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Original Article

Thyroid Transcription Factor-1, Hepatocyte Nuclear Factor-3β, Surfactant Protein B, C, and Clara Cell Secretory Protein in Developing Mouse Lung[1]

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We used immunohistochemical analysis to localize thyroid transcription factor-1 (TTF-1), hepatocyte nuclear factor-3β (HNF-3β), prosurfactant proteins B and C (pro-SP-B, pro-SP-C), surfactant protein B (SP-B), and Clara cell secretory protein (CCSP) in developing mouse lung. TTF-1 and HNF-3β were expressed at the onset of lung morphogenesis (gestational Day 10) and throughout fetal lung development, being detected in the nuclei of airway epithelial cells. TTF-1 was most prominent in distal airway epithelial cells in embryonic lung and HNF-3β in proximal bronchial and bronchiolar epithelial cells. Pro-SP-B and pro-SP-C were first detected on gestational Day 11, being localized to the cytoplasm of airway epithelial cells. Expression of both pro-proteins was confined to distal airway epithelial cells from gestational Day 12 to Day 16. From gestational Day 17 and thereafter, pro-SP-B was detectable in Type II cells and bronchiolar epithelial cells, whereas pro-SP-C was restricted to Type II cells. SP-B peptide was first detected on gestational Day 17 in the cytoplasm of Type II cells and within the lumen of distal airways. SP-B peptide was detectable only in the cytoplasm of Type II cells in adult lung. CCSP was first detected on gestational Day 17, being localized to the cytoplasm of columnar epithelial cells lining the conducting airways. Pro-SP-B, SP-B, pro-SP-C, and CCSP staining increased before birth. The early expression of TTF-1 and HNF-3β, preceding and overlapping that of pro-SP-B, mature SP-B, pro-SP-C, and CCSP, supports a regulatory role for TTF-1 and HNF-3β in lung-specific gene expression. (*J Histochem Cytochem 44:1183–1193, 1996*)

KEY WORDS: Mouse lung development; Transcription factors; Surfactant proteins; Clara cell secretory protein; Immunohistochemistry.

Introduction

Pulmonary surfactant is a lipoprotein complex synthesized and secreted by Type II cells that reduces surface tension at the air–liquid interface (Weaver and Whitsett, 1991). Surfactant-associated proteins, including SP-A, SP-B, SP-C, and SP-D, are believed to play important roles in surfactant synthesis, storage, secretion, and functions (Clark et al., 1995; Weaver and Whitsett, 1991). SP-A and SP-D have been implicated in host defense (Weaver and Whitsett, 1991). CCSP, a secretory protein identified from Clara cells lining pulmonary conducting airways, may contribute to the anti-inflammatory activities of the respiratory epithelium (Dodge et al., 1993; Singh et al., 1984,1988; Bedetti et al., 1987).

Expression of SP-A, SP-B, SP-C, and CCSP is determined at the transcriptional level by mechanisms that are mediated by transcription factors TTF-1 and HNF-3β (Bingle et al., 1995; Bruno et al., 1995; Bohinski et al., 1994; Clevidence et al., 1994; Sawaya et al., 1994). TTF-1, a member of the Nkx2 family of homeodomain transcription factors, plays important roles in lung (Bruno et al., 1995; Bohinski et al., 1994; Clevidence et al., 1994) and thyroid (Civitareale et al., 1989) epithelial cell gene expression. In vitro, TTF-1 transactivates promoter activities of the SP-A, SP-B, SP-C, CCSP, thyroglobulin, and thyroperoxidase genes via the binding of TTF-1 to DNA binding sites located within the promoters of each of these genes (Bruno et al., 1995; Bohinski et al., 1994; Clevidence et al., 1994; Civitareale et al., 1989). HNF-3β, a member of HNF-3/forkhead winged helix family of transcription factors, modulates lung- (Bohinski et al., 1994; Clevidence et al., 1994; Sawaya et al., 1994) and liver- (Simone et al., 1991) specific gene expression. HNF-3 DNA binding sites were identified in promoters of SP-A, SP-B, and CCSP genes, where they contribute to the regulation of these genes (Bohinski et al., 1994; Clevidence et al., 1994; Sawaya et al., 1994). Because TTF-1 and HNF-3β are not restricted to lung epithelial cells, the combined effects of TTF-1, HNF-3β, and other transcription

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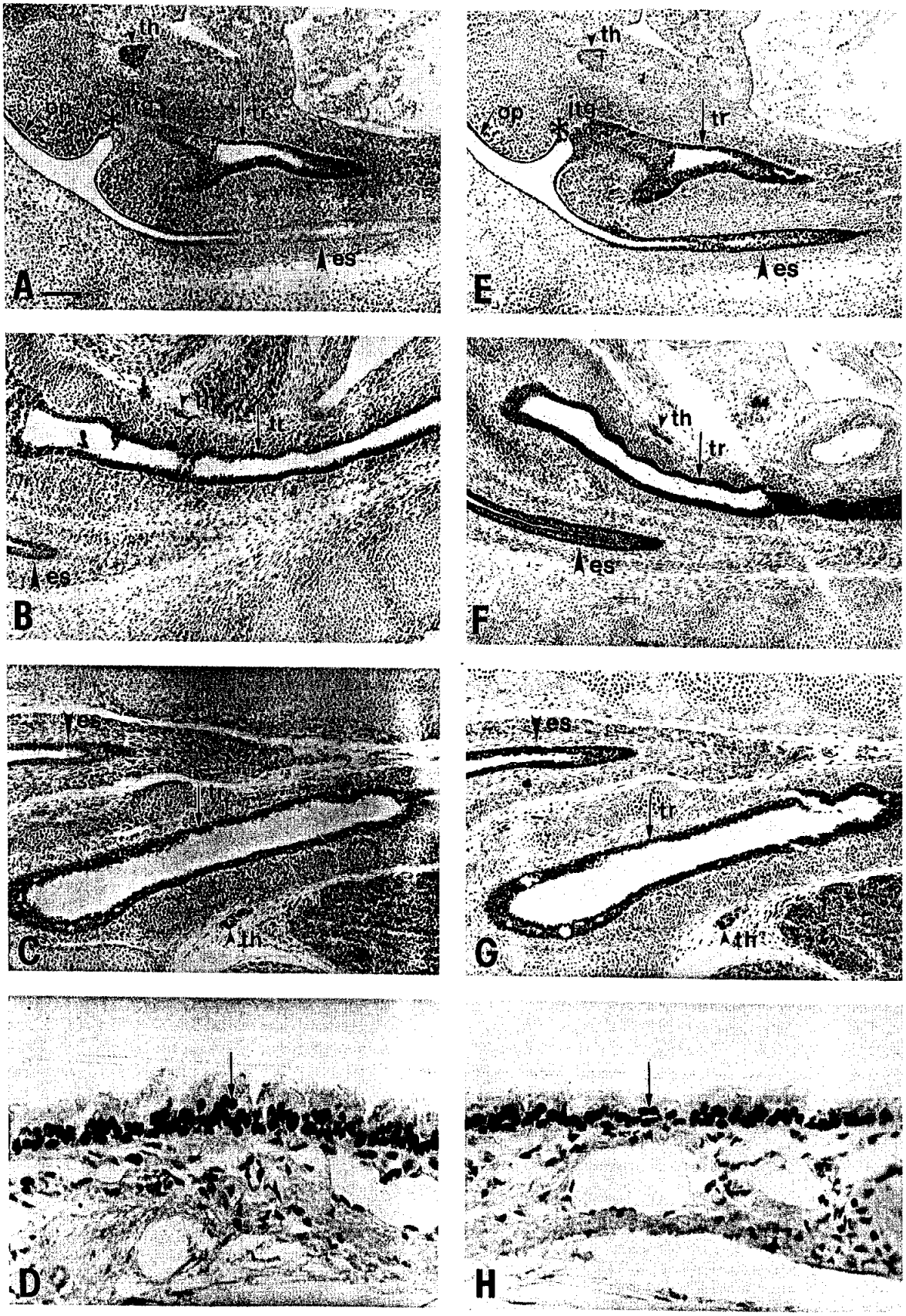
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factors, including ubiquitous factors, may influence respiratory epithelial cell gene expression.

Consistent with the importance of the TTF-1 and HNF-3 family members in lung-specific gene expression, these transcription factors are expressed in fetal and mature lung epithelial cells in patterns overlapping those of SP-A, SP-B, SP-C, and CCSP (Ikeda et al., 1995; Khoor et al., 1993,1994; Ang et al., 1993; Monaghan et al., 1993; Lazzaro et al., 1991; Singh et al., 1988). TTF-1 was expressed at the onset of rat lung morphogenesis (gestational Day 10.5), being localized to the nuclei of airway epithelial cells (Lazzaro et al., 1991). TTF-1 protein was detected in fetal human lung as early as 11 weeks of gestation (Ikeda et al., 1995), preceding the expression of SP-A (Khoor et al., 1993), SP-B (Khoor et al., 1994), SP-C (Khoor et al., 1994), and CCSP (Singh et al., 1988), which were first detected in human lung epithelial cells at 13 weeks, 12 weeks, 15 weeks, and 21 weeks of gestation, respectively. SP-A, SP-B, SP-C, and CCSP mRNAs and proteins were assessed previously by in situ hybridization and immunohistochemistry in the lungs of various species. SP-A, SP-B, and SP-C were detected in terminal airway epithelial cells, including differentiated Type II cells (Khoor et al., 1993,1994; Wert et al., 1993; Stahlman et al., 1992; Wohlford–Lenane et al., 1992; Glasser et al., 1991). SP-A and SP-B were also detected in epithelial cells lining conducting airways (Khoor et al., 1993,1994; Stahlman et al., 1992). The sites and extent of expression varied from species to species and with development. CCSP was detected primarily in Clara cells lining conducting airways and was not readily detected in alveolar Type II cells (Dodge et al., 1993; Margraf et al., 1993; Wert et al., 1993; Hackett et al., 1992; Singh et al., 1988; Bedetti et al., 1987). TTF-1 mRNA and protein were localized to Type II cells and epithelial cells of conducting airways, overlapping the expression of SP-A, SP-B, SP-C, and CCSP, providing further support for a regulatory role for TTF-1 in the expression of these genes. The sites and extent of expression of HNF-3β in the lung are more controversial. HNF-3β mRNA was detected in airway epithelial cells in fetal and adult mouse lungs (Ang et al., 1993; Monaghan et al., 1993) but in smooth muscle cells surrounding the adult mouse airway and pulmonary vessels in the rat (Clevidence et al., 1994).

Although the expression patterns of TTF-1, HNF-3β, pro-SP-B, mature SP-B, pro-SP-C, and CCSP have been assessed in various species, the developmental expression patterns of these proteins have not been determined in mouse lungs. Immunohistochemical analysis was therefore used to clarify the temporal and spatial patterns of these proteins in developing mouse lung. The overlapping expression patterns of TTF-1, HNF-3β, surfactant proteins, and CCSP support a role for these transcription factors in the control of lung-specific gene expression.

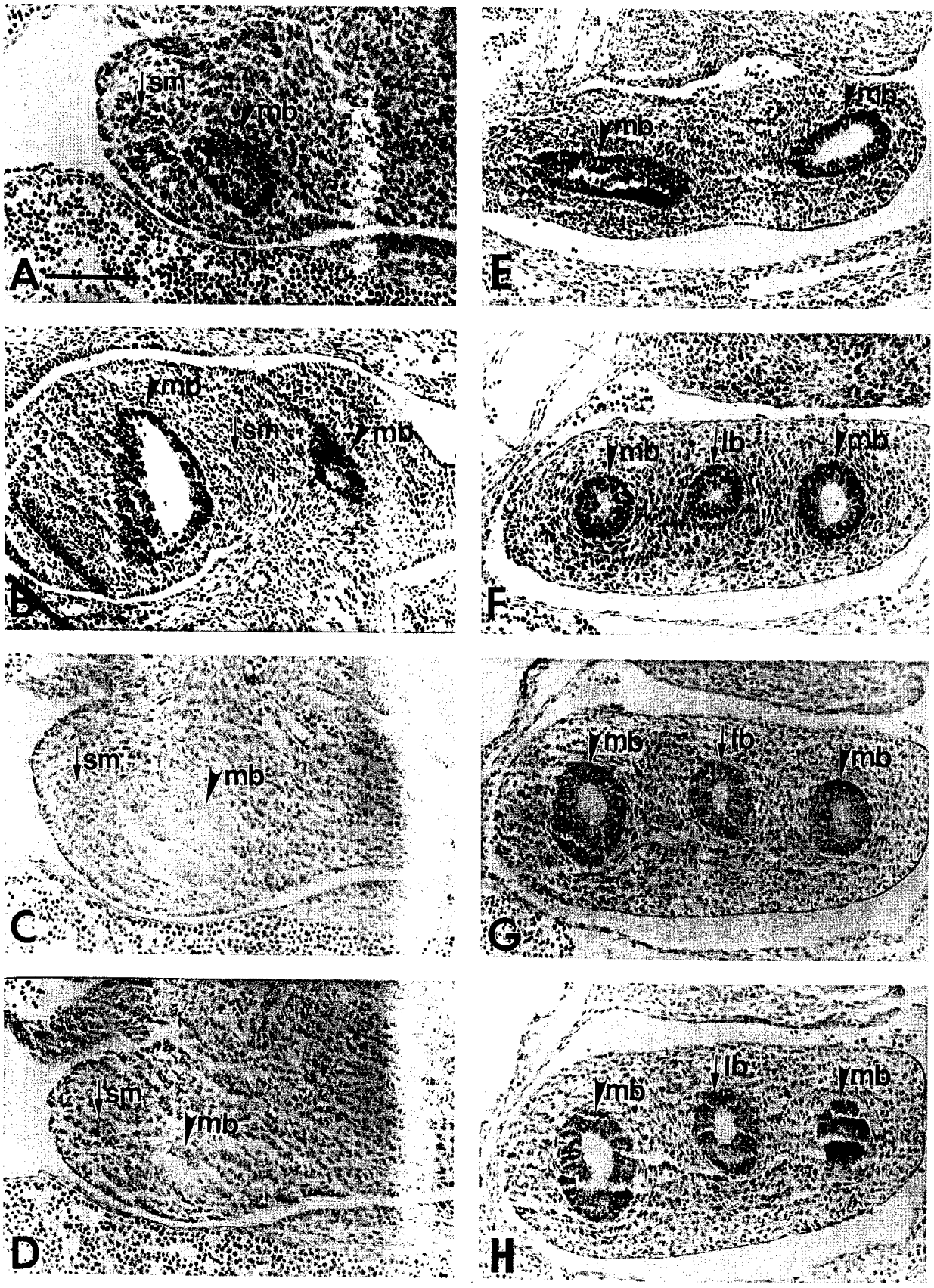
Materials and Methods

Tissue Preparation. Mouse embryos were obtained by breeding FVB/N mice. The presence of a vaginal plug at noon was considered as gestational Day 0.5. The embryos and lungs from adult FVB/N mice were fixed in neutral buffered 4% paraformaldehyde for 16 hr at 4°C. Tissues were dehydrated through a graded series of ethanol solutions and embedded in paraffin. Sagittal sections of the embryos and sections of adult lungs were placed on poly-lysine-coated slides. Embryos and postnatal samples reflect results from at least two individual animals.

Antibodies and Immunohistochemistry. Rabbit polyclonal antibody, generated against a synthetic peptide spanning amino acid residues 110–122 of rat TTF-1 (Guazzi et al., 1990), was kindly provided by Dr. Roberto Di Lauro (Stazione Zoologica 'Anton Dohrn'; Naples, Italy) and was used at a dilution of 1:5000 (Ikeda et al., 1995; Lazzaro et al., 1991). Affinity-purified rabbit polyclonal antibody against amino acids 7–86 of rat HNF-3β was generated in Dr. Robert H. Costa's laboratory (Jacob et al., 1994) and was used at a dilution of 1:100. Rabbit polyclonal antisera against human pro-SP-B (R4633 and R96189), bovine mature SP-B (R28031), and human pro-SP-C (R68514) were generated in this laboratory and used at dilutions of 1:1000, 1:500, 1:1000, and 1:1000, respectively. R4633 was generated against the full-length recombinant human SP-B proprotein (O'Reilly et al., 1989a,b; Weaver and Whitsett, 1989). R96189 was generated against a recombinant peptide containing 102 residues of the C-terminal fragment of the human SP-B proprotein (Lin et al., in press). R28031 was generated to the mature active SP-B peptide isolated from bovine lung (Lin et al., 1996; Baatz et al., 1990). R68514 was generated against a recombinant protein containing amino acids 1–20 from the N-terminal of the human SP-C pro-protein (Vorbroker et al., 1992,1995). These antisera crossreact with and immunostain mouse surfactant proteins (Huffman et al., 1996; Zhou et al., in press; Clark et al., 1995). Rabbit antiserum against rat CCSP was a gift from Dr. Gurmukh Singh (1988) and was used at a dilution of 1:5000 (Zhou et al., 1996).

Immunohistochemical staining for TTF-1 and HNF-3β was performed as follows: 5-μm paraffin sections were deparaffinized and microwaved in buffer containing 0.1 M citric acid, 0.1 m sodium citrate (pH 6.0) at high-power for 8 min to boil and at mid-low power for 15 min (Gown et al., 1993; Shi et al., 1991). This antigen retrieval method was used to unmask antigens and enhance immunohistochemical staining. Sections were then treated with 3% hydrogen peroxide for 30 min, blocked with 5% normal serum at room temperature for 2 hr, and incubated overnight at 4°C with primary antibody and developed using the Vector Elite ABC method (Vector Laboratories; Burlingame, CA). Antigen localization was enhanced with nickel–DAB, followed by incubation with Tris–cobalt and counterstaining with Nuclear Fast Red. The immunohistochemical staining for pro-SP-B, mature SP-B, pro-SP-C, and CCSP was performed as described above without microwave antigen retrieval. The lack of primary antibody treatment was used as a negative control for each immunostaining. Specificity of antibodies has been established previously (Ikeda et al., 1995; Jacob et al., 1994; Khoor et al., 1994; Singh et al., 1988).

Figure 1. Immunohistochemical staining of TTF-1 and HNF-3β in developing mouse trachea. TTF-1 was detected in nuclei of tracheal epithelial cells (**A–D**, long arrows), at high levels on gestational Days 11 (**A**), 13 (**B**), and 16 (**C**) and at lower levels in the adult (**D**, long arrow). TTF-1 was also detected in thyroid (**A–C**, small arrowheads) but was not detectable in oral–pharyngeal region (**A**, short arrow) and esophagus (**A–C**, large arrowheads). HNF-3β was detected in tracheal epithelial cells (**E–H**, long arrows) at high levels on gestational Days 11 (**E**), 13 (**F**), and 16 (**G**) and at lower levels in the adult (**H**, long arrow). HNF-3β was also detected in the oral–pharyngeal region (**E**, short arrow), laryngotracheal groove (**E**, asterisk), and esophagus (**E–G**, large arrowheads) but was not detectable in thyroid (**E–G**, small arrowheads). tr, trachea; th, thyroid; es, esophagus; op, oral–pharyngeal region; ltg, laryngotracheal groove. Bars: **A–C, E–G** = 32 μm; **D–H** = 192 μm.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Results

Distribution of TTF-1 in Developing Lung

TTF-1 was readily detected on gestational Day 10 during early lung bud formation, where it was localized to the nuclei of the main bronchial epithelial cells (Figure 2A). No staining was seen in splanchnic mesenchymal cells surrounding the main bronchi (Figure 2A). On gestational Day 11, TTF-1 was abundantly expressed in the trachea (Figure 1A) and in main and lobar bronchi (Figure 2E). Immunostaining for TTF-1 was also detected in the ventral region of the diencephalon, in the pars nervosa portion of pituitary (data not shown), and in thyroid primordium (Figure 1A), but was not detectable in esophagus (Figure 1A) and other organs (data not shown). On gestational Days 12–16, TTF-1 was expressed at high levels in trachea (Figures 1B and 1C), bronchi, bronchioles, and distal acinar lung buds, being prominent in newly formed distal airway tubules (Figures 3A and 3E). On gestational Day 17, TTF-1 was still strongly expressed in epithelial cells lining conducting airways. In the distal lung at this stage, staining was restricted to subsets of epithelial cells (Type II cells), in which staining was more intense than in cells in conducting airways (Figure 4A). This spatial pattern of TTF-1 expression was maintained on gestational Day 18 and in adulthood, however, Type II cells and epithelial cells lining bronchi and bronchioles being equally stained (Figures 4E and 4I). TTF-1 staining was weaker in epithelial cells lining adult trachea (Figure 1D).

Distribution of HNF-3β in Developing Lung

Like TTF-1, HNF-3β was detected on gestational Day 10 at high levels in the nuclei of main bronchial epithelial cells but was not present in the surrounding mesenchymal cells (Figure 2B). On gestational Day 11, HNF-3β was strongly expressed in epithelial cells of trachea (Figure 1E) and in main and lobar bronchi (Figure 2F). HNF-3β was also readily detected in the oral–pharyngeal region (Figure 1E), in liver primordium, in gut, and in the wall of the midbrain and the floor of the hindbrain (data not shown), but was not detectable in thyroid primordium (Figure 1E). During gestational Days 12–16, HNF-3B was detected at high levels in the epithelial cells of trachea (Figures 1F and 1G), bronchi (Figure 3B), bronchioles, and terminal acinar lung buds (Figure 3F), being prominent in proximal bronchi and bronchioles (Figures 3B and 3F). Intense staining was also noted in esophagus (Figures 1F and 1G) and pancreas at this stage of development (data not shown). On gestational Day 17, HNF-3β was detected in epithelial cells of conducting airways and was restricted to subsets of epithelial cells (Type II cells) in the distal lung (Figure 4B). On gestational Day 18, HNF-3β expression decreased in epithelial cells of both proximal and distal airways (Figure 4F). In adult lung, HNF-3β was expressed in epithelial cells of bronchi, bronchioles, and Type II cells at similarly high levels (Figure 4J) and at lower levels in trachea (Figure 1H). The distribution pattern of HNF-3β protein was consistent with that of HNF-3β mRNA in fetal mouse lungs as determined by in situ hybridization (data not shown).

Distribution of Pro-SP-B and SP-B in Developing Lung

Pro-SP-B was not detected in gestational Day 10 lung (Figure 2C) but was detected at low levels in the cytoplasm of epithelial cells in both main and lobar bronchi on gestational Day 11 (Figure 2G). During gestational Days 12–16, pro-SP-B was detected at low levels in epithelial cells of newly formed distal tubules but was not detected in large proximal airways (Figures 3C and 3G). From gestational Day 17 and thereafter, pro-SP-B staining was restricted to Type II cells in the distal lung. It was also detectable in tracheal (data not shown) and bronchiolar epithelial cells by antibody R96189 (Figures 4C and 4G) but not by antibody R4633 (Figure 4K). The intensity of pro-SP-B staining increased from gestational Day 16 to adulthood with antibody R4633 (data not shown) but not with antibody R96189 (Figures 4C and 4G). Mature SP-B was not detectable during the pseudoglandular period (gestational Days 10–16) (Figure 5A). However, by gestational Day 17 trace immunostaining was detected in Type II cells as well as in the distal airway lumen (Figure 5B). On gestational Day 18, staining was heavily deposited in the cytoplasm of Type II cells and in the distal airway lumen (Figure 5C). In adult lung, SP-B was detected only in Type II cells but was not readily detected in the alveolar lumen (Figure 5D). A dramatic increase in SP-B staining was noted before birth (Figures 5B and 5C).

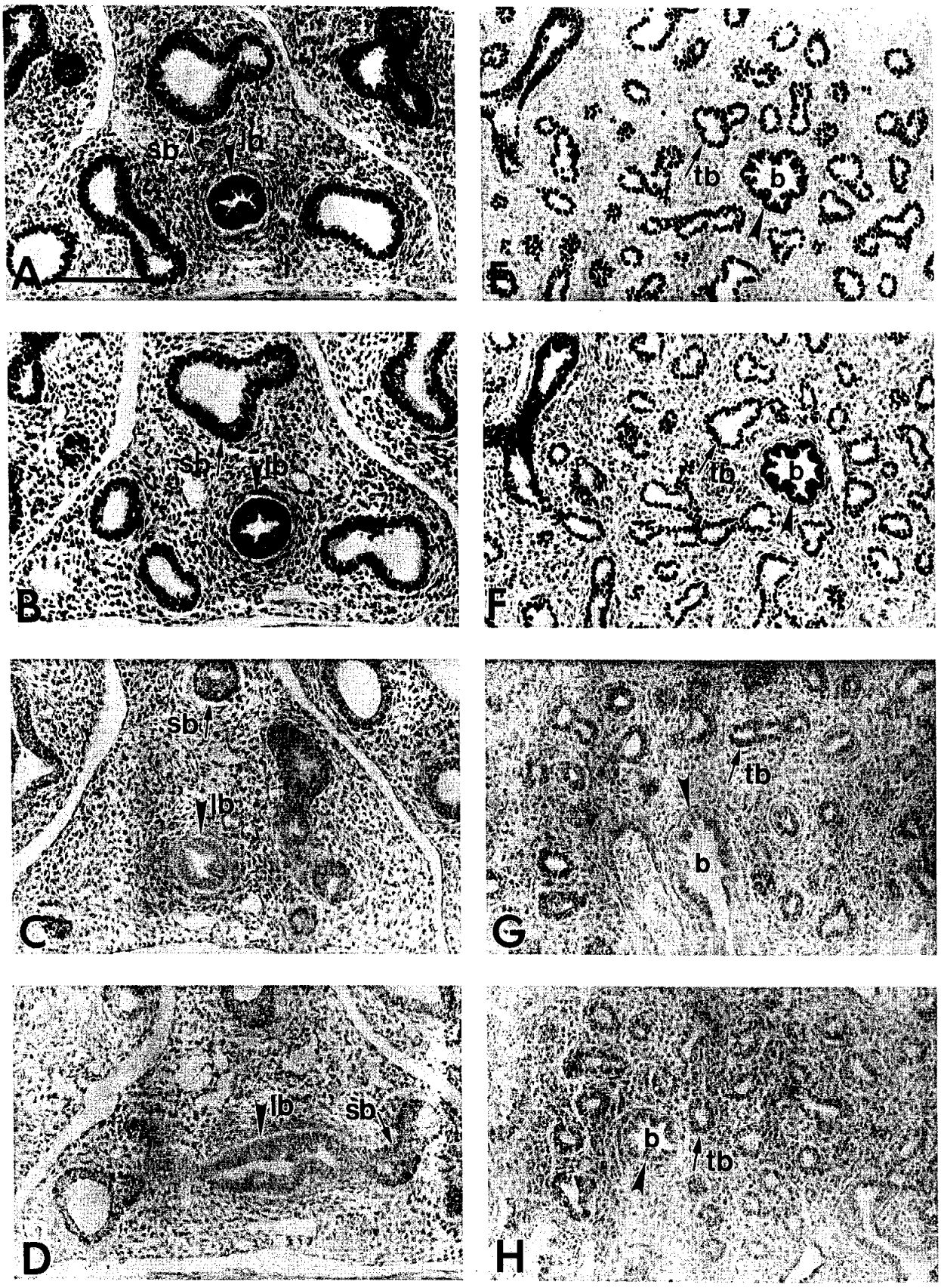
Distribution of Pro-SP-C in Developing Lung

During the pseudoglandular stage, the expression patterns of pro-SP-C were similar to those of pro-SP-B, being detected from gestational Day 11 and restricted to the cytoplasm of epithelial cells in distal lung buds (Figures 2D, 2H, 3D, and 3H). From gestational Day 17 and thereafter, pro-SP-C was restricted to Type II epithelial cells, the intensity of staining increasing with advancing gestational (Figures 4D, 4H, and 4L).

Distribution of CCSP in Developing Lung

CCSP was not detectable in pseudoglandular mouse lung on gestational Days 10–16 (Figure 5E). From gestational Day 17 and there-

Figure 2. Immunohistochemical staining of TTF-1, HNF-3β, pro-SP-B, and pro-SP-C in lungs at gestational Days 10 and 11. Sections of lungs from mouse embryos at gestational Days 10 (**A–D**) and 11 (**E–H**) were stained with antibodies against TTF-1 (**A,E**), HNF-3β (**B,F**), pro-SP-B (**C,G**), and pro-SP-C (**D,H**). TTF-1 and HNF-3β were detected at high levels in the epithelial cells lining main bronchi (arrowheads) on gestational Day 10 (**A,B**) and in main (arrowheads) and lobar (arrows) bronchi on gestational Day 11 (**E,F**), being localized to the nuclei of lung epithelial cells. TTF-1 and HNF-3β were not detectable in splanchnic mesenchymal cells (**A,B**, arrows). Pro-SP-B (**C**) and pro-SP-C (**D**) were not detected on gestational Day 10. Pro-SP-B (**G**) and pro-SP-C (**H**) were detected at low levels in the cytoplasm of epithelial cells lining main (arrowheads) and lobar (arrows) bronchi on gestational Day 11. mb, main bronchus; lb, lobar bronchus; sm, splanchnic mesenchyme. Bar = 64 μm.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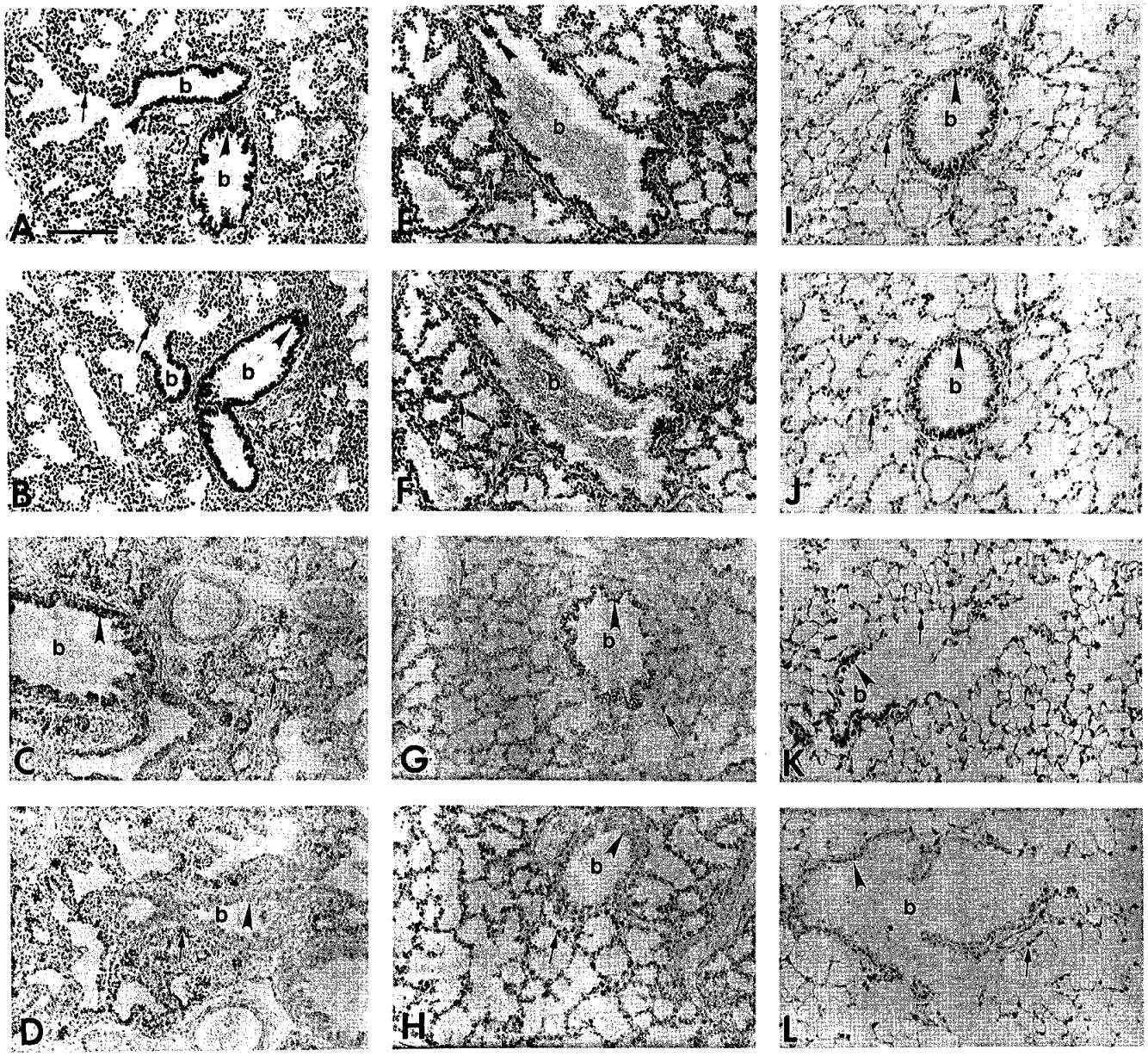
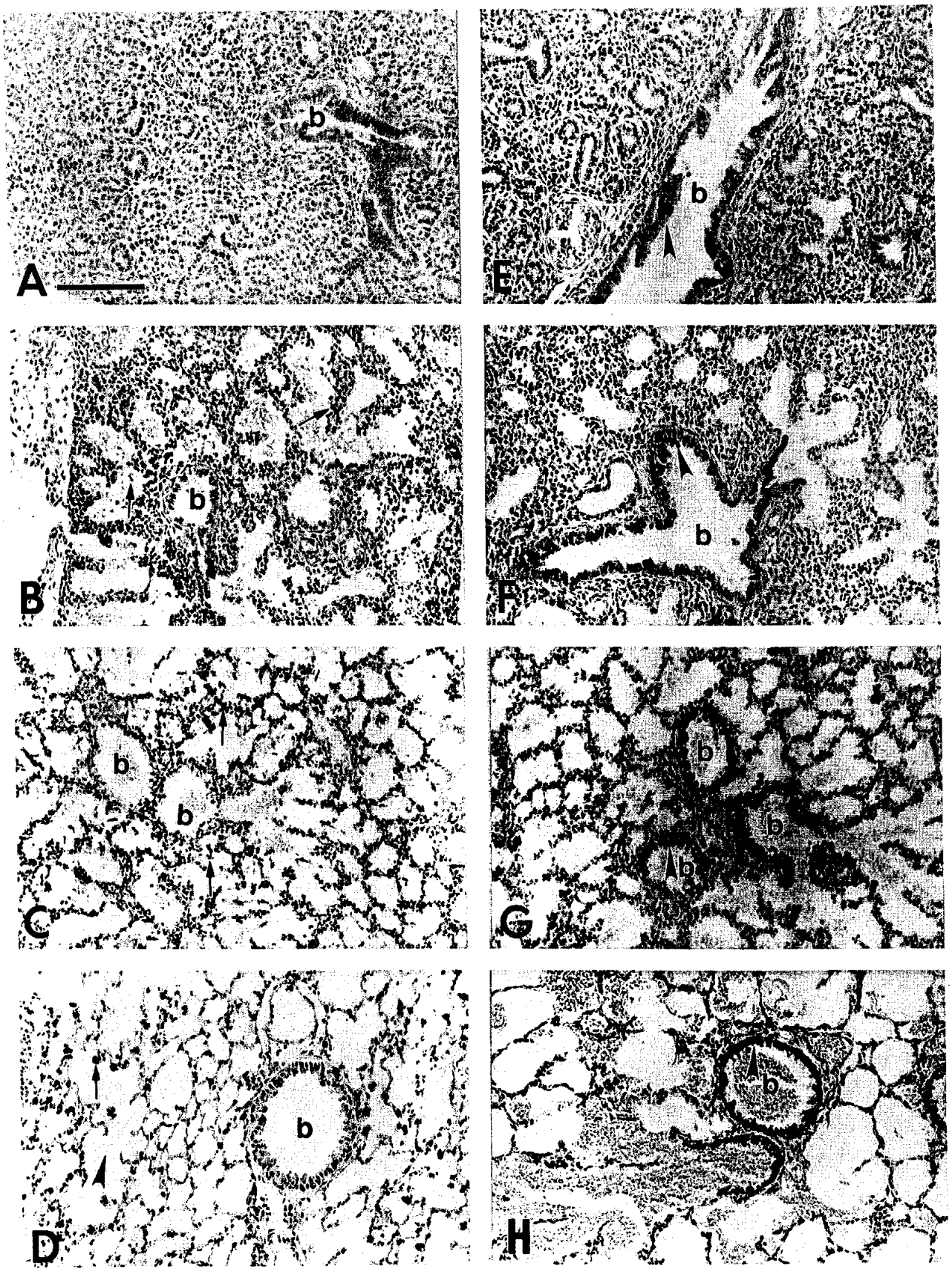


Figure 4. Immunohistochemical staining of TTF-1, HNF-3β, pro-SP-B, and pro-SP-C in gestational Days 17 and 18 and in adult mouse lungs. Sections of lungs from Day 17 (**A–D**), Day 18 (**E–H**), and adult mice (**I–L**) were stained with antibodies against TTF-1 (**A,E,I**), HNF-3β (**B,F,J**), pro-SP-B (**C,G,K**), and pro-SP-C (**D,H,L**). TTF-1 (**A,E,I**) and HNF-3β (**C,G,K**) were detected at high levels in the epithelial cells of bronchi (not shown), bronchioles (arrowheads), and differentiated Type II cells (arrows). HNF-3β (**F**) expression decreased in both bronchiolar epithelial cells (arrowhead) and distal Type II cells (arrow) on gestational Day 18. Pro-SP-B (**C,G**) was detected in distal Type II cells (arrows) and bronchiolar epithelial cells (arrowheads) with antibody R96189. Pro-SP-B (**K**) was not detected in bronchiolar epithelial cells (arrowhead) with antibody R4633. Pro-SP-C (**D,H,L**) staining was restricted to the distal Type II cells (arrows). b, bronchiole. Bar = 64 μm.

Figure 3. Immunohistochemical staining of TTF-1, HNF-3β, pro-SP-B, and pro-SP-C in lungs at gestational Days 13 and 16. Sections of lungs from mouse embryos at Day 13 (**A–D**) and Day 16 (**E–H**) were stained with antibodies against TTF-1 (**A,E**), HNF-3β (**B,F**), pro-SP-B (**C,G**), and pro-SP-C (**D,H**). TTF-1 and HNF-3β were detected at high levels in the epithelial cells of both proximal and distal airways, TTF-1 (**A,E**) being more abundant in newly formed distal lung tubules (arrows) and HNF-3β (**B,F**) being prominent in columnar epithelial cells lining proximal tubules (arrowheads). Pro-SP-B (**C,G**) and pro-SP-C (**D,H**) were detected at low levels in newly formed distal tubules (arrows) but were not detectable in proximal columnar epithelial cells (arrowheads). (**D**) A lobar bronchus lacking pro-SP-C staining (arrowhead) branches into a segmental bronchus expressing pro-SP-C (arrow). lb, lobar bronchus; sb, segmental bronchus; b, bronchiole,; tb, terminal lung buds. Bar = 64 μm.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after, CCSP was detected in the cytoplasm of respiratory epithelial cells lining trachea, bronchi (data not shown), and bronchioles, but was not detected in Type II cells (Figures 5F–5H). CCSP staining also increased with advancing gestation (Figures 5F–5H).

Discussion

The temporal and spatial patterns of TTF-1, HNF-3β, pro-SP-B, mature SP-B, pro-SP-C, and CCSP were assessed in developing mouse lung by immunohistochemical analysis. TTF-1 and HNF-3β were detected in nuclei of respiratory epithelial cells throughout fetal lung development. The expression of TTF-1 and HNF-3β preceded and overlapped that of pro-SP-B, mature SP-B, pro-SP-C, and CCSP. The present findings and previous data, demonstrating the role of TTF-1 and HNF-3β in transcriptional control of surfactant protein and CCSP genes, support the concept that TTF-1 and HNF-3β play critical roles in lung morphogenesis, lung epithelial cell differentiation, and lung epithelial cell gene expression.

The early expression of TTF-1 and HNF-3β in the developing lung suggests that these two transcription factors contribute to the initiation of lung morphogenesis. TTF-1 is expressed at the onset of mouse lung morphogenesis, its expression being restricted to the endodermally derived epithelial cells. These findings are consistent with the early expression of TTF-1 in lung epithelial cells in rat (Lazzaro et al., 1991) and human (Ikeda et al., 1995), suggesting that TTF-1 may participate in early lung morphogenesis. Recently, the TTF-1 gene was knocked out and the homozygous knockout mice were born dead and lacked peripheral lung parenchyma (Kimura et al., 1996), supporting the concept that TTF-1 is essential for lung organogenesis. HNF-3β is expressed very early in mouse development, being detected in the node, notochord, floor plate, and gut, suggesting that HNF-3β plays a role in body axis formation, neural tube patterning, and definitive endoderm formation (Sasaki and Hogan, 1993,1994; Ang et al., 1993; Monaghan et al., 1993). HNF-3β deficient mouse embryos were generated by homologous recombination (Ang and Rossant, 1994; Weinstein et al., 1994). HNF-3β-deficient embryos die around gestational Day 10 and lack node, notochord, floor plate, and gut tube, further supporting the essential role of HNF-3β in the morphogenesis of notochord and foregut from which lung is formed. HNF-3β is expressed in neural tissues and endoderm-derived epithelial cells in lung, liver, pancreas, and intestine. In contrast, TTF-1 is expressed in thyroid and lung epithelial cells and in the brain, in a pattern distinct from that of HNF-3β expression. Therefore, TTF-1 and HNF-3β are co-localized only in lung epithelial cells, suggesting that the co-expression of these two transcription factors may contribute to respiratory epithelial cell lineage specificity.

The expression of TTF-1 and HNF-3β overlaps that of SP-B, SP-C, and CCSP, consistent with the concept that these two transcription factors regulate the expression of these genes. Whereas CCSP was restricted to respiratory epithelial cells of the conducting airways, immunostaining of SP-B and SP-C was more restricted to distal airway epithelial cells, consistent with the distribution of their mRNAs (Wert et al., 1993). However, TTF-1 and HNF-3β are expressed in epithelial cells in both proximal and distal airways, supporting in vitro findings demonstrating that TTF-1 and HNF-3β regulate surfactant protein and CCSP gene expression by binding to *cis*-acting elements in the promoter region of each gene (Bingle et al., 1995; Bruno et al., 1995; Bohinski et al., 1994; Sawaya et al., 1994). The restricted expression patterns of SP-B, SP-C, and CCSP, and the relative broad expression of TTF-1 and HNF-3β in respiratory epithelial cells, also indicate that other transcription factors, including ubiquitous factors and/or distinct combinations of transcription factors in different types of cells, may further specify proximal and distal lung epithelial cell differentiation. In support of this concept, AP-1 sites altered TTF-1-dependent activation of the mouse SP-B gene promoter (Sever et al., 1995). The expression of TTF-1 and HNF-3β precedes that of SP-B, SP-C, and CCSP, supporting the potential upstream roles of these two transcription factors in lung-specific gene expression.

Expression of SP-B, SP-C, and CCSP increased markedly before birth, consistent with the developmental increases in their mRNAs (Wert et al., 1993; Hackett et al., 1992; Wohlford–Lenane et al., 1992; Glasser et al., 1991; Korfhagen et al., 1990). The increase of SP-B is consistent with the finding that SP-B is required for adaptation to air breathing at birth. SP-B-deficient mice die from respiratory failure immediately after birth, in association with defects in tubular myelin, lamellar body formation, and pro-SP-C processing (Clark et al., 1995). This developmental increase in surfactant proteins may reflect their requirement for lung function at birth.

Whereas pro-SP-B was detected in Type II cells by both pro-SP-B antisera, the expression of pro-SP-B in bronchiolar epithelial cells was detectable with antibody R96189 and not with antibody R4633, indicating that the intracellular processing, storage, or antigen presentation of pro-SP-B may be distinct in conducting airway epithelial cells vs Type II cells. The presence of mature SP-B peptide in Type II cells but not in the conducting airway epithelial cells supports the concept that pro-SP-B may not be completely processed in conducting airway epithelial cells to produce mature SP-B peptide.

In summary, TTF-1 and HNF-3β are expressed in respiratory epithelial cells throughout lung development. The pattern of their expression precedes and overlaps that of SP-B, SP-C, and CCSP, consistent with their role in morphogenesis, differentiation, and control of gene expression in the developing respiratory epithelium.

Figure 5. Immunohistochemical staining of SP-B and CCSP in developing lungs. SP-B was undetectable in pseudoglandular lungs including gestational Day 16 (**A**). SP-B was first detected at low levels in Type II cells and within the distal airway lumen on Day 17 (**B**, arrows). Staining for SP-B increased in gestational Day 18 lung (**C**, arrows). In adult lung (**D**), SP-B was detected at high levels in Type II cells (arrow) but was not detected in the alveolar lumen (arrowhead). CCSP was undetectable in pseudoglandular lungs including gestational Day 16 (**E**). CCSP was detected at low levels in bronchiolar epithelial cells on gestational Day 17 (**F**, arrowhead). CCSP staining was more intense on gestational Day 18 (**G**, arrowhead). CCSP signal intensity further increased on postnatal Day 1 (**H**, arrowhead). b, bronchiole. Bar = 64 μm.

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Literature Cited

Ang SL, Rossant J (1994) HNF-3β is essential for node and notochord formation in mouse development. Cell 78:561–574

Ang SL, Wierda A, Wong D, Stevens KA, Cascio S, Rossant J, Zaret KS (1993) The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development 119:1301–1315

Baatz JE, Elledge B, Whitsett JA (1990) Surfactant SP-B induces ordering at the surface of model membrane bilayers. Biochemistry 29:6714–6720

Bedetti CD, Singh J, Singh G, Katyal SL, Wong-Chong ML (1987) Ultrastructural localization of rat Clara cell 10 KD secretory protein by the immunogold technique using polyclonal and monoclonal antibodies. J Histochem Cytochem 35:789–794

Bingle CD, Hackett BP, Moxley M, Longmore M, Gitlin JD (1995) Role of hepatocyte nuclear factor-3α and hepatocyte nuclear factor-3β in Clara cell secretory protein gene expression in the bronchiolar epithelium. Biochem J 308:197–202

Bohinski RJ, Di Lauro R, Whitsett JA (1994) 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

Bruno MD, Bohinski RJ, Huelsman KM, Whitsett JA, Korfhagen TR (1995) 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

Civitareale RD, Lonigro AJ, Sinclair C, DiLauro R (1989) A thyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. EMBO J 8:2537–2542

Clark JC, Wert SE, Bachurski CJ, Stahlman MT, Stirpp BR, Weaver TE, Whitsett JA (1995) Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. Proc Natl Acad Sci USA 92:7794–7798

Clevidence DE, Overdier DG, Peterson RS, Porcella A, Ye H, Paulson KE, Costa RH (1994) 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

Dodge DE, Rucker RB, Singh G, Plopper CG (1993) Quantitative comparison of intracellular concentration and volume of Clara cell 10 KD protein in rat bronchi and bronchioles based on laser scanning confocal microscopy. J Histochem Cytochem 41:1171–1183

Glasser SW, Korfhagen TR, Wert SE, Bruno MD, McWilliams KM, Vorbroker DK, Whitsett JA (1991) Genetic element from human surfactant protein SP-C gene confers bronchiolar-alveolar cell specificity in transgenic mice. Am Physiol Soc 261:L349–356

Gown AM, De Wever N, Battifora H (1993) Microwave-based antigenic unmasking: a revolutionary new technique for routine immunohistochemistry. Appl Immunohistochem 14:256–266

Guazzi S, Price M, De Felice M, Damante G, Mattei M-G, Di Lauro R (1990) Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. EMBO J 9:3631–3639

Hackett BP, Shimizu N, Gitlin JD (1992) Clara cell secretory protein gene expression in bronchiolar epithelium. Am Physiol Soc 262:L399–404

Huffman JA, Hull WM, Dranoff G, Mulligan RC, Whitsett JA (1996) Pulmonary epithelial cell expression of GM-CSF corrects the alveolar proteins in GM-CSF-deficient mice. J Clin Invest 97:649–655

Ikeda K, Clark JC, Shaw-White JR, Stahlman MT, Boutell CJ, Whitsett JA (1995) Gene structure and expression of human thyroid transcription factor-1 in respiratory epithelial cells. J Biol Chem 270:8108–8114

Jacob A, Budhiraja S, Qian X, Clevidence D, Costa RH, Reichel RR (1994) Retinoid acid-mediated activation of HNF-3α during ES stem cell differentiation. Nucleic Acids Res 22:2126–2133

Khoor A, Gary ME, Hull WM, Whitsett JA, Stahlman MT (1993) 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

Khoor A, Stahlman MT, Gray ME, Whitsett JA (1994) Temporal-spatial distribution of SP-B and SP-C proteins and mRNA in developing respiratory epithelium of human lung. J Histochem Cytochem 42:1187–1199

Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, Gonzalez FJ (1996) The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Gen Dev 10:60–69

Korfhagen TR, Glasser SW, Wert SE, Bruno MD, Daugherty CC, McNeish JD, Whitsett JA (1990) Cis-acting sequences from a human surfactant protein gene confer pulmonary-specific gene expression in transgenic mice. Proc Natl Acad Sci USA 87:6122–6126

Lazzaro D, Price M, De Felice M, Di Lauro R (1991) 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

Lin S, Phillips KS, Wilder MR, Weaver TE Structural requirements for intracellular transport of pulmonary surfactant B (SP-B). Biochim Biophys Acta, in press

Margraf LR, Finegold MJ, Stanley LA, Major A, Hawkins HK, DeMayo FJ (1993) Cloning and tissue-specific expression of the cDNA for the mouse Clara cell 10 KD protein: comparison of endogenous expression to rabbit uteroglobin promoter-driven transgene expression. Am J Respir Cell Mol Biol 9:231–238

Monaghan AP, Kaestner KH, Grau E, Schutz G (1993) Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3α, β and γ genes in determination of the definitive endoderm, chordomesoderm and neuroectoderm. Development 119:567–578

O'Reilly MA, Gazdar AF, Clark JC, Pilot-Matias TJ, Wert SE, Hull WM, Whitsett JA (1989a) Glucocorticoids regulate surfactant protein synthesis in a pulmonary adenocarcinoma cell line. Am J Physiol 157:L385–392

O'Reilly MA, Weaver TE, Pilot-Matias TJ, Sarin VK, Gazdar AF, Whitsett JA (1989b) In vitro translation, post-translational processing and secretion of pulmonary surfactant protein B precursor. Biochim Biophys Acta 1011:140–148

Sasaki H, Hogan BLM (1994) HNF-3β as a regulator of floor plate development. Cell 76:103–115

Sasaki H, Hogan BLM (1993) Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. Development 118:47–59

Sawaya PL, Luse DS (1994) 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 the Clara cell secretory protein (CCSP) promoter. J Biol Chem 269:22211–22216

Sever Z, Yan C, Whitsett JA (1995) Regulation of murine surfactant B gene transcription by TTF-1 and AP1. Mol Biol Cell 6:416a

Shi SR, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on oven heating of tissue sections. J Histochem Cytochem 39:741–748

Simone D, Corese R (1991) Transcription regulation of liver specific gene expression. Curr Opin Cell Biol 3:960–965

Singh G, Katyal SL (1984) An immunologic study of the secretory products of rat Clara cells. J Histochem Cytochem 32:49–54

Singh G, Singh J, Katyal SJ, Brown WE, MacPherson JA, Squeglia N (1988) Identification, cellular localization, isolation and characterization of human Clara cell-specific 10 KD protein. J Histochem Cytochem 36:73–80

Stahlman MT, Gray ME, Whitsett JA (1992) The ontogeny and distribution of surfactant protein B in human fetus and newborns. J Histochem Cytochem 40:1471–1480

Vorbroker DK, Dey C, Weaver TE, Whitsett JA (1992) Surfactant protein C precursor is palmitoylated and associates with subcellular membrane. Biochim Biophys Acta 1105:161–169

Vorbroker DK, Profitt SA, Nogee LM, Whitsett JA (1995) Aberrant processing of surfactant protein C (SP-C) in hereditary SP-C deficiency. Am J Physiol 268:L647–656

Weaver TE, Whitsett JA (1991) Function and regulation of expression of pulmonary surfactant-associated proteins. Biochem J 273:249–264

Weaver TE, Whitsett JA (1989) Processing of hydrophobic pulmonary surfactant protein B in rat Type II cells. Am Physiol Soc 257:L100–108

Weinstein DC, Altaba ARI, Chen WS, Hoodless P, Prezioso VR, Jessel TM, Darnell JE (1994) The winged-helix transcription factor HNF-3β is required for notochord development in the mouse embryo. Cell 78:575–588

Wert SE, Glasser SW, Korfhagen TR, Whitsett JA (1993) Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. Dev Biol 156:426–443

Wohlford–Lenane CL, Durham PL, Snyder JM (1992) Localization of surfactant-associated protein C (SP-C) mRNA in fetal rabbit lung tissue by in situ hybridization. Am J Respir Cell Mol Biol 6:225–234

Zhou L, Dey C, Wert SE, Whitsett JA (1996) Arrested lung morphogenesis in transgenic mice bearing an SP-C-TGF-β1 chimeric gene. Dev Biol 175: 227–238