are heparin-binding growth factors for

alveolar type II cells in fibroblast-conditioned medium. *J. Clin. Invest.* 92: 969–977, 1993.

134. Peters, K., S. Werner, X. Liao, S. Wert, J. Whitsett, and L. Williams. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO J.* 13: 3296–3301, 1994.
135. Peterson, R.L., L. Lim, H.G. Ye, H.P. Zhou, D.G. Overdier, and R.H. Costa. The winged helix transcriptional factor HFH-8 is expressed in the mesoderm of the primitive streak stage of mouse embryos and its cellular derivatives. *Mech. Dev.* 69: 53–69, 1997.
136. Pierrou, S., M. Hellqvist, L. Samuelsson, S. Enerbäck, and P. Carlsson. Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA binding. *EMBO J.* 13: 5002–5012, 1994.
137. Rannels, S.R., C. Stinson Fisher, L.J. Heuser, and D.E. Rannels. Culture of type II pneumocytes on a type II-cell derived fibronectin-rich matrix. *Am. J. Physiol.* 253 (*Cell Physiol.* 22): C759–C765, 1987.
138. Rannels, S.R., J.A. Yarnell, C. Stinson Fisher, J.P. Fabisiak, and D.E. Rannels. Role of laminin in maintenance of type II pneumocyte morphology and function. *Am. J. Physiol.* 253 (*Cell Physiol.* 22): C835–C845, 1987.
139. Ray, M.K., C.-Y. Chen, R.J. Schwartz, and F.J. De Mayo. Transcriptional regulation of a mouse Clara cell-specific protein (mCC10) gene by the NKx transcription factor family members thyroid transcription factor 1 and cardiac muscle-specific homeobox protein (CSX). *Mol. Cell. Biol.* 16: 2056-2064, 1996.
140. Rindfleisch, M.S., J.D. Hasday, V. Taciak, K. Broderick, and R.M. Viscardi. Potential role of interleukin-1 in the development of bronchopulmonary dysplasia. *J. Interferon Cytokine Res.* 16: 365–373, 1996.
141. Ruocco, S., A. Lallemand, J.M. Tournier, and D. Gaillard. Expression and localization of epidermal growth factor, transforming growth factor-α, and localization of their common receptor in fetal human lung development. *Pediatr. Res.* 39: 448–455, 1996.
142. Rust, K., L. Bingle, W. Mariencheck, A. Persson, and E.C. Crouch. Characterization of the human surfactant protein D promoter: transcriptional regulation of SP-D gene expression by glucocorticoids. *Am. J. Respir. Cell Mol. Biol.* 14: 121–130, 1996.
143. Ryan, R.M., M.M. Mineo-Kuhn, C.M. Kramer, and J.N. Finkelstein. Growth factors alter neonatal type II alveolar epithelial cell proliferation. *Am. J. Physiol.* 266 (*Lung Cell. Mol. Physiol.* 10): L17–L22, 1994.
144. Sanford, L.P., I. Ormsby, A.C. Gittenberg-de Groot, H. Sariola, R. Friedman, G.P. Boivin, E.L. Cardell, and T. Doetschman. TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development* 124: 2659–2670, 1997.
145. Sawai, S., A. Shimono, Y. Wakamatsu, C. Palmes, K. Hanaoka, and H. Kondoh. Defects of embryonic organogenesis resulting from targeted disruption of the N-*myc* gene in the mouse. *Development* 117: 1445–1455, 1993.
146. Sawaya, P.L. and D.S. Luse. Two members of the HNF-3 family have opposite effects on a lung transcriptional element; HNF-3α stimulates and HNF-3β inhibits activity of region I from Clara cell secretory protein (CCSP) promoter. *J. Biol. Chem.* 268: 22211–22216, 1994.
147. Sawaya, P.L., B.R. Stripp, J.A. Whitsett, and D.S. Luse. The lung-specific CC10 gene is regulated by transcription factors from the AP-1, octamer, and hepatocyte nuclear factor 3 families. *Mol. Cell. Biol.* 13: 3860–3871, 1993.

148. Schellase, D.E., P.A. Emrie, J.H. Fisher, and J.M. Shannon. Ontogeny of surfactant apoproteins in the rat. *Pediatr. Res.* 26: 167–174, 1989.
149. Schmid, P., D. Cox, G. Bilbe, R. Maier, and G.K. McMaster. Differential expression of TGF β1, β2 and β3 genes during mouse embryogenesis. *Development* 111: 117–130, 1991.
150. Serra, R., R.W. Pelton, and H.L. Moses. TGFβ1 inhibits branching morphogenesis and N-*myc* expression in lung bud organ cultures *Development* 120: 2153–2161, 1994.
151. Shahar, I., E. Fireman, M. Topilsky, J. Grief, S. Kivity, Z. Spirer, and S. Ben Efraim. Effect of IL-6 on alveolar fibroblast proliferation in interstitial lung diseases. *Clin. Immunol. Immunopathol.* 79: 244–251, 1996.
152. Shannon, J.M. Induction of alveolar type II cell differentiation in fetal tracheal epithelium by grafted distal lung mesenchyme. *Dev. Biol.* 166: 600–614, 1994.
153. Shannon, J.M., P.A. Emrie, J.H. Fisher, Y. Kuroki, S.D. Jennings, and R.J. Mason. Effect of a reconstituted basement membrane on expression of surfactant apoproteins in cultured adult rat alveolar type II cells. *Am. J. Respir. Cell Mol. Biol.* 2: 183–192, 1990.
154. Shannon, J.M., S.D. Jennings, and L.D. Nielsen. Modulation of alveolar type II cell differentiated function in vitro. *Am. J. Physiol.* 262 (*Lung Cell. Mol Physiol.* 6): L427–L436, 1992.
155. Shannon, J.M., R.J. Mason, and S.D. Jennings. Functional differentiation of alveolar type II epithelial cell in vitro: effects of cell shape, cell-matrix interaction and cell-cell interactions. *Biochim. Biophys. Acta* 931: 143–156,1987.
156. Shannon, J.M., L.D. Nielsen, S.A. Gebb, and S.H. Randell. Mesenchyme specifies epithelial differentiation in reciprocal recombinants of embryonic lung and trachea. *Dev. Dyn.* 212: 482–494, 1998.
157. Shiratori, M., E. Oshika, L.P. Ung, G. Singh, H. Shinozuka, D. Warburton, G. Michalopoulos, and S.L. Katyal. Keratinocyte growth factor and embryonic rat lung morphogenesis. *Am. J. Respir. Cell Mol. Biol.* 15: 328–338, 1996.
158. Simonet, W.S., M.L. DeRose, N. Bucay, H.Q. Nguyen, S.E. Wert, L. Zhou, T.R. Ulich, A. Thomason, D.M. Danilenko, and J.A. Whitsett. Pulmonary malformation in transgenic mice expressing human keratinocyte growth factor in the lung. *Proc. Natl. Acad. Sci. USA* 92: 12461–12465, 1995.
159. Singh, G., J. Singh, S.L. Katyal, W.E. Brown, J.A. Kramps, I.L. Paradis, J.H. Dauber, T.A. MacPherson, and N. Squeglia. Identification, cellular localization, isolation, and characterization of human Cara cell-specific 10 KD protein. *J. Histochem. Cytochem.* 36: 73–80, 1988.
160. Smith, C.I., E. Rosenberg, S.R. Reisher, F. Li, P. Kefalides, A.B. Fisher, and S.I. Feinstein. Sequence of rat surfactant protein A gene and functional mapping of its upstream region. *Am. J. Physiol.* 269 (*Lung Cell. Mol. Physiol.* 13): L603–L612, 1995.
161. Sonnenberg, E., D. Meyer, K.M. Weidner, and C. Birchmeier. Scatter factor/hepatocyte growth factor and its receptor, the c-*met* tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J. Cell Biol.* 123: 223–235, 1993.
162. Stahlman, M.T., M.E. Gray, and J.A. Whitsett. Expression of thyroid transcription factor-1 (TTF-1) in fetal and neonatal human lung. *J. Histochem. Cytochem.* 44: 673–678, 1996.
163. Stahlman, M.T., M.E. Gray, and J.A. Whitsett. Temporal-spatial distribution of hepatocyte nuclear factor-3β in developing human lung and other foregut derivatives. *Histochem. Cytochem.* 46: 955–962, 1998.

164. Stanton, B.R., A.S. Perkins, L. Tessarollo, D.A. Sassoon, and L.F. Parada. Loss of N-*myc* function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev.* 6: 2235–2247,1992.
165. Strayer, D.S., S. Yang, and H.H. Jerng. Surfactant protein A–binding proteins. Characterization and structures. *J. Biol. Chem.* 268: 18679–18684, 1993.
166. Stripp, B.R., P.L. Sawaya, D.S. Luse, K.A. Wikenheiser, S.E. Wert, J.A. Huffman, D.L. Lattier, G. Singh, S.L. Katyal, and J.A. Whitsett. *Cis*-acting elements that confer lung epithelial cell expression of the CC_{10} gene. *J. Biol. Chem.* 267: 14703–14712, 1992.
167. Strum, J.M., R.S. Compton, S.L. Katyal, and G. Singh. The regulated expression of mRNA for Clara cell protein in the developing airways of the rat, as revealed by in situ hybridization. *Tissue Cell* 24: 461–474, 1992.
168. Stuempfle, K.J., M. Koptides, P.G. Quinn, and J. Floros. In vitro analysis of rat surfactant protein A gene expression. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L504–L516, 1996.
169. Sugahara, K., J.S. Rubin, R.J. Mason, E.L. Aronsen, and J.M. Shannon. Keratinocyte growth factor increases mRNAs for SP-A and SP-B in adult rat alveolar type II cells in culture. *Am. J. Physiol.* 269 (*Lung Cell. Mol. Physiol.* 13): L344–L350, 1995.
170. Ten Have-Opbroek, A.A.W. The development of the lungs in mammals: an analysis of concepts and findings. *Am. J. Anat.* 162: 201–219, 1981.
171. Toonen, R.F.G., S. Gowan, and C.D. Bingle. The lung enriched transcription factor TTF-1 and the ubiquitously expressed proteins Sp-1 and Sp-3 interact with elements located in the minimal promoter of the rat Clara cell secretory protein gene. *Biochem. J.* 316: 467–473, 1996.
172. Torday, J.S. and S. Kourembanas. Fetal rat lung fibroblasts produce a TGF β homolog that blocks alveolar type II cell maturation. *Dev. Biol.* 139: 35–41, 1990.
173. Ulich, T.R., E.S. Yi, K. Longmuir, S. Yin, R. Blitz, C. F. Morris, R. Housley, and G.F. Pierce. Keratinocyte growth factor is a growth factor for type II pneumocytes in vivo. *J. Clin. Invest.* 93: 1298–1306, 1994.
174. Venkatesh, V.C., B.C. Planer, M. Schwartz, J.N. Vanderbilt, R.T. White, and P.L. Ballard. Characterization of the promoter of human pulmonary surfactant protein B gene. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L674–L682, 1995.
175. Wang, J., M. Kuliszewski, W. Yee, L. Sedlackova, J. Xu, I. Tseu, and M. Post. Cloning and characterization of glucocorticoid-induced genes in fetal rat lung fibroblasts: transforming growth factor β_3. *J. Biol. Chem.* 270: 2722–2728, 1995.
176. Wang, J., P. Souza, M. Kuliszewski, A.K. Tanswell, and M. Post. Expression of surfactant proteins in embryonic rat lung. *Am. J. Respir. Cell Mol. Biol.* 10: 222–229, 1994.
177. Weigel, D., G. Jurgens, F. Kuttner, E. Seifert, and H. Jäckle. The homeotic gene *fork head* encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo. *Cell* 57: 645–658, 1989.
178. Weinstein, D.C., A. Ruiz i Altaba, W.S. Chen, P. Hoodless, V.R. Prezioso,T.M. Jessell, and J.E. Darnell. The winged-helix transcription factor *HNF-3β* is required for notochord development in the mouse embryo. *Cell* 78:575–585, 1994.
179. Wert, S.E., S.W. Glasser, T.R. Korfhagen, and J.A. Whittsett. Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev. Biol.* 156: 426–443, 1993.
180. White, R.T., D. Damm, J. Miller, K. Spratt, J. Schilling, S. Hawgood, B. Benson, and

B. Cordell. Isolation and characterization of the human pulmonary surfactant apoprotein gene. *Nature* 317: 361–363, 1985.

181. Whitsett, J.A. and J.E. Baatz. Hydrophobic surfactant proteins SP-B and SP-C: molecular biology, structure and function. In: *Pulmonary Surfactant, from Molecular Biology to Clinical Practice*, edited by B. Robertson, L.M.G. van Golde, and J.J. Batenburg. Amsterdam: Elsevier, 1992, p. 55–75.
182. Whitsett, J.A., J.C. Clark, J.R. Wispé, and G.S. Pryhuber. Effects of TNF-α and phorbol ester on human surfactant protein and MnSOD gene transcription in vitro. *Am. J. Physiol.* 262 (*Lung Cell. Mol. Physiol.* 6): L688–L693, 1992.
183. Whitsett, J.A., T.E. Weaver, M.A. Lieberman, J.C. Clark, and C. Daugherty. Differential effects of epidermal growth factor and transforming growth factor-β on synthesis of Mr = 35,000 surfactant-associated protein in fetal lung. *J. Biol. Chem.* 262: 7908–7913, 1987.
184. Wikenheiser, K.A. and J.A. Whitsett. Tumor progression and cellular differentiation of pulmonary adenocarcinomas in SV40 large T transgenic mice. *Am. J. Respir. Cell Mol. Biol.* 16: 713–723, 1997.
185. Williams, M.C., Y.-X. Cao, A. Hinds, A.K. Rishi, and A. Wetterwald. T1α protein is developmentally regulated and expressed by alveolar type I cells, choroid plexus, and ciliary epithelia of adult rats. *Am. J. Respir. Cell Mol. Biol.* 14: 577–585, 1996.
186. Wolf, M., J. Klug, R. Hackenberg, M. Gessler, K.-H. Grzeschik, M. Beato, and G. Suske. Human CC10, the homologue of rabbit uteroglobin: genomic cloning, chromosomal localization and expression in endometrial cell lines. *Hum. Mol. Genet.* 1: 371–378, 1992.
187. Wu, J.E. and S.A. Santoro. Differential expression of integrin α subunits supports distinct roles during lung branching morphogenesis. *Dev. Dyn.* 206: 169–181, 1996.
188. Wuenschell, C.W., M.E. Sunday, G. Singh, P. Minoo, H.C. Slavkin, and D. Warburton. Embryonic mouse lung epithelial progenitor cells co-express immunohistochemical markers of diverse mature cell lineages. *J. Histochem. Cytochem.* 44: 112–123, 1996.
189. Yan, C., M. Ghaffari, J.A. Whitsett, X. Zeng, Z. Sever, and S. Lin. Retinoic acid receptor activation of SP-B gene transcription in respiratory epithelial cells. *Am. J. Physiol.* 275 (*Lung Cell. Mol. Physiol.* 19): L239–L246, 1998.
190. Yan, C., Z. Sever, and J.A. Whitsett. Upstream enhancer activity in the human surfactant protein B gene is mediated by thyroid transcription factor 1. *J. Biol. Chem.* 270: 24852–24857, 1995.
191. Yan, C. and J.A. Whitsett. Protein kinase A activation of the surfactant protein B gene is mediated by phosphorylation of thyroid transcription factor 1. *J. Biol. Chem.* 272: 17327–17332, 1997.
192. Yee, W., J. Wang, J. Liu, I. Tseu, M. Kuliszewski, and M. Post. Glucocorticoid-induced tropoelastin expression is mediated via transforming growth factor-β_3. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L992–L1001, 1996.
193. Zhao, Y. and S.L. Young. Expression of transforming growth factor-β type II receptor in rat lung is regulated during development. *Am. J. Physiol.* 269 (*Lung Cell. Mol. Physiol.* 13): L419–L426, 1995.
194. Zhou, L., C.R. Dey, S.E. Wert, and J.A. Whitsett. Arrested lung morphogenesis in transgenic mice bearing an SP-C-TGF-β1 chimeric gene *Dev. Biol.* 175: 227–238, 1996.
195. Zhou, L., L. Lim, R.H. Costa, and J.A. Whitsett. Thyroid transcription factor-1, hepatocyte nuclear factor-3β, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. *J. Histochem. Cytochem.* 44:1183–1193, 1996.