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The Bone Morphogenic Protein Antagonist Gremlin Regulates Proximal-Distal Patterning of the Lung

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ABSTRACT **The proximal-distal patterning of lung epithelium involves a complex series of signaling and transcriptional events resulting in the programmed differentiation of highly specialized cells for gas exchange and surfactant protein expression essential for postnatal lung function. The BMP signaling pathway has been shown to regulate cellular differentiation in the lung as well as other tissues. In this report, we show that the *can* family of related BMP antagonists, including gremlin, cer-1, PRDC, and Dan are expressed in the lung during embryonic development with gremlin expression observed in the proximal airway epithelium. The role of gremlin in lung development was explored by overexpressing it in the distal lung epithelium of transgenic mice using the human SP-C promoter. SP-C/gremlin transgenic mice exhibited a disruption of the proximal-distal patterning found in the airways of the mammalian lung. Expanded expression of the proximal epithelial cell markers CC10 and HFH-4 (Foxj1) was observed in the distal regions of transgenic lungs. Furthermore, smooth muscle α-actin expression was observed surrounding the distal airways of SP-C/gremlin mice, indicating a proximalization of distal lung tubules. These data suggest that gremlin plays an important role in lung morphogenesis by regulating the proximal-distal patterning of the lung during development.**

Key words: gremlin; BMP; lung epithelium; lung development; proximal-distal patterning

INTRODUCTION

During embryonic development, the lung epithelium differentiates from a uniform columnar epithelial cell type lining the airways to a diverse population of specialized epithelial cells that differentiate along a proximal-distal axis (for review see Warburton et al., 2000). This proximal-distal patterning results in the development of specialized distal airway epithelial cells, type I and II pneumocytes, which are involved in gas exchange and surfactant protein expression, respectively. Upon differentiation of the proximal and distal epithelial cell types, marker genes such as the Clara Cell 10-kDa protein (CC10) and human forkhead homologue 4 (HFH-4 also known as Foxj1) are expressed exclusively in the proximal epithelium, whereas genes including surfactant protein C and GATA-6 are expressed in the distal but not proximal epithelium (Clevidence et al., 1994; Morrisey et al., 1996; Zhou et al., 1996). One family of proteins that has been implicated in regulating this proximal-distal patterning in the lung is the bone morphogenetic proteins (BMPs). The BMP signaling pathway transmits signals from the cell surface to the nucleus by means of Smad proteins, which in turn govern the development and morphogenesis of various tissues. This pathway involves the secreted BMP molecules (of which at least 16 are known), two different receptor subunits, and the 10 known Smad transcriptional regulator proteins (for review see Hogan, 1996; Kawabata et al., 1998).

The importance of BMP signaling during lung development has been demonstrated in several studies. BMP-4, -5, and -7 exhibit complex expression patterns within the developing lung with BMP-4 and -7 expression observed in the developing epithelium and BMP-5 expression observed in the lung mesenchyme (King et al., 1994; Bellusci et al., 1996). Forced expression of BMP-4 in the distal epithelium of the lung using the human SP-C promoter results in an expansion of terminal air sac size in transgenic mice (Bellusci et al., 1996). These lungs also exhibited decreased cell proliferation in the distal epithelium. Moreover, inhibition of BMP signaling by overexpression of the BMP antagonist *xnoggin* or a dominant-negative Alk6 BMP receptor (dnAlk6) resulted in a severe disruption of the proximal-distal patterning of epithelial cell types within the lung (Weaver et al., 1999). These data suggest that modulation of BMP expression and activity is required for normal morphogenesis and correct patterning of the mouse lung epithelium. However, the mechanism by which BMP signals are regulated along the proximal-distal axis remains unclear.

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Recently, a group of unique but structurally related secreted BMP antagonists has been identified. This group of factors includes the mammalian orthologues of the *Xenopus* cerberus (cer-1) and Drm/gremlin genes and the Dan and PRDC (Protein Related to Dan and Cerberus) proteins (Ozaki and Sakiyama, 1993; Belo et al., 1997; Nakamura et al., 1997; Biben et al., 1998; Hsu et al., 1998; Minabe-Saegusa et al., 1998). The name *can* has been proposed for this family of proteins (derived from cerberus and Dan) (Pearce et al., 1999). All of these proteins contain a cysteine knot motif similar to that observed in other TGF-β superfamily members (McDonald and Hendrickson, 1993). In addition, cer-1 and gremlin have been shown to act as inhibitors of BMP signaling in *Xenopus* (Biben et al., 1998; Hsu et al., 1998; Piccolo et al., 1999). The mechanism of this inhibition was demonstrated to involve direct binding of cer-1 and gremlin to BMP-2 and BMP-4, respectively. Gremlin was recently shown to be important for regulation of limb development in vertebrates (Capdevila et al., 1999; Merino et al., 1999). However, a complete understanding of the role of this gene family during vertebrate development remains to be elucidated. Recent reports describing the lack of a detectable phenotype in cer-1 null mice suggest that there may be redundant functions for some or all of these proteins (Simpson et al., 1999; Stanley et al., 2000).

In this report, we present evidence that gremlin, a member of the *can* family of BMP antagonists, regulates proximal-distal patterning of the lung. Gremlin protein expression was restricted to the proximal epithelium during late lung development, suggesting that it plays a role in the proximal-distal patterning of the lung. To further address the role of gremlin in lung development, transgenic mice were generated that overexpressed gremlin in the distal epithelium of the mouse lung using the 3.7-kb human SP-C promoter. Lungs from transgenic embryos exhibited a disruption of the proximal-distal patterning normally observed within the mammalian lung. The expression patterns of CC10 and the forkhead transcription factor HFH-4 (Foxj1), both markers of distinct proximal epithelial cell types, were expanded into the distal epithelium of transgenic mice. Finally, the presence of ectopic smooth muscle surrounding the distal airways further supports the hypothesis that the proximal-distal axis in the embryonic lung is disrupted in SP-C/gremlin mice. Thus, gremlin expression is sufficient to modify proximal-distal airway identity in the lung.

RESULTS

Expression of Gremlin and Other *can* Family Members During Lung Development

We used previously characterized rabbit polyclonal antisera to analyze gremlin protein expression by immunohistochemistry (Topol et al., 2000). Histologic sections from fixed and embedded embryonic day (E) 12.5 and E18.5 wild-type mouse embryos were stained with a rabbit anti-rat gremlin antisera, and the resulting signal was detected by the use of a secondary anti-rabbit FITC conjugated antibody. Specific staining of esophageal but not bronchial epithelium is observed at E12.5 (Fig. 1A). In addition, as a positive control, specific staining of the ectodermal layer of the forming limb bud at E12.5 was observed (Fig. 1D). Gremlin expression has been reported previously in this region of the forming limb bud by in situ hybridization (Pearce et al., 1999). At E18.5, gremlin protein expression is observed in the proximal airway epithelium of the developing mouse lung (Fig. 1B,C). Gremlin expression is not observed in the distal airways or as the proximal airways branch further and form distal airway tubules (Fig. 1B,C). Of note, these expression patterns were also observed with a rabbit polyclonal antisera raised against the human gremlin protein (data not shown). Nonimmune rabbit antisera did not produce a signal in any of the tissues examined (Fig. 1E,F). Although the pattern of gremlin mRNA expression in the lung was not discernible by in situ hybridization at the developmental time points tested (Fig. 4C and data not shown), gremlin transcripts were detected by RT-PCR in the embryonic lung at E17.5 (Fig. 2). These data show that gremlin, or an antigenically related protein, is expressed in the proximal but not distal airway epithelium of the lung during development.

To define the temporal and spatial patterns of gene expression of *can* family members other than gremlin in the developing lung, we performed in situ hybridization by using sense and antisense riboprobes for mouse Dan, PRDC, and cer-1 on staged mouse embryos. At E12.5 and E14.5, cer-1 was expressed within the airway epithelium (Fig. 3A,B, arrows). Expression of cer-1 in the epithelium was not homogenous but patchy and irregular, a pattern that was also reported in the cer-1/β-galactosidase knock-in mice (Stanley et al., 2000). At E16.5, however, expression of cer-1 was not observed within the lung (data not shown). A sense cer-1 probe did not reveal noticeable hybridization (Fig. 3C). Of note, these data differ from the β-galactosidase knock-in at the cer-1 locus, which results in β-galactosidase expression in the epithelium of the lung through postnatal development. This discrepancy could be due to the lower sensitivity of the in situ hybridization assay used in our studies or to the persistence of β-galactosidase protein in epithelial cells after cessation of gene expression. Expression of Dan and PRDC was observed at low levels in the mesenchymal cells immediately adjacent to the proximal airway epithelium from E12.5 (Fig. 3D,G) through E16.5 (Fig. 3E,H). This expression pattern overlaps that of SM22α, a marker of bronchial smooth muscle cells (Fig. 3J and Morrisey et al., 1997). Expression was not observed in the mesenchyme surrounding the pulmonary vasculature (Fig. 3E,H, arrowheads). Of note, sense probes for Dan and PRDC Cer-1 did not produce any specific signal in the lung (Fig. 3F,I). Therefore, coexpression of PRDC, Dan, and SM22α correlates with the development of the bronchial smooth muscle layer surrounding the proximal airways. This pattern of expression suggests that

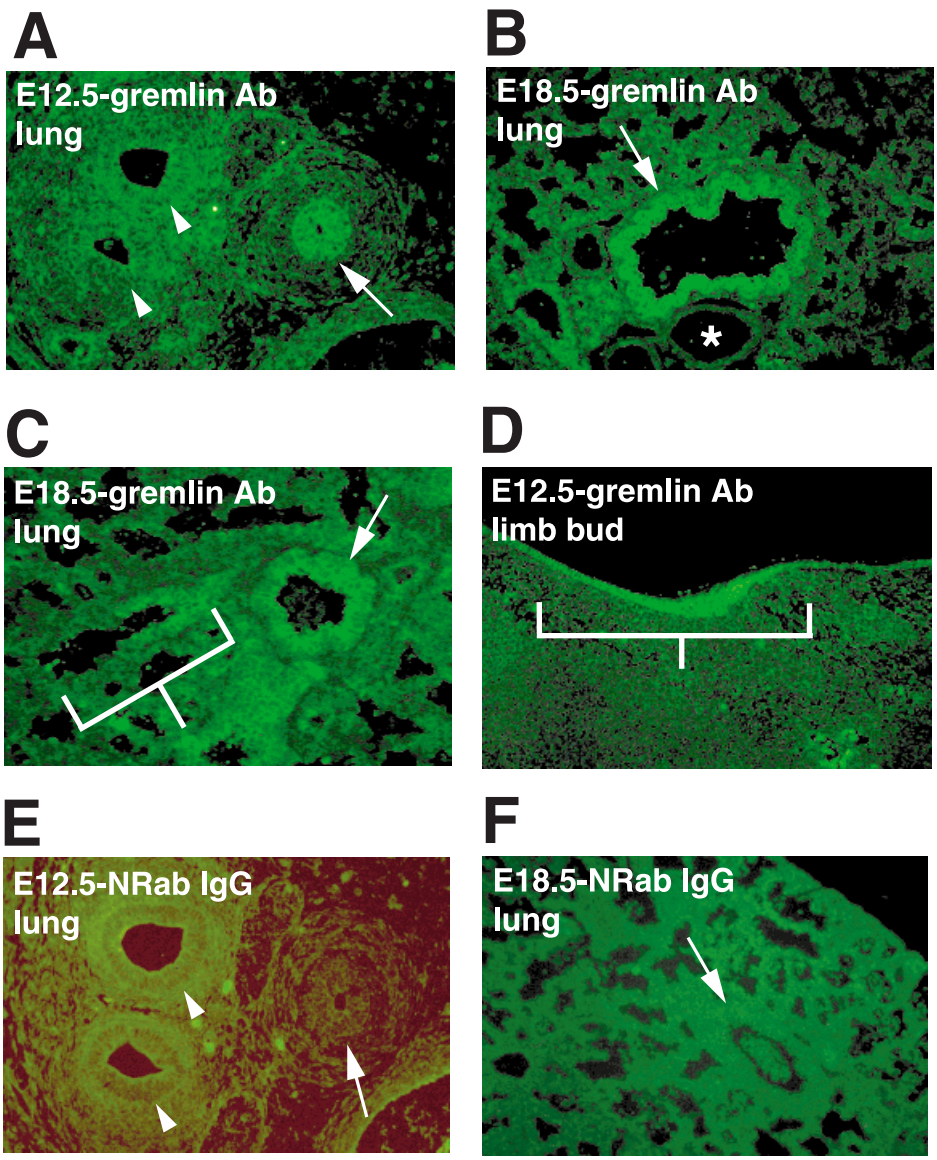

Fig. 1. Gremlin protein expression during lung development. Immunohistochemistry was performed on embryonic day (E) 12.5 and 18.5 wild-type mouse embryos using a previously characterized rabbit anti-gremlin polyclonal antibody (Topol et al., 2000). At E12.5, gremlin protein expression was observed in the epithelium of the developing esophagus (**A**, arrow) and the limb bud (**D**, bracket). However, gremlin expression was not observed at this time in the airway epithelium of the lung (A, arrowheads). **B,C:** At E18.5, gremlin expression was observed in the epithelium of the proximal bronchial airways (arrow) and was also noticed in proximal columnar epithelium but not squamous epithelium as the major proximal airways branch into distal airways (C, bracket) or in the pulmonary vasculature (B, asterisk). Original magnifications: 200×.

PRDC and Dan may regulate important BMP signaling events between the endodermal and mesodermal components of the lung and may play a role in bronchial smooth muscle development. In addition, lack of PRDC and Dan expression in the pulmonary vasculature also suggests that these genes differentially regulate bronchial versus vascular smooth muscle development in the lung (Fig. 3E,H, arrowhead). Taken together, these data show that gremlin, cer-1, PRDC, and Dan are expressed in unique patterns that suggest important roles in regulating lung development.

Morphologic Abnormalities in SP-C/Gremlin Transgenic Lungs

To further investigate the role of gremlin in lung development, we generated transgenic mice that overexpressed gremlin in the distal lung epithelium using the human 3.7-kb SP-C promoter. This promoter has been shown to direct expression of genes specifically within the distal epithelium of the lung (Wert et al., 1993). As anticipated at E17.5, the transgene was expressed in the distal lung airways in 10 of 12 independent F0 genotype-positive animals as determined by in situ hybridization analysis using probes for the SV40 polyadenylation sequence (Fig. 4A,B) and mouse gremlin (Fig. 4C,D). Morphologically, the transgenic lungs were slightly smaller and contained a more condensed mesenchyme than wild-type littermates (Fig. 4E,F). The distal epithelium of the mouse lung at E17.5 is normally lined with squamous and cuboidal epithelial cells composed of type I and type II pneumocytes, respectively (Fig. 4G). However, the distal airways of transgenic lungs were populated primarily with columnar epithelial cells that closely resemble proximal air-

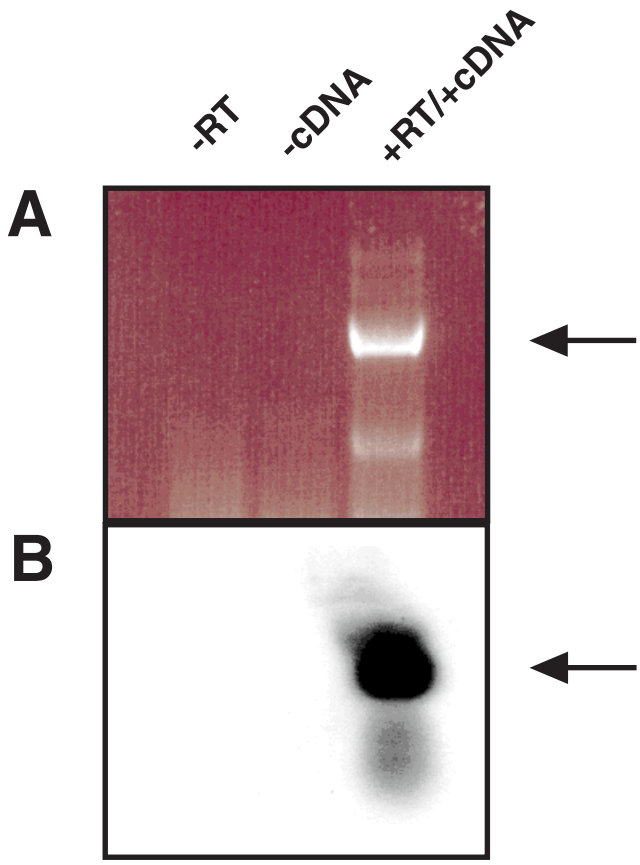

Fig. 2. Gremlin expression can be detected by reverse transcriptase-polymerase chain reaction (RT-PCR) of embryonic day (E) 17.5 mouse lung cDNA. RT-PCR was performed on cDNA derived from embryonic day (E) 17.5 wild-type whole lung RNA. Reactions were carried out without RT (-RT), without E17.5 lung cDNA (-cDNA), or with both RT and E17.5 lung cDNA (+RT/+cDNA). The top panel represents the ethidium bromide stained agarose gel of the resulting PCR reactions, whereas the bottom panel represents the same gel blotted and hybridized to a gremlin-specific probe internal to the PCR product. Arrows indicate the gremlin-specific amplification product.

way epithelium and were deficient in squamous epithelium that is normally present at this gestational age (Fig. 4H). This morphologic difference was observed in all 10 of the transgenic embryos that expressed the gremlin transgene. This disruption of distal epithelial differentiation was not noticed earlier than E16.5, which correlates both with the initiation of the proximal-distal differentiation process that results in the appearance of Clara cells and ciliated epithelium in the proximal airways and with the appearance of squamous epithelium in the distal airways of the lung (data not shown) (Warburton et. al., 2000). In addition, the 3.7-kb human SP-C promoter does not drive high levels of transgene expression before E15.5 of mouse development (Wert et. al., 1993). Together, these data suggest that the normal proximal-distal patterning of epithelial cell types observed in the lung is disrupted by expression of gremlin in the distal epithelium. Of note, no live genotype positive animals were detected out of 78 potential transgenic pups at 2 weeks of age, suggesting that the disruption of epithelial development in the lung caused by gremlin overexpression is lethal before this time (data not shown).

Analysis of Surfactant Protein Expression in SP-C/Gremlin Transgenic Lungs

To characterize whether expression of gremlin within the distal epithelium interfered with surfactant protein expression and expression of the proximal epithelial cell marker gene CC10, in situ hybridization was performed using radiolabeled riboprobes specific for surfactant proteins A, B, C, and CC10. The wild-type and transgenic-positive mice shown in Figure 5 were obtained from the same litter, and the results were consistent in all 10 of the SP-C/gremlin mice that expressed the transgene. At E17.5 in the mouse, surfactant proteins A and B are expressed by both distal and proximal epithelial cells, surfactant protein C is expressed solely in distal epithelium, and CC10 is expressed only in the nonciliated Clara cells of the proximal airways (Wert et al., 1993; Zhou et al., 1996). Expression of SP-A, -B, and -C was observed in both wild-type and transgenic lungs at E17.5 (Fig. 5A–F). However, at higher magnification, SP-C was not expressed in most of the distal epithelium of SP-C/gremlin mice that contain the abnormal columnar epithelium, indicating that most of these cells are not type II pneumocytes (Fig. 6D). The expression of SP-A, SP-B, and SP-C indicates that the lungs of SP-C/gremlin mice are not arrested in the pseudoglandular stage of lung development, which might otherwise have explained, at least partially, our histologic findings. In contrast to surfactant expression, the pattern of CC10 expression was markedly different in transgenic and wild-type lungs. Although expression of CC10 is normally confined to Clara cells of the proximal airways, transgenic lungs displayed ectopic expression within the distal regions of the lung (Fig. 5H, red arrowheads). These data are consistent with the morphologic abnormalities shown in Figure 4H demonstrating columnar epithelial cells (a proximal epithelial cell type) in the distal airways of SP-C/gremlin mice. Furthermore, these data support the hypothesis that gremlin expression in the distal epithelium disrupts the proximal-distal patterning of lung epithelium.

Expression of Transcriptional Regulators Important for Lung Development in SP-C/Gremlin Transgenic Mice

Several transcription factors have been implicated in regulating lung-specific gene expression, including the Nkx homeodomain protein TTF-1/Nkx2.1 and the forkhead/winged-helix transcription factors HFH-4 (Foxj1) and HNF-3α (Foxa1). To determine whether expression of any of these transcriptional regulators was altered in SP-C/gremlin transgenic

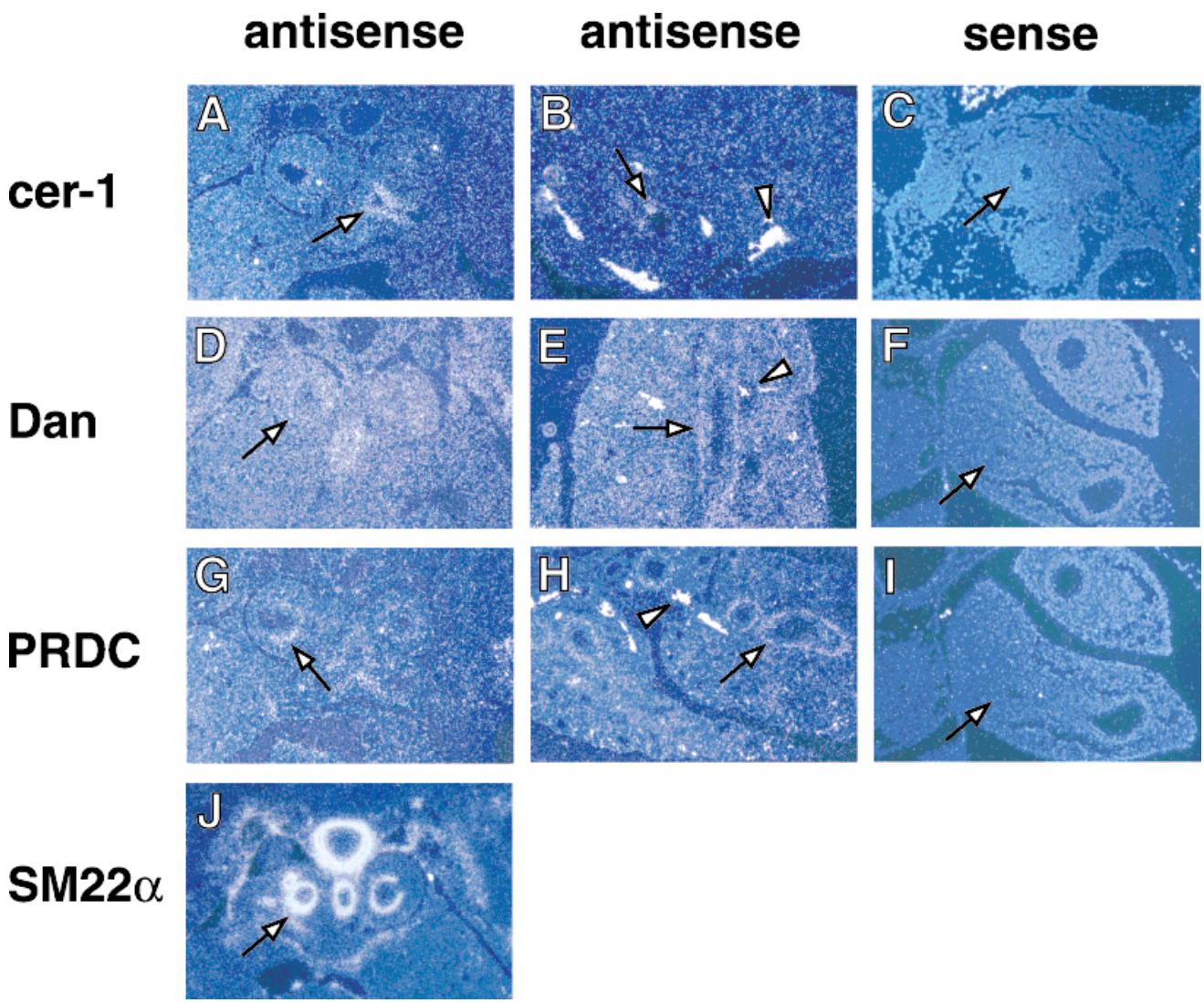

Fig. 3. Expression of cer-1, PRDC, and Dan during lung development. Radioactive in situ hybridization was performed on embryonic day (E) 12.5 (**A,D,G,J**), E14.5 (**B**), and E16.5 (**E,H**) staged mouse embryos with antisense riboprobes specific for cer-1 (A,B), Dan (D,E), PRDC (G,H), and SM22α (J). Sense riboprobes for cer-1 at E12.5 (**C**), Dan at E12.5 (**F**), and PRDC (**I**) at E12.5 are shown as negative controls. Arrows denote regions of hybridization in the epithelium for cer-1 (A,B) and the mesenchyme surrounding the epithelium for Dan (D,E) and PRDC (G,H). Note lack of hybridization to the sense probes for cer-1, Dan, and PRDC (C,F,I, arrows). Arrowheads indicate the pulmonary vasculature, which contains nonspecific autofluorescent blood (B,E,H). Original magnifications: 200×.

lungs, in situ hybridization was performed on wild-type and transgenic lung sections using radioactive riboprobes for TTF-1/Nkx2.1, HFH-4 (Foxj1), and HNF-3α (Foxa1). Of note, the wild-type and transgenic positive mice shown in Figure 7 were obtained from the same litter, and the results were consistent in all 10 of the E17.5 SP-C/gremlin mice that expressed the transgene. Expression of the transcription factors TTF-1/Nkx2.1 and HNF-3α (Foxa1) was unchanged in the transgenic lungs compared with the wild-type lungs, with TTF-1/Nkx2.1 expression observed in the proximal and distal airway epithelium and HNF-3α (Foxa1) expression observed predominantly in the proximal epithelium (Fig. 7A–D). HFH-4 (Foxj1) expression, which is normally confined to ciliated epithelial cells of the proximal airways, was expanded into the distal lung epithelium of SP-C/gremlin transgenic mice in a manner similar to that observed with the proximal Clara cell marker gene CC10 (Figs. 7E, 5F) (Hackett et al., 1995; Chen et al., 1998). These data demonstrate that expression of HFH-4 (Foxj1) was altered in SP-C/gremlin transgenic lungs. This alteration in HFH-4 (Foxj1) expression is consistent with a disruption of proximal-distal epithelial cell patterning in the lungs of SP-C/gremlin mice.

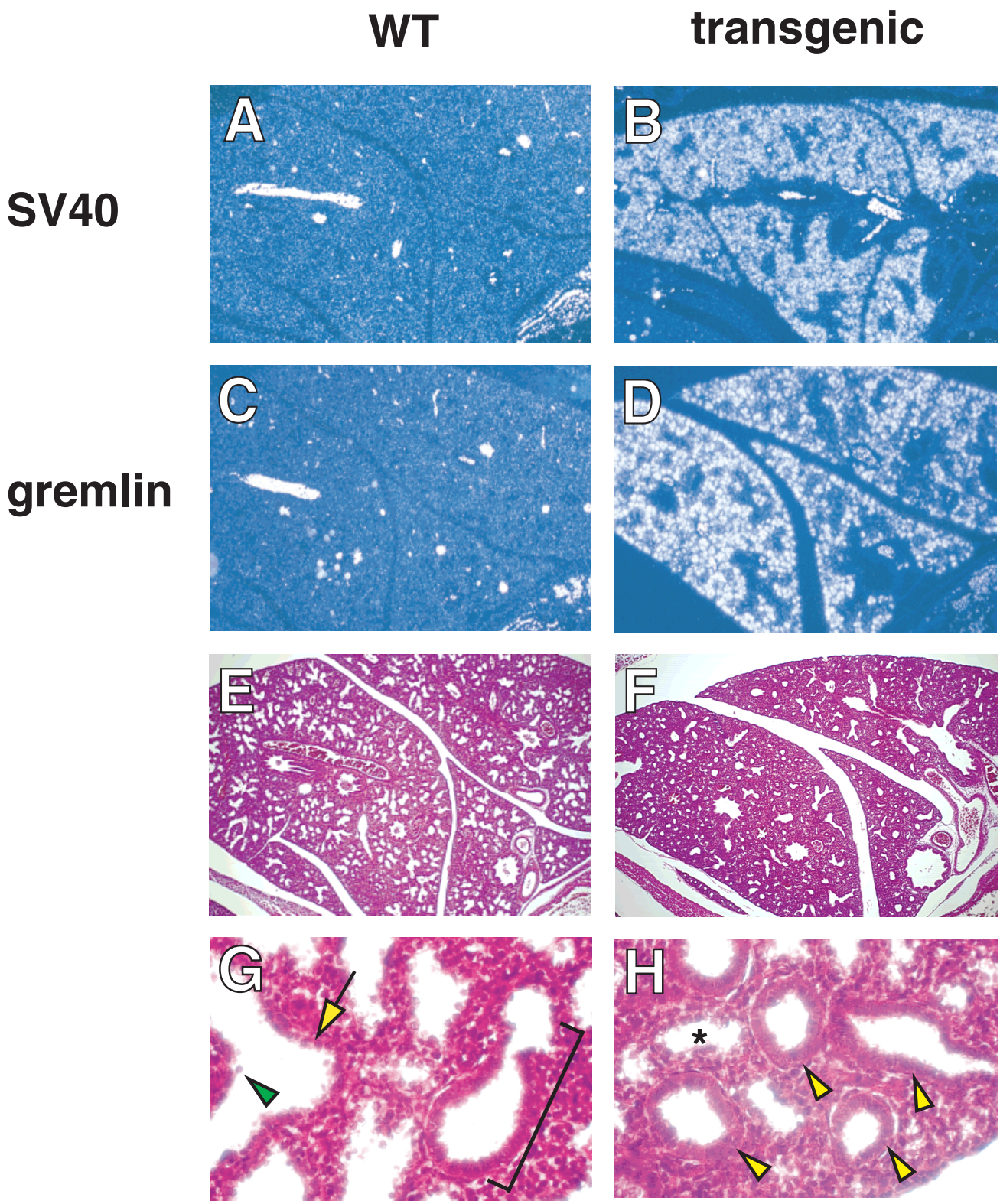

Fig. 4. Expression of the SP-C/gremlin transgene in the lungs of embryonic day (E) 17.5 genotype-positive mice. Radioactive in situ hybridization was performed on E17.5 wild-type (WT; **A,C**) and transgenic (**B,D**) littermates by using antisense riboprobes specific for the SV40 polyadenylation sequence (A,B) or to mouse gremlin (C,D). The wild-type lungs did not exhibit hybridization to either probe, whereas the transgenic lungs showed robust hybridization to both probes. H&E staining of adjacent histologic sections showed that the SP-C/gremlin transgenic lungs were slightly but reproducibly smaller and contained a condensed mesenchyme (**E** vs. **F**). At E17.5, the distal airways of wild-type lungs are lined with squamous cell types (**G**, green arrowhead) or cuboidal cells similar to type II pneumocytes (G, yellow arrow). Some of the airways in the wild-type lungs do display airway epithelium transitioning from the columnar epithelial cell type to the cuboidal/squamous cell types in the distal regions (G, bracket). However, the distal airways of SP-C/gremlin mice lacked squamous epithelium but instead were lined primarily with columnar epithelium (**H**, yellow arrowheads), with a few airways lined with cuboidal epithelium (H, asterisk). Original magnifications: 100× in A–F, 500× in G,H.

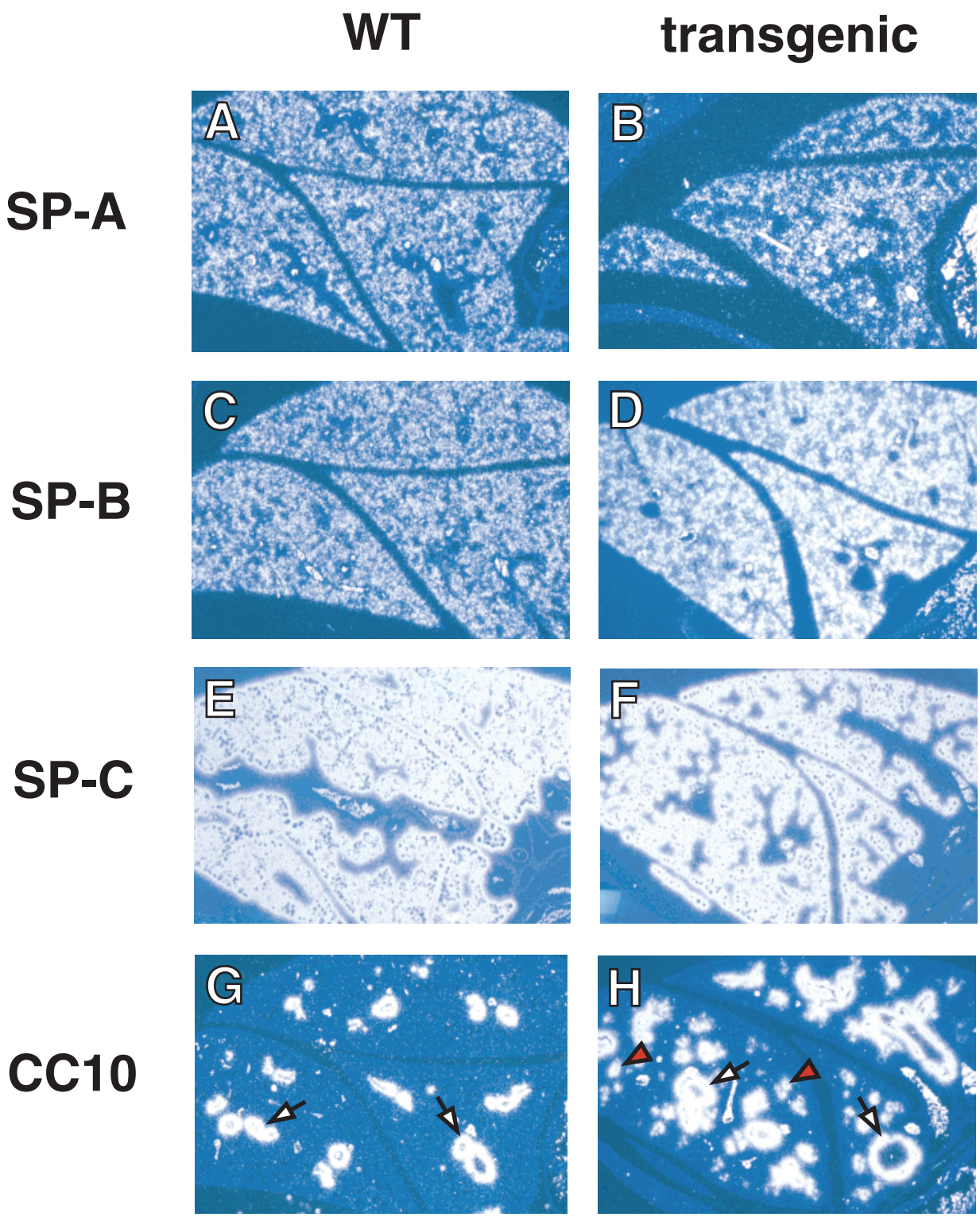

Fig. 5. Surfactant protein and CC10 expression in SP-C/gremlin transgenic lungs. Radioactive in situ hybridization was performed on embryonic day (E) 17.5 wild-type (WT; **A,C,E,G**) and SP-C/gremlin transgenic (**B,D,F,H**) littermates by using antisense riboprobes specific for SP-A (A,B), SP-B (C,D), SP-C (E,F), and CC10 (G ,H). Expression of SP-A and SP-C is observed predominantly in the distal airway epithelium at this time, whereas SP-B is expressed in both distal and proximal epithelium. No difference is observed in the expression of SP-A, SP-B, or SP-C between wild-type or SP-C/gremlin transgenic lungs. Expression of CC10 is observed in the proximal airways of wild-type mice (G, arrows), whereas expression of CC10 is observed in both the proximal (H, arrows) and the distal (H, red arrowheads) airways. Original magnifications: 100×.

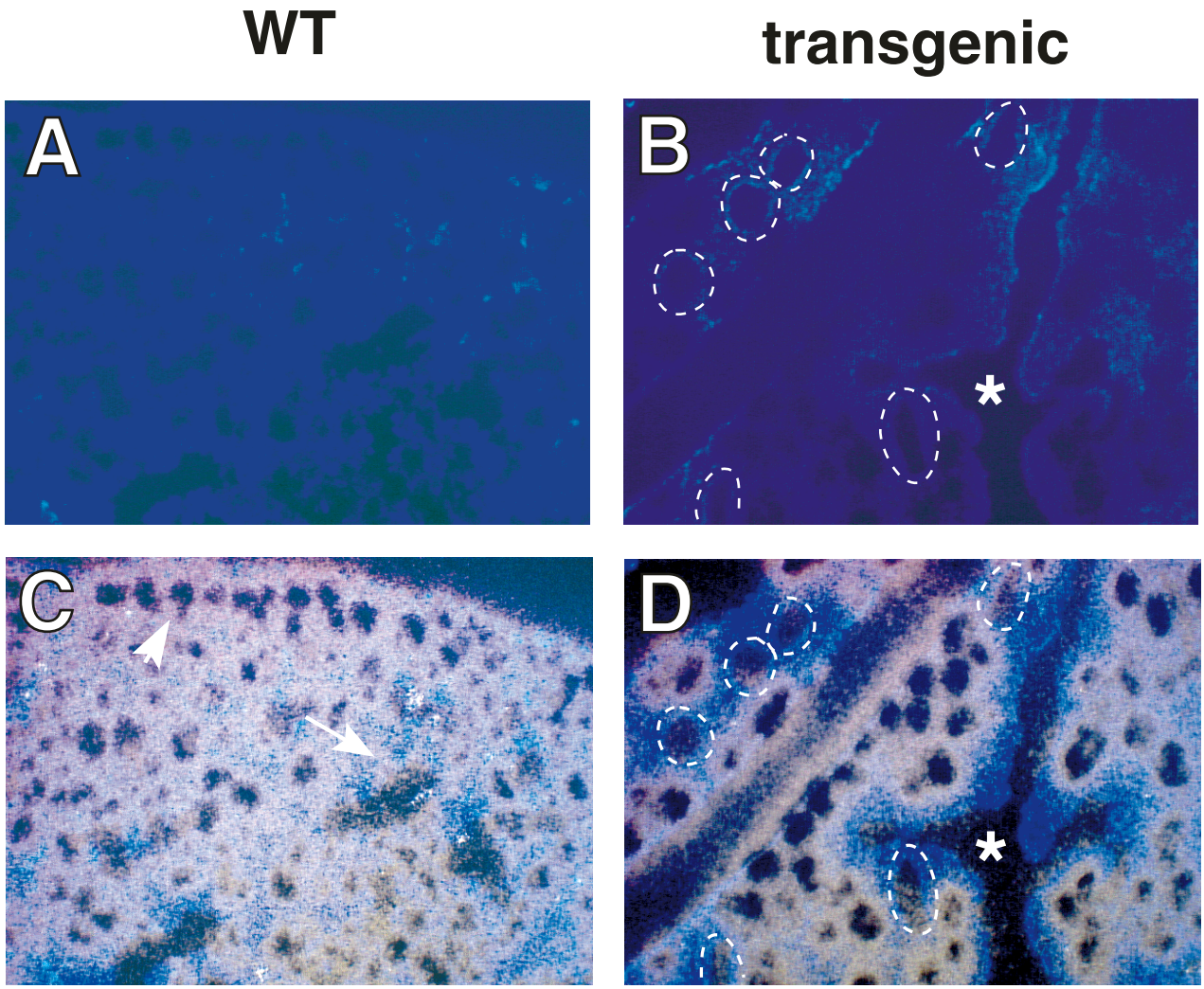

Fig. 6. SP-C was not expressed in the abnormal columnar distal airway epithelium of SP-C/gremlin mice. High magnification photomicrographs (400×) of Hoescht-stained slides (**A**,**B**) and slides processed for in situ hybridization analysis (**C**,**D**) by using a mouse SP-C riboprobe on sections of wild-type (WT; A,C) and SP-C/gremlin (B,D) embryonic day (E) 17.5 lung tissue. The abnormal columnar epithelium observed in the small, most distal regions of SP-C/gremlin lungs is highlighted by the white dashed lines, whereas a larger distal airway is denoted with an asterisk (B,D). Note the absence of SP-C expression in most of these distal airways populated with this columnar epithelium as well as the larger airways in SP-C/gremlin mice (D), whereas wild-type mice express SP-C in all of the alveolar airways (C, arrowhead) and the larger branched airways (C, arrow).

Smooth Muscle Expression Is Expanded to Surround the Distal Airways in SP-C/Gremlin Mice

To ascertain whether differentiation of the lung mesenchyme was affected by gremlin overexpression in the distal epithelium, histologic sections from wild-type and SP-C/gremlin transgenic lungs were immunohistochemically stained with a monoclonal antibody that recognizes smooth muscle α-actin. Of note, bronchial smooth muscle differentiates from the pulmonary mesenchyme and normally surrounds the proximal but not distal airways in wild-type mice. At E17.5, smooth muscle α-actin expression was observed in the mesenchyme immediately surrounding the proximal airways of wild-type lungs, which contain columnar epithelium but is absent in the distal airways, which are lined with squamous epithelium (Fig. 8A,C). However, smooth muscle α-actin expression was expanded to surround the distal airways of E17.5 SP-C/gremlin mice (Fig. 8D, arrowheads). As noted above, these airways are lined with columnar epithelium similar to proximal airways. These results were consistent in all 10 of the E17.5 SP-C/gremlin mice that expressed the transgene. These data suggest that gremlin overexpression in the distal airway epithelium disrupts mesenchymal differentiation in the lung and promotes smooth muscle formation around the distal airway tubules of SP-C/gremlin mice.

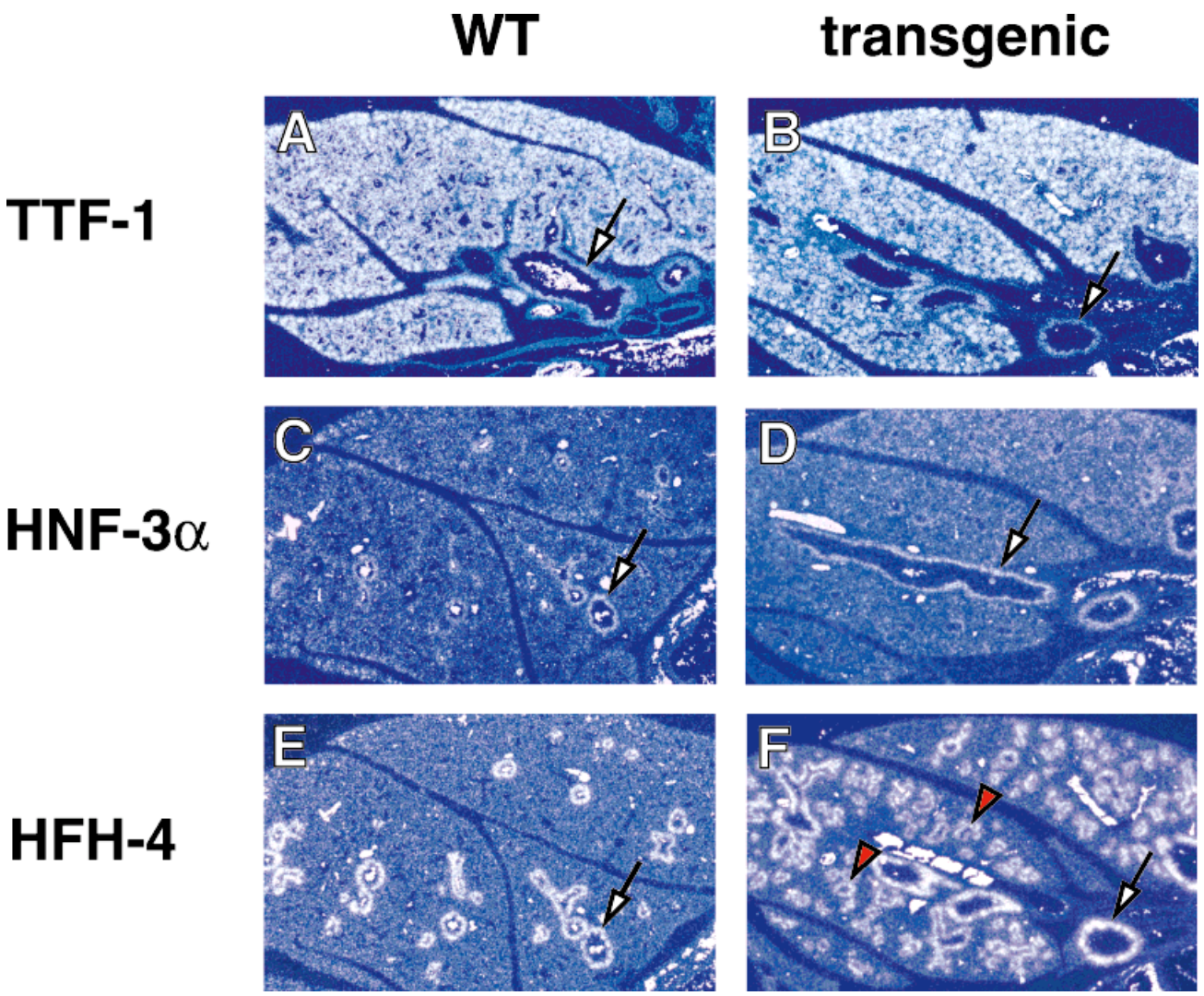

Fig. 7. Expression of cell lineage restricted transcriptional regulators in the lungs of SP-C/gremlin mice. In situ hybridization was used to examine the expression of the transcription factors TTF-1/Nkx2.1 (**A,B**), HNF-3α (Foxa1) (**C,D**), and HFH-4 (Foxj1) (**E,F**) in wild-type (WT; A,C,E) and SP-C/gremlin transgenic (B,D,F) lungs. Arrows indicate the proximal epithelium in each section. Red arrowheads indicate expanded expression of HFH-4 (Foxj1) in the distal airways of SP-C/gremlin mice (F). Original magnifications: 100×.

DISCUSSION

The mechanisms involved in the proximal-distal patterning of the lung epithelium are not well understood. The importance of this patterning process is underscored by the differentiation of distal epithelial cell types required for surfactant protein expression and gas exchange. In this report, we show that gremlin, a member of the *can* family of BMP antagonists, is expressed in the proximal epithelium of the lung during development. This pattern of expression suggests that gremlin may play a role in the proximal-distal patterning of the embryonic lung. To examine this hypothesis, we overexpressed gremlin in the distal epithelium of transgenic mice by using the human 3.7-kb SP-C promoter. Analysis of SP-C/gremlin transgenic lungs showed that the normal proximal-distal patterning of the lung had been disrupted. Molecular markers such as CC10 and HFH-4 (Foxj1), which are normally expressed exclusively in the proximal epithelium, were expressed in the distal lung epithelium of SP-C/gremlin mice. SP-C/gremlin mice expressed SP-A, SP-B, and SP-C indicating that lung development had not been arrested in the pseudoglandular stage of development. However, in the case of SP-C, expression was not observed in the abnormal columnar distal epithelium, showing that these cells do not exhibit characteristics of type II pneumocytes. Expression of CC10 and HFH-4 (Foxj1) and lack of SP-C expression indicates that these abnormal distal epithelial cells closely resemble proximal airway epithelium. Smooth muscle α-actin staining was extended to surround the distal airways in SP-C/gremlin mice, further supporting the hypothe-

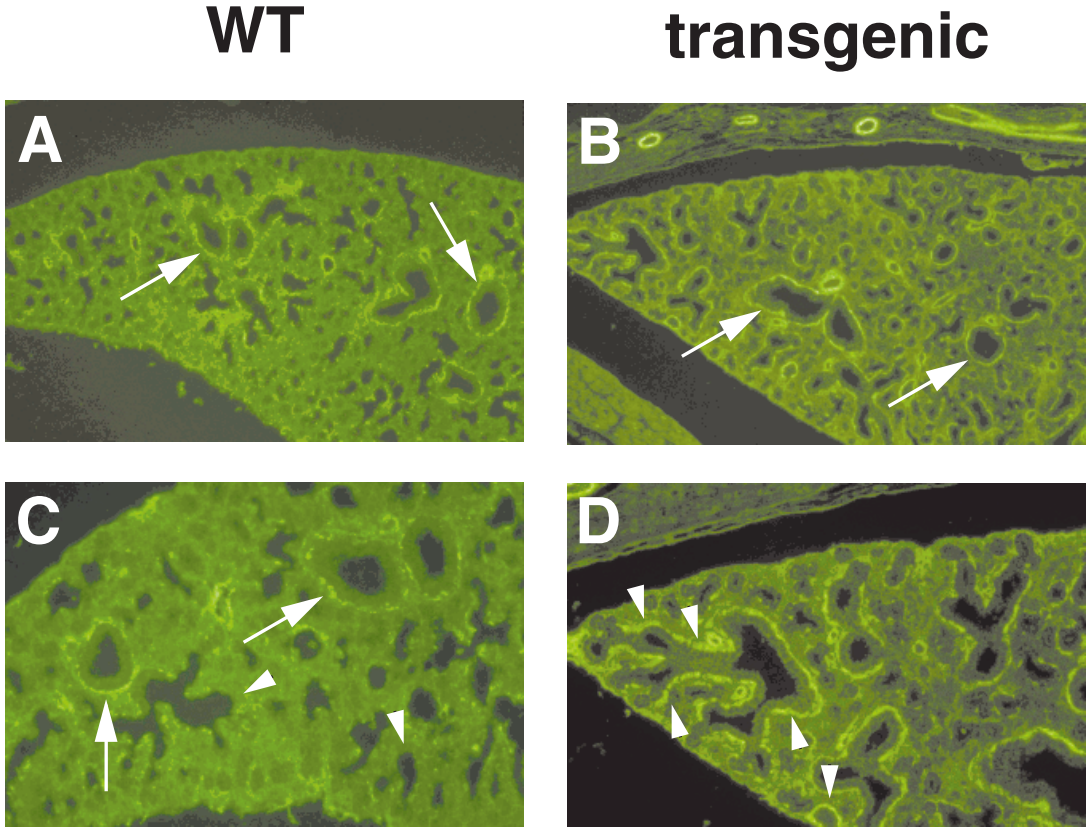

Fig. 8. Expression of smooth muscle α-actin in the lungs of SP-C/gremlin mice. Immunohistochemistry using a monoclonal antibody that recognizes smooth muscle α-actin was performed on histologic sections of wild-type (WT; **A,C**) and SP-C/gremlin transgenic (**B,D**) embryos. Expression of smooth muscle α-actin was observed surrounding the proximal airways in wild-type animals (A,C, arrows) but is absent in the mesenchyme surrounding the distal airways (C, arrowheads). However, in SP-C/gremlin mice, smooth muscle α-actin expression was observed in both the proximal airways (B, arrows) and was expanded to surround the distal airways of SP-C/gremlin mice (D, arrowheads). Original magnifications: 100× in A,B, 200× in C,D.

sis that overexpression of gremlin disrupted the proximal-distal patterning of the lung. These results suggest that gremlin regulates the proximal-distal patterning of the lung and that overexpression of gremlin in the distal epithelium by using the human SP-C promoter disrupts this patterning.

The phenotype of SP-C/gremlin transgenic lungs is very similar to that observed in a recent report describing the phenotype of mice overexpressing the BMP antagonist *xnoggin* and a dominant-negative Alk6 BMP receptor by using the human SP-C promoter (Weaver et al., 1999). The authors of this report also concluded that inhibition of BMP signaling by *xnoggin* and dnAlk6 resulted in a disruption of the proximal-distal patterning normally observed in the epithelial cells of the lung. Transgenic lungs from these animals displayed a dramatic increase in the expression domain of distal epithelial marker genes CC10 and HFH-4 (Foxj1) that corresponded to a change in the distal epithelium from a squamous cell type to a columnar cell type. One important difference between the studies presented here and those reported by Weaver et al. is that endogenous gremlin is expressed in the proximal airway epithelium, whereas mouse noggin is normally expressed in the distal mesenchyme of the embryonic lung (Weaver et al., 1999). These data, along with previous reports that gremlin inhibits signaling by BMP-4, strongly implicate gremlin as an important regulator of proximal-distal patterning during lung development and suggests that gremlin may program proximal lung epithelial cell fate through inhibition of BMP signaling (Hsu et al., 1998; Topol et al., 2000).

Endogenous expression of gremlin in the proximal but not distal epithelium of the lung indicates that it may restrict BMP activity exclusively in the proximal epithelium of the lung. Noggin, a well-characterized BMP antagonist, is expressed in the distal mesenchyme of the lung from E10.5 through E13.5 (Weaver et al., 1999). In addition, BMP-4, -5, and -7 are expressed in overlapping patterns during lung development (King et al., 1994; Bellusci et al., 1996). Therefore, gremlin and noggin together may regulate BMP activity along the proximal-distal axis of the lung, promoting the proper differentiation of both proximal and distal lung cell types (Fig. 9). This regulation not only involves spatial restriction of BMP activity but also

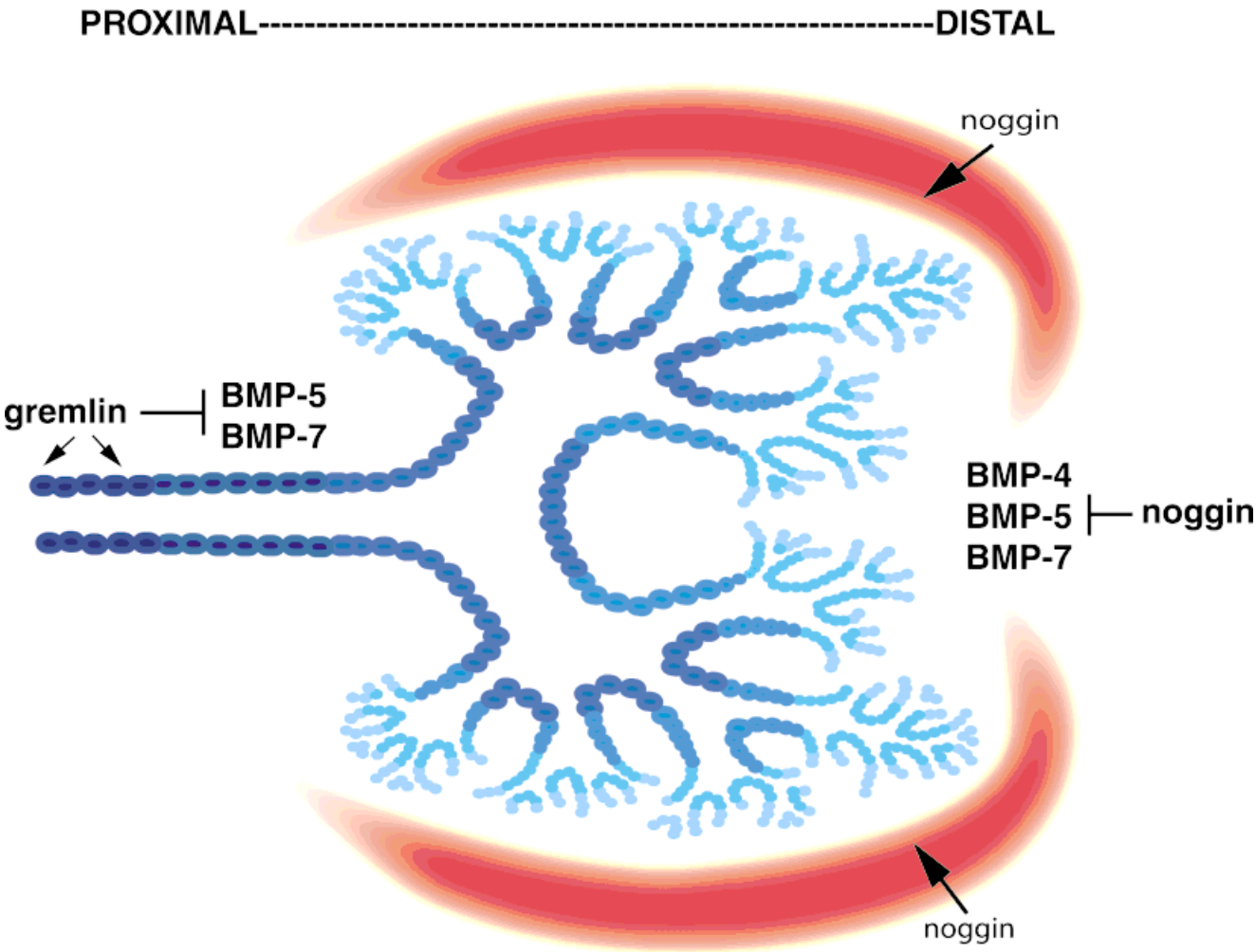

Fig. 9. Regulation of bone morphogenic protein (BMP) signaling in the developing lung. A schematic showing the complex interplay of BMP, gremlin, and noggin expression during lung development. Proximal epithelium is indicated as dark blue, whereas distal epithelium is indicated as light blue. The mesenchyme is shown in red. The distal epithelium is exposed to high levels of BMP-4, whereas the proximal epithelium is not. The activity of BMPs is regulated by gremlin in the proximal regions of the lung, whereas noggin regulates BMP activity in the distal regions. As noted in the text, noggin and gremlin are also expressed in a restricted temporal manner with noggin expression observed only between embryonic day (E) 10.5 and E13.5 and gremlin expression observed only later in lung development (E18.5) (Weaver et al., 1999).

temporal restriction, because noggin is expressed early in lung development, whereas gremlin is expressed in the latter stages of lung development (this report and Weaver et al., 1999). BMP-4, which is expressed in the distal epithelium and mesenchyme but not proximal regions of the lung, may also play a dominant role in promoting distal epithelial cell differentiation during development (Bellusci et al., 1996). The expression of other *can* proteins such as cer-1, PRDC, and Dan further suggests that BMP activity is regulated in a complex and potentially redundant manner during lung development. Thus, a fine-tuned balance of BMP activity and inhibition, through spatial and temporal expression of the various BMPs and their antagonists, may be required for proper proximal-distal patterning to occur in the lung during embryonic development.

In transgenic mice, the human SP-C promoter drives expression of genes exclusively in the distal epithelium of the lung, making BMP-4 and BMP-7 the likely targets for gremlin inhibition in the epithelium of SP-C/gremlin mice (Wert et al., 1993). Signaling by BMP-5, which is expressed in the mesenchyme, may also be disrupted due to the secreted nature of gremlin (King et al., 1994). However, because the cell-autonomous dnAlk6 BMP receptor transgenic mice exhibited a very similar phenotype to our SP-C/gremlin transgenics, we favor the concept that BMP-4 and/or BMP-7 are the best candidates for inhibition by gremlin in the distal lung epithelium of SP-C/gremlin mice (Weaver et al., 1999). Little is know about the specificity of gremlin or other *can* family members for different BMPs, although a recent report shows that gremlin can bind to

BMP-4 with relatively high affinity, which gives further credence to our hypothesis that gremlin inhibits BMP-4 signaling in SP-C/gremlin mice (Topol et al., 2000). Interestingly, BMP-4 has been shown to regulate proximal-distal patterning during tooth development, suggesting that this regulatory role for BMP-4 may be generalized to tissues other than lung (Tucker et al., 1998; Cheifetz, 1999). Further exploration into the specificity between gremlin and different BMPs should help to clarify this regulatory system in the lung.

As stated above, a previous report using transgenic mice overexpressing a dominant-negative Alk6 BMP receptor in the distal lung epithelium resulted in a similar phenotype as that observed in our SP-C/gremlin mice. Together, these data suggest cell-autonomous mechanisms for disruption of proximal-distal pattering of the lung in these two transgenic models. However, the alteration of smooth muscle α-actin expression in SP-C/gremlin mice suggests possible noncell autonomous affects from gremlin overexpression in the distal epithelium. Noncell autonomous affects are not unanticipated, considering that gremlin is a secreted protein and would be expected to affect surrounding cells. In addition, the high level of expression normally observed in transgenic mice using the human SP-C promoter further increases the chances of noncell autonomous affects (Wert et al., 1993). Therefore, the observed phenotype in SP-C/gremlin mice could be due to a direct inhibition of BMP signaling in the mesenchyme or proximal epithelium by secreted gremlin. Alternatively, the phenotype could result from disruption of distal epithelial cell differentiation which, in turn, generates an incorrect signaling event that is communicated to the adjacent mesenchyme. This "incorrect" signal may result in the reprogramming of cell fate, resulting in a phenotypic change in cell types. Results from the SP-C/dominant-negative Alk6 receptor transgenic mice support this latter hypothesis (Weaver et al., 1999). Thus, the increased smooth muscle α-actin expression surrounding the distal airways may be caused by a cell-autonomous defect in signaling produced by the distal epithelium of the lung.

The absence of SP-C expression and the presence of CC10 and HFH-4 (Foxj1) expression in the abnormal distal epithelium of SP-C/gremlin mice also indicates that these cells are not a hybrid of type II pneumocytes and proximal epithelium. In addition, much of the abnormal distal epithelium observed in SP-C/gremlin mice no longer expresses the transgene, suggesting that a permanent phenotypic switch has occurred upon expression of gremlin in the distal epithelium, producing proximalized epithelium in the distal regions of SP-C/gremlin airways and extinguishing transgene expression. Again this hypothesis is supported by the model put forth by Weaver et. al. (Weaver et. al. 1999). Alternatively, the distal epithelium in SP-C/gremlin mice may cycle between a type II pneumocyte phenotype that expresses the gremlin transgene and a proximalized epithelial cell where transgene expression is absent (because the human SP-C promoter is not active in proximal lung epithelium). These proximalized epithelial cells may then revert back to a type II pneumocyte phenotype after loss of gremlin expression. Further investigation into the role that BMP signaling plays in the development of the epithelial and mesenchymal components of the lung will be required to further characterize these observations.

Shi et al. recently reported that adenoviral overexpression of gremlin in lung explant cultures reduced branching morphogenesis (Shi et al., 2001). The data presented in that report suggests that the phenotype observed in SP-C/gremlin mice could result from a disruption of branching morphogenesis. However, a simple disruption in branching morphogenesis should not result in the ectopic appearance of columnar epithelium in the distal airways of SP-C/gremlin mice. We believe that SP-C/gremlin mice highlight a new and potentially important role for gremlin in the regulation of proximal-distal patterning of the lung by restricting the activity of BMPs, such as BMP-4, in the proximal airways. Together, these data provide further evidence that unique signaling mechanisms have evolved to differentiate proximal and distal lung epithelium along specific cell lineage pathways and that gremlin and the BMP signaling pathway play an important role in this developmental process.

EXPERIMENTAL PROCEDURES

Immunohistochemistry

The rabbit polyclonal anti-rat gremlin and anti-human gremlin antibodies have been previously described (Topol et al., 2000). The mouse monoclonal anti-smooth muscle α-actin was obtained from Roche Biochemicals. Paraformaldehyde-fixed tissue sections on slides were deparaffinized and rehydrated into distilled water through a series of decreasing ethanol washes. The slides were then washed with PBS + 0.1% Tween 20 and blocked with 10% goat serum for 1 hr. Slides were incubated with primary antisera overnight at 4°C at the following dilutions: rabbit anti-rat gremlin 1:100, rabbit anti-human gremlin 1:50, normal rabbit serum 1:50, mouse monoclonal anti-smooth muscle α-actin 1:40. All antibodies were diluted in PBS + 0.1% Tween 20 with 5% goat serum. Slides were then washed with PBS + 0.1% Tween 20 and incubated with a 1:200 dilution of biotinylated goat anti-rabbit or goat anti-mouse IgG (Vector Laboratories, Inc.) for 1 hr at room temperature. Slides were washed again and incubated with a 1:200 dilution of streptavidin FITC (NEN Biochemicals) for 1 hr at room temperature. After a final series of washes, slides were mounted with Vectashield (Vector Laboratories, Inc.) and viewed under a Zeiss Axiophot microscope. Amplification of gremlin transcripts from mouse lung cDNA.

Amplification of Gremlin Transcripts From E17.5 Mouse Lung cDNA

Total RNA was extracted from E17.5 wild-type whole lungs by using Trizol (Life Technologies, Inc.). This RNA, along with a commercially available kit (Life Technologies, Inc.), were used to generate cDNA for PCR amplification. Gremlin-specific oligos that were used to generate the in situ probe were used with this cDNA along with the Advantage cDNA PCR kit (Clontech) in a two-step cycling reaction as follows: 94°-30 sec, 68°-3 min, 30 cycles total. The PCR reactions were resolved on a 2% agarose gel and blotted to Hybond nylon membranes (Amersham, Inc.). The internal gremlin fragment used as a probe for the Southern blot was generated from gremlin cDNA by PCR amplification by using the following oligos: sense 5′-CTGAGCAGACCCAGTCCCCACC-3′; antisense 5′-TGTGACCATCATGGTGGTGAAC-3′. The resulting 334-bp fragment was radiolabeled and used to probe the PCR Southern blot, which was then washed and exposed to film.

Construction of SP-C/Gremlin Transgenic Vector and Generation of Transgenic Mice

The full-length mouse gremlin cDNA was generated by RT-PCR from total mouse embryo (E14.5) RNA by using the following primers: sense 5′-CACGTCGACATGAATCGCACCGCATACACTG-3′, antisense 5′-CACGAATTCTTAATCCAAGTCGATGGATATGC-3′. The resulting 573-bp PCR product was cloned into the *Sal*I/*Eco*RI sites of the human SP-C 3.7-kb promoter/enhancer transgenic construct to generate the SP-C/gremlin transgenic vector (Wert et al., 1993). The fidelity of the PCR reaction was confirmed by DNA sequence analysis. The SP-C/gremlin vector was digested with *Nde*1 and *Not*1 and the resulting 4.8-kb fragment was purified by using the Elutip DNA purification system (Schlicher and Schuell). Purified DNA was injected into FVB/N oocytes, which were transplanted back into pseudopregnant female mice. Of 72 embryos collected on E17.5, 12 were genotype positive by Southern blot analysis of yolk sac DNA by using a 0.7-kb fragment of the human SP-C promoter as a probe and analyzed further. DNA from tail samples of weaned mice 2 weeks of age were genotyped by using Southern blot analysis with the same probe.

Histology and In Situ Hybridization Analysis

E17.5 mouse embryos were fixed in 4% paraformaldehyde for 48 hr and processed as previously described to produce histologic sections for hematoxylin and eosin (H&E) staining and in situ hybridization (Morrisey et al., 1996). The HNF-3α (Foxa1) and SM22α probes have been described previously (Kuo et al., 1997; Morrisey et al., 1997; Morrisey et al., 1998). Other probes were generated by RT-PCR from mouse lung cDNA (surfactant proteins A [SP-A], B [SP-B], and C [SP-C], HFH-4 [Foxj1], and Nkx2.1/TTF-1) or E14.5 total mouse embryo cDNA (cer-1, Dan, PRDC, and gremlin) by using the following oligonucleotides: SP-A sense 5′-GATTGGGAGAAACCACTGGTACAG-3′, antisense 5′-CCACGCGCTCTGGTACACATCTC-3′; SP-B sense 5′-GTTCCTGGAACAAGAATGTGATATCC-3′, antisense 5′-GCACACCTGGGACACAGCCACAG-3′; SP-C sense 5′-AATGGACATGAGTAGCAAAGAGG-3′, antisense 5′-GATATAGTAGAGTGGTAGCTCTCC-3′; CC10 sense 5′-CATCTACAGACACCAAAGCCTCC-3′, antisense 5′-GAGACACAGGGCAGTGACAAGGC-3′; Nkx2.1/TTF-1 sense 5′-CTGCAACGGCAACCTGGGCAAC-3′, antisense 5′-GAATCATGCTGGCCAGATGC-3′; HFH-4 (Foxj1) sense 5′-AGCACCTTACTGCTGACCCAGG-3′, antisense 5′-CAAGAAGGTCTCATCAAAG-3-3′; Dan sense 5-3′-GGTAGAGAAGATTGTACACTGCAG-3-3′, antisense 5-3′-CACTCATCCTCAGCAGCTCAG-3-3′; Cer-1 sense 5-3′-AGGAGGAAGCCAAGAGGTTCTG-3-3′, antisense 5-3′-GGAAGTCCAGGGATGAAGG-3-3′; PRDC sense 5-3′-CCTTGTGTACTCAGAGTGCA-3-3′, antisense 5-3′-ACTGTGAGGCTGAGATGTG-3-3′; gremlin sense 5-3′-CCGCCTCCTGACAAGGCTCAG-3-3′, antisense 5-3′-TTGGTGGGTGGCTGTAGCTC-3-3′. Sense riboprobes had a T7 RNA polymerase binding site engineered into the 5-3′ end and antisense had a T7 site engineered into the 3-3′ end of the PCR products. [^{35}S]UTP-labeled riboprobes were hybridized to histologic sections, washed, and exposed to emulsion for 7 days before developing. Adjacent sections were stained with H&E for morphologic comparison. Photographs were taken on a Zeiss Axiophot microscope.

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