Development 129, 2233-2246 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV4604

GATA6 regulates differentiation of distal lung epithelium

Honghua Yang[1], Min Min Lu[1], Lili Zhang[1], Jeffrey A. Whitsett[2] and Edward E. Morrisey[1,*]

[1]Department of Medicine, Molecular Cardiology Research Center, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA
[2]Department of Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, Ohio 45229, USA
*Author for correspondence (e-mail: emorrise@mail.med.upenn.edu)

Accepted 4 February 2002

SUMMARY

GATA6 is a member of the GATA family of zinc-finger transcriptional regulators and is the only known GATA factor expressed in the distal epithelium of the lung during development. To define the role that GATA6 plays during lung epithelial cell development, we expressed a GATA6-Engrailed dominant-negative fusion protein in the distal lung epithelium of transgenic mice. Transgenic embryos lacked detectable alveolar epithelial type 1 cells in the distal airway epithelium. These embryos also exhibited increased *Foxp2* gene expression, suggesting a disruption in late alveolar epithelial differentiation. Alveolar epithelial type 2 cells, which are progenitors of alveolar epithelial type 1 cells, were correctly specified as shown by normal thyroid transcription factor 1 and surfactant protein A gene expression. However, attenuated endogenous surfactant protein C expression indicated that alveolar epithelial type 2 cell differentiation was perturbed in transgenic embryos. The number of proximal airway tubules is also reduced in these embryos, suggesting a role for GATA6 in regulating distal-proximal airway development. Finally, a functional role for GATA factor function in alveolar epithelial type 1 cell gene regulation is supported by the ability of GATA6 to trans-activate the mouse *aquaporin-5* promoter. Together, these data implicate GATA6 as an important regulator of distal epithelial cell differentiation and proximal airway development in the mouse.

Key words: GATA6, Lung development, Distal epithelium, Surfactant proteins, Fox genes, Mouse

INTRODUCTION

Embryonic lung development in the mouse can be divided into four overlapping stages: embryonic (E9.5-E12.5), pseudoglandular (E12.5-E16.0), canalicular (E16.0-E17.5), and saccular (E17.5-birth) (for reviews, see Costa et al., 2001; Warburton et al., 2000). During embryonic development, the epithelium lining the airways differentiates from a uniform cell type to a diverse population of specialized epithelial cells that differentiate along a proximal-distal axis (Warburton et al., 2000; Weaver et al., 1999). Much of this differentiation occurs in the canalicular (E16.0-E17.5) and saccular (E17.5-birth) stages of lung development when normal proximal-distal patterning results in the development of specialized distal airway epithelium, which consists of type 1 and 2 alveolar epithelial cells (AEC-1 and -2) required for gas exchange and expression of surfactant proteins, respectively (for a review, see Warburton et al., 2000). Current paradigms in lung epithelial cell biology suggest that AEC-2 cells can self-renew and trans-differentiate into AEC-1 cells (Evans et al., 1975; Warburton et al., 2000). During later stages of lung development (E16.5 and later), genes encoding proteins such as the Clara cell-specific 10 kDa protein (CC10) and Foxj1 (previously known as human forkhead homologue 4), are expressed exclusively in the proximal epithelium, while genes encoding proteins including surfactant protein C (SP-C), GATA6, and Foxp2 are expressed in the distal but not proximal epithelium (Morrisey et al., 1996; Shu et al., 2001; Tichelaar et al., 1999b; Zhou et al., 1996b).

Several transcription factors have been implicated in regulating lung epithelial development including thyroid transcription factor-1 (TTF-1) (also known as Nkx2.1 and Titf1), several Fox gene products including Foxa2 and Foxj1, and the zinc finger transcription factor GATA6. TTF-1 is a member of the Nkx homeodomain gene family and has been shown to bind and trans-activate sequences present in many lung-specific promoters including the surfactant proteins A (*SP-A/Sftpa*), B (*SP-B/Sftpb*), and C (*SP-C/Sftpc*) and *CC10* (*Utg*) (Bruno et al., 1995; Margana and Boggaram, 1997; Toonen et al., 1996). Mice harboring a null mutation in the *TTF-1* gene exhibit severely attenuated lung epithelial development with a dramatic decrease in airway branching (Kimura et al., 1996). In addition, lung epithelial cells present in these mice lack expression of certain putative target genes such as *SP-C* suggesting that TTF-1 is an important and essential transcription factor for lung epithelial gene expression (Minoo et al., 1999). Members of the winged-helix/forkhead or Fox gene family are expressed in a wide range of cells in the lung including the distal epithelium (*Foxp2* and *Foxa2*), proximal epithelium (*Foxj1* and *Foxa2*), and mesenchyme (*Foxf1* and *Foxf2*) and Fox DNA binding sites are located in the promoters and enhancers of several lung specific genes

including *CC10*, *TTF-1* and *SP-B* (Braun and Suske, 1998; Clevidence et al., 1994; Ikeda et al., 1996; Kaestner et al., 1994; Mahlapuu et al., 1998; Margana and Boggaram, 1997; Miura et al., 1998; Shu et al., 2001; Tichelaar et al., 1999b; Zhou et al., 1996b). Furthermore, gain-of-function and loss-of-function experiments have demonstrated the necessity of wild-type expression levels of some of these Fox genes during lung differentiation and development including *Foxf1*, *Foxa2* and *Foxj1* (Kalinichenko et al., 2001; Mahlapuu et al., 2001; Tichelaar et al., 1999a; Zhou et al., 1997).

The zinc finger transcription factor GATA6 has recently been implicated in regulating lung gene expression and development. GATA6 belongs to the GATA family of zinc finger transcriptional regulators, several of which have been shown to play important roles in tissue specification and gene expression (Simon, 1995). GATA6 is the only known GATA factor expressed in the distal epithelium of the developing lung where its expression is observed as early as E10.5 (Bruno et al., 2000; Keijzer et al., 2001; Morrisey et al., 1996; Morrisey et al., 1997). Binding sites for GATA6 are present in several lung-specific promoters and enhancers including *SP-A*, *SP-C*, and *TTF-1* (Bruno et al., 2000; Shaw-White et al., 1999). In addition, GATA6 is able to trans-activate these promoters in non-lung cells suggesting a role in the transcriptional regulation of these genes (Bruno et al., 2000; Shaw-White et al., 1999). Previous experiments implicated an essential role for GATA6 in early lung epithelial development because *Gata6*$^{-/-}$ embryonic stem (ES) cells were unable to contribute to the airway epithelium of *Gata6*$^{-/-}$/C57BL6 chimeric mice at E13.5 (Morrisey et al., 1998). However, a recent report suggests that under certain in vivo experimental conditions, *Gata6*$^{-/-}$ ES cells can differentiate down the lung epithelial cell lineage pathway (Keijzer et al., 2001). These *Gata6*$^{-/-}$/C57BL6 chimeric lungs displayed defects in branching morphogenesis and down-regulation of putative target genes such as *SP-C* (Keijzer et al., 2001). However, the specific defects in epithelial cell differentiation were not reported. Therefore, much is still not known about the cell intrinsic function(s) of GATA6 transcriptional regulation in lung epithelium, which is important because of the expression of GATA6 in the adjacent mesenchyme of the lung (Bruno et al., 2000; Keijzer et al., 2001; Morrisey et al., 1996).

We have examined the role of GATA6 in distal lung epithelial development by expressing a GATA6/Engrailed dominant-negative fusion protein (GATA6/En) in transgenic mice using the well-characterized human 3.7 kb *SP-C* promoter which drives expression exclusively in distal lung epithelium (Koutsourakis et al., 2001; Lu et al., 2001; Weaver et al., 1999; Wert et al., 1993; Zhou et al., 1997). Several reports, from our laboratory and others, have shown that the GATA6/En fusion protein can repress GATA6 transcriptional activation (Bruno et al., 2000; Liang et al., 2001). In addition, similar Engrailed repressor domain/transcription factor fusion protein strategies have been used to perturb the function of c-myb and Nkx2.5 (Badiani et al., 1994; Fu et al., 1998). Although the GATA6/En dominant-negative may repress the function of most if not all GATA factors, GATA6 is the only known GATA factor expressed in distal lung epithelium (Morrisey et al., 1996; Morrisey et al., 1997). The morphological characteristics of the surfactant protein C/GATA6-Engrailed transgenic (*SP-C/G6en*) embryos are distinct from other lung transgenic models previously reported. *SP-C/G6en* transgenic mice exhibited a lack of squamous epithelium in the distal regions of the lung during late alveolar development indicating a defect in AEC differentiation. In particular, AEC-1 cells were absent in the distal airways of *SP-C/G6en* embryos as demonstrated by electron microscopy and attenuated *aquaporin 5* gene expression. AEC-2 cells had been properly specified as shown by normal *TTF-1* gene expression in these embryos. Furthermore, the presence of lamellar bodies, secreted surfactant and normal expression of SP-A suggests that AEC-2 differentiation had progressed beyond the pseudoglandular stage (E12.5-E16.0) of lung development in *SP-C/G6en* embryos. However, decreased endogenous SP-C expression as well as increased *Foxp2* gene expression after E17.5 indicates that certain aspects of AEC-2 cell gene expression were perturbed. Proximal airway development was also disrupted as shown by a decrease in CC10- and Foxj1-positive proximal airway tubules. Finally, we show that binding sites for GATA factors are conserved in the aquaporin 5 promoters from mouse and rat and that GATA6 can trans-activate the mouse *aquaporin 5* promoter, supporting a role for GATA function in AEC-1 cell gene expression. Taken together, these data implicate a role for GATA6 in the regulation of distal airway epithelial differentiation, leading to a disruption in AEC-1 differentiation and proximal airway development.

MATERIALS AND METHODS

Generation of *SP-C/Gata6/engrailed* transgenic mice

The GATA6/Engrailed chimeric protein was generated by cloning together, in frame, cDNAs encoding the DNA binding domain of the mouse GATA6 protein (aa233-532) and aa1-298 of the *Drosophila* Engrailed (En) transcriptional regulator, which has been shown to confer transcriptional repression when fused to heterologous DNA binding domains (Badiani et al., 1994; Fu et al., 1998). This fusion construct was further cloned into the human 3.7 kb SP-C transgenic construct with a modified multiple cloning site resulting in *SP-C/G6en* (Wert et al., 1993). The 298 amino acid Engrailed repressor domain containing a nuclear localization signal at the amino terminus (MKRKKK) was also cloned into the same SP-C transgenic vector resulting in *SP-C/en*. The resulting constructs, *SP-C/G6en* and *SP-C/en*, were digested with *Asc*I to release the transgenes away from the backbone plasmid, and purified using Geneclean (Bio 101, Inc.). Purified transgenic DNA was injected into FVB/N fertilized oocytes and transferred into pseudopregnant females. For histological analysis, six independent founders at E17.5, five at E19.5, and two deceased pups just after birth (P0) were examined for the *SP-C/G6en* construct while five independent founder lines were examined at E19.5 for the *SP-C/en* construct. All transgenic embryos examined contained at least 15 copies of the transgene and the phenotypes reported were consistent in all of the founders tested.

Histology

E17.5 embryos were dissected from the uterus and fixed in 4% paraformaldehyde for 48 hours. The lungs from E19.5 embryos and P0 neonates were dissected and removed from the chest cavity and fixed for 24 hours in 4% paraformaldehyde. All tissue samples were dehydrated in a series of ethanol washes and embedded in paraffin blocks. Hematoxylin and Eosin (H+E) staining was performed as previously described (Soudais et al., 1995). The in situ probes for *Gata6*, SV40 poly(A)+ intron, *engrailed*, *SP-A*, *SP-C*, *Foxp2*, *CC10* and *Foxj1* have been described (Lu et al., 2001; Morrisey et al., 1996; Shu et al., 2001). The in situ probe for mouse aquaporin 5 was

generated by RT-PCR from adult mouse lung mRNA using the following primers: sense 5′-GCTGTGGTCAAAGGCACATATGAG-3′, antisense w/T7 site 5′-TGTAATACGACTCACTATAGGG-CGACTGAACTGCTGTGAGCTTGCAC-3′. Radioactive in situ hybridization was performed as previously described (Soudais et al., 1995). To quantitate the numbers of CC10-positive airways in wild-type and *SP-C/G6en* mice, five sections corresponding to the same region of the left lung from three wild-type and three *SP-C/G6en* transgenic littermates at E17.5 and E19.5 were probed with the *CC10* riboprobe and the resulting positive airways were counted and analyzed using the NIH Image 1.62 software.

Non-radioactive in situ hybridization was performed by probing deparaffinized tissue sections with a digoxigenin-labeled riboprobe and developing and immunologically detecting the positive signal with an anti-digoxigenin monoclonal antibody (Roche Biochemicals, Inc.). Non-radioactive in situ hybridization was used to detect *SP-C* gene expression because the signal from radioactive in situ hybridization was too intense for high resolution analysis of expression. Periodic acid-Schiff staining (PAS) was performed by soaking deparaffinized embryo sections in 0.5% Periodic acid for 5 minutes, rinsing with dH_2O and then staining in Schiff's reagent for 60 seconds. PAS-stained slides were then counterstained with Hematoxylin. For amylase treatment, slides were predigested in diastase (with α-amylase) solution for 15 minutes prior to PAS staining. Further details of all histological procedures can be obtained at the University of Pennsylvania Molecular Cardiology Research Center Web page-Histology Core Facility Protocols (http://www.med.upenn.edu/mcrc/histology/ histologyhome.html).

Ultrastructural analysis of mouse lung tissue

Electron microscopy procedures were performed as previously described (Soudais et al., 1995). Briefly, lung tissue was dissected from E19.5 *SP-C/G6en*, *SP-C/en* and wild-type littermates and fixed in 2% glutaraldehyde with 0.1 M sodium cacodylate pH 7.4 for 48 hours at 4°C. Samples were then incubated with 2% osmium tetroxide and 0.1 M sodium cacodylate pH 7.4 for 1 hour at 4°C. Ultrathin sections were stained with lead citrate and uranyl acetate and viewed on a JEM 1010 microscope. Digital images were captured on a Hamamatsu HamC4742-95-12 CCD camera using AMT Advantage software.

Northern blot analysis

SP-C/G6en transgenic mice were harvested at E19.5 and the whole lung was dissected from the fetus and frozen at –80°C. Two genotype positive and wild-type lung samples from the same litter were homogenized in Trizol and total RNA was extracted according to the manufacturers protocol (Invitrogen, Inc.). 10 μg of total RNA was resolved on a 1.5% formaldehyde agarose gel and blotted to a Hybond-N membrane (Amersham, Inc.). Generation of radiolabeled probes and hybridization was performed as described previously (Morrisey et al., 1996). The DNA fragments used to generate probes for *aquaporin 5* (*Aqp5*), *Foxp2* and *engrailed* were the same as described for the in situ hybridization analysis.

Co-transfection experiments

The –1437 bp mouse *Aqp5* promoter was amplified from mouse genomic DNA and subcloned into the *Mlu*I/*Xho*I sites of pGL3basic (Promega) to generate pGL3/Aqp5-1.4 using the following primers: sense 5′-CACACGCGTAAACCTAGAAGGTCCTCCCTCC-3′, antisense 5′-CACCTCGAGTTCTGCGAGACGTGCGGTGCCC-3′. A truncated version lacking the four potential GATA6 binding sites (pGL3/Aqp5-0.5) was generated by PCR from pGL3/Aqp5-1.4kb using the same antisense primer described above and the following sense primer: 5′-CACACGCGTGGCGCTGTCCTCAGAAACT-CATC-3′. The fidelity of both constructs was verified by DNA sequence analysis. These reporter constructs (0.5 μg) were co-transfected into NIH-3T3 cells along with the pcDNA3G6 construct (Morrisey et al., 1996) (2.5 μg) containing the entire open reading frame of the mouse *Gata6* cDNA using Fugene 6 (Roche Biochemicals, Inc.). To test whether the GATA6 activation of the *Aqp5* promoter could be repressed with the GATA6/Engrailed fusion protein, pcDNA3 plasmids containing either the GATA6/Engrailed fusion protein (pcDNA3G6/En), the Engrailed repressor domain (pcDNA3En) or a mutant GATA6/Engrailed fusion protein (pcDNA3G6mut/En), which contains mutations changing aa293-294 of GATA6 cysteine-alanine to serine-arginine (eliminating DNA binding), were transfected along with the pGL3/Aqp5-1.4kb reporter plasmid and the pcDNA3G6 expression plasmid (1 μg). All transfections contained 0.5 μg of the pMSVβgal vector to control for transfection efficiency. Forty-eight hours after transfection, cells were harvested and luciferase and β-galactosidase assays were performed using commercially available kits (Promega).

RESULTS

Analysis of *SP-C/G6en* transgenic mice

To determine the role of GATA6 during lung epithelial development, we generated transgenic mice using the human 3.7 kb *SP-C* promoter to express a fusion protein consisting of the DNA binding domain of the mouse GATA6 protein (aa233-352) fused to the well-characterized repression domain of the *Drosophila* Engrailed protein (aa1-298) followed by the SV40 intron/poly(A)+ sequence (Fig. 1A). In addition, a construct consisting of the human *SP-C* promoter driving expression of the *Drosophila* Engrailed repressor domain (aa1-298) with a nuclear localization signal was also injected and used as a control for specificity of the GATA6/Engrailed fusion protein (Fig. 1B). The human *SP-C* promoter was chosen for its ability to restrict high level expression of transgenes to the distal

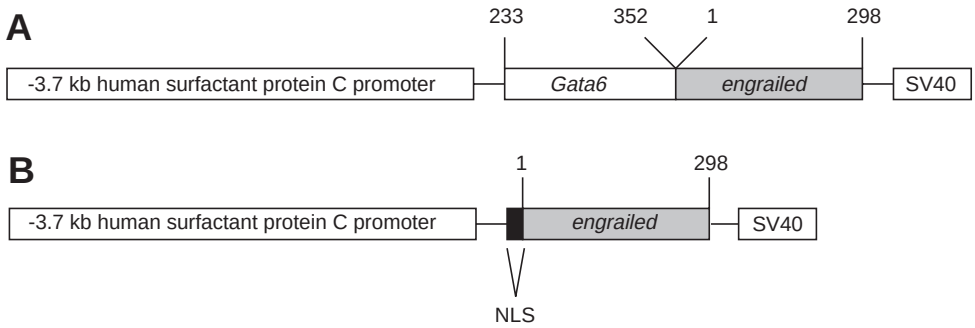

Fig. 1. Schematic of the *SP-C/G6en* and *SP-C/en* transgenic constructs. (A) The 3.7 kb human SP-C promoter/enhancer was used to drive expression of a fusion cDNA consisting of the DNA binding domain of mouse GATA6 (aa233-352) fused to the repression domain of the *Drosophila* Engrailed protein (aa1-298). This was followed by the SV40 poly(A)+ sequence. (B) The 3.7 kb human SP-C promoter/enhancer was also used to drive expression of the *Drosophila* Engrailed repression domain (aa1-298) containing a nuclear localization sequence at the amino-terminus (MKRKKK). This was followed by the SV40 poly(A)+ sequence.

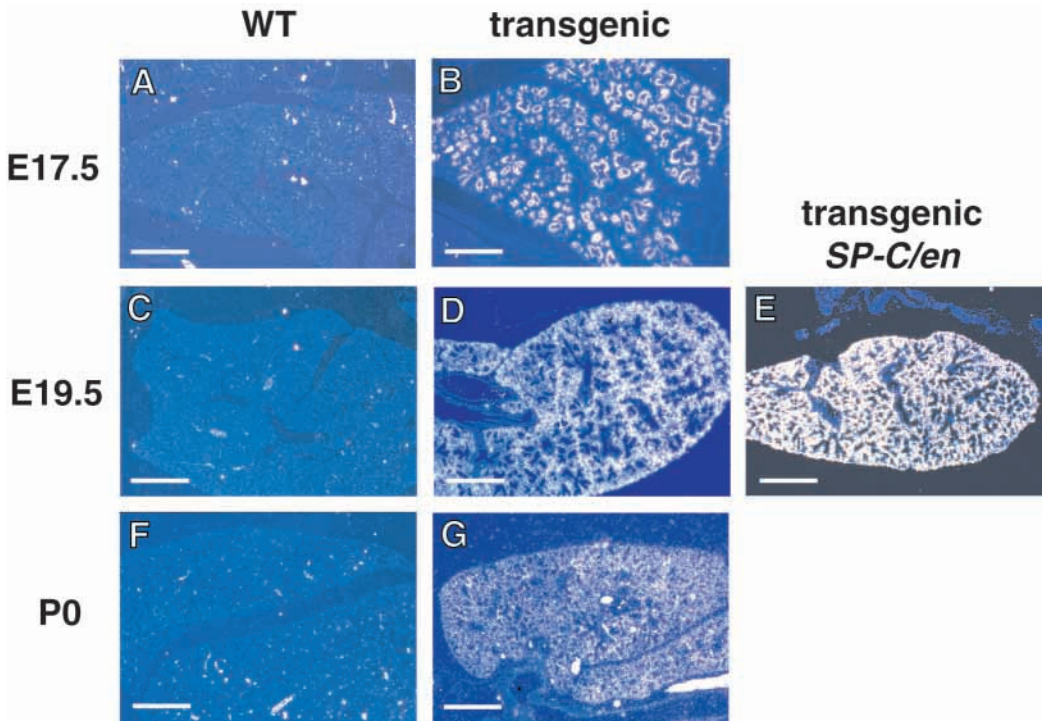

Fig. 2. Expression of the *SP-C/G6en* and *SP-C/en* transgenes during lung development. The SV40 poly(A)+ sequence was used as an in situ hybridization probe to detect the expression pattern of the *SP-C/G6en* (A-D,F,G) and *SP-C/en* (E) transgenes at the following time points during lung development: E17.5 (A,B), E19.5 (C-E), and P0 (F,G). There was no hybridization of the sense probe to the SV40 sequence (data not shown). Notice the restriction of transgene expression to the distal airway epithelium during lung development as has been reported previously with the human *SP-C* promoter (Wert et al., 1993). Scale bar: 250 μm.

airway epithelium in mice (Wert et al., 1993). Initially, we attempted to generate adult founder transgenic lines with the *SP-C/G6en* construct. However, 121 potential F0 founders were genotyped at 2 weeks of age and no *SP-C/G6en*-positive animals were obtained (data not shown). These data suggest that expression of the GATA6/Engrailed fusion protein in the distal epithelium of the lung is lethal in postnatal mice. Therefore, six independent F0 founder embryos were harvested at E17.5 and five at E19.5. In addition, two transgene-positive P0 neonates that died soon after birth were also analyzed. *SP-C/G6en* embyros harvested at E17.5, E19.5 and P0 displayed high levels of transgene expression in the distal airway epithelium as determined by in situ hybridization using probes that recognize the SV40 poly(A)+ sequence or the *engrailed* repressor domain (Fig. 2 and data not shown). Of note, the expression pattern of the *SP-C/en* transgene was similar to that of the *SP-C/G6en* transgene (Fig. 2).

At E17.5, wild-type distal airway epithelium differentiates to assume a squamous morphology as a result of the differentiation of AEC-1 cells from AEC-2 cells (Fig. 3A,C). The lungs of *SP-C/G6en* embryos were approximately equal in size to wild-type littermates at E17.5 but the distal airways were dilated and lacked squamous epithelium (Fig. 3B,D). Instead, the distal airways of E17.5 *SP-C/G6en* embryos were lined completely with thick, cuboidal AEC-2-like cells (Fig. 3D, yellow arrowhead). At E19.5 and P0, the number of interalveolar septa was decreased with thicker mesenchyme between the distal airways in *SP-C/G6en* embryos and neonates suggesting a defect in distal airway branching or delayed maturation (Fig. 3F,H,J,L). In contrast, at E19.5, the *SP-C/en* control transgenic embryos displayed normal morphology in the distal epithelium of the lung (Fig. 3N,P). All other tissues appeared normal histologically, consistent with the tissue-restricted expression pattern of the human SP-C promoter (Koutsourakis et al., 2001; Lu et al., 2001; Weaver et al., 1999; Wert et al., 1993; Zhou et al., 1996a; Zhou et al., 1997) and data not shown). These data suggest that distal airway epithelial cell differentiation and development is specifically disrupted in *SP-C/G6en* mice.

Defects in distal lung epithelial differentiation in *SP-C/G6en* embryos

To examine the ultrastructural morphology of the AECs in *SP-C/G6en* embryos, transmission electron microscopy was performed on *SP-C/G6en* transgenic and non-transgenic littermates and *SP-C/en* transgenic embryos at E19.5. Normal distal epithelium of the lung at this time point is lined with squamous (flattened) AEC-1 cells and cuboidal AEC-2 cells with AEC-1 cells covering a majority of the surface area in the airways (Ten Have-Opbroek, 1991). Electron micrographs show normal morphology in the lungs of wild-type and *SP-C/en* embryos at E19.5 with squamous AEC-1 cells covering most of the airway tubules (Fig. 4A,B,G,H). However, *SP-C/G6en* embryos exhibit a deficiency in these squamous AEC-1 cells when compared to non-transgenic littermates and *SP-C/en* mice (Fig. 4C-F). Instead, the distal airways in *SP-C/G6en* embryos were populated almost completely with large cuboidal AEC-2-like cells that contained large vacuoles reminiscent of the glycogen storage vesicles seen in immature AEC-2 cells during early lung development (Fig. 4D,E arrowheads and (Ten Have-Opbroek, 1991). To confirm that these large vacuoles represented an increase in glycogen content in these cells, Periodic acid-Schiff (PAS) staining was performed on histological sections from transgenic and wild-type littermates at E19.5. As shown in Fig. 5, *SP-C/G6en* mice exhibit an increase in PAS staining in the distal airway epithelium indicating an increase in glycogen content (Fig. 5B). Pretreatment with amylase eliminated the staining, indicating the specificity of the staining for glycogen (Fig. 5C).

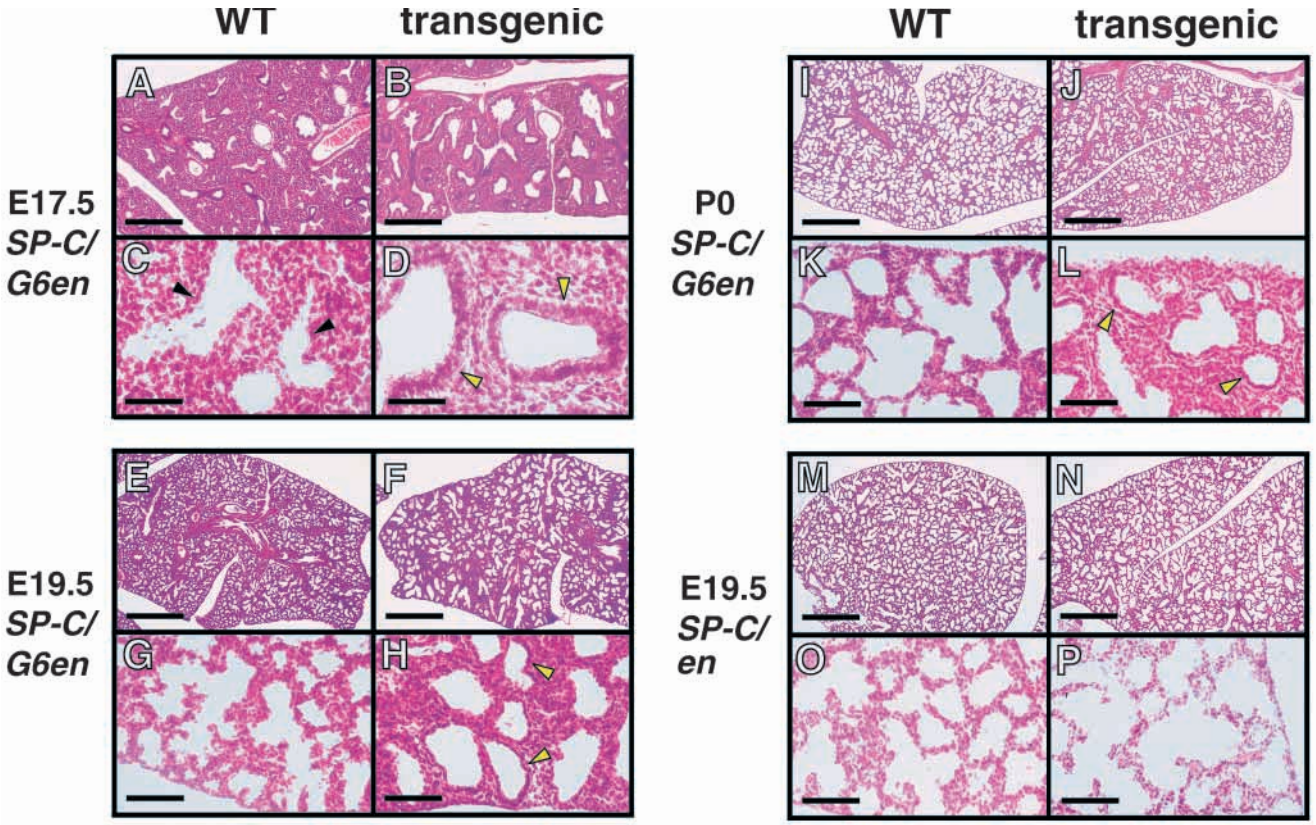

Fig. 3. *SP-C/G6en* mice display abnormal lung morphology. Histological sections through E17.5 (A-D), E19.5 (E-H,M-P), or P0 (I-L) *SP-C/G6en* (A-L) and *SP-C/en* (M-P) transgenic embryos and neonates. At E17.5, the distal airways of *SP-C/G6en* embryos are lined with abnormal cuboidal epithelium (D, yellow arrowheads) while the distal airways of wild-type littermates are lined primarily with squamous epithelium (C, black arrowheads). Similar morphological differences are observed at E19.5 (H, yellow arrowheads) and P0 (L, yellow arrowheads). Increased ratio of mesenchyme to airway space is also noted in *SP-C/G6en* embryos (compare G and H, K and L). However, *SP-C/en* mice display normal distal epithelial morphology and branching as in the wild-type littermates (M-P). Scale bar: 500 μm (E, F,I,J,M,N), 250 μm (A,B), 125 μm (G,H,K,L,O,P) and 67.5 μm (C,D).

The presence of lamellar bodies in many of these abnormal distal airway cells in *SP-C/G6en* embryos confirms their identity as AEC-2 cells and also suggests that AEC-2 cell differentiation had progressed beyond the pseudoglandular stage (Fig. 4F, black arrow). The presence of secreted surfactant in the distal airways of *SP-C/G6en* mice also supports these findings (Fig. 4F, white arrow). In addition, pulmonary vascular development was normal in *SP-C/G6en* embryos as demonstrated by a normal capillary plexus closely adjoining the distal airways (Fig. 4C-E). Together, these data indicate that *SP-C/G6en* mice have a specific disruption in AEC differentiation and development resulting in a lack of squamous AEC-1 cells in the distal airways.

Expression of *TTF-1* and *SP-C* in *SP-C/G6en* embryos

To further investigate pulmonary epithelial cell differentiation in *SP-C/G6en* embryos, in situ hybridization utilizing gene-specific riboprobes for *SP-C*, a marker for AEC-2 cells, and *TTF-1* an important transcription factor expressed in both AEC-2 cells and in proximal Clara epithelial cells, was performed (Glasser et al., 1991; Wert et al., 1993; Zhou et al., 1996b). Both of these genes have been used previously as specific markers of lung epithelial cell specification (Zhou et al., 1996b). *TTF-1* null embryos die during gestation and exhibit severe defects in lung epithelial development and lack *SP-C* expression (Minoo et al., 1999). In addition, the mouse *TTF-1* promoter contains an important GATA factor DNA binding site suggesting that GATA factors may regulate its expression (Shaw-White et al., 1999). *TTF-1* expression was observed in both wild-type and *SP-C/G6en* lung epithelium at E17.5 and E19.5 (Fig. 6A-D). No differences were observed in the level or pattern of *TTF-1* expression between wild-type and transgenic littermates (Fig. 6A-D). At E17.5, expression of endogenous *SP-C* was observed throughout the distal airway epithelium of wild-type embryos (Fig. 6E,G). Interestingly, endogenous *SP-C* expression was not observed in the thick cuboidal epithelium where the *SP-C/G6en* transgene was expressed (Fig. 6H, bracket). This observation was consistent in all five *SP-C/G6en* embryos examined. However, endogenous *SP-C* was expressed in regions of the distal lung epithelium that lacked expression of the *SP-C/G6en* transgene (Fig. 6F,H arrowheads). These data suggest that pulmonary epithelium has been specified but expression of endogenous *SP-C* is attenuated in *SP-C/G6en* embryos. However, attenuated endogenous *SP-C* expression in *SP-C/G6en* embryos is not due to the loss of *TTF-1*, which is expressed at wild-type levels.

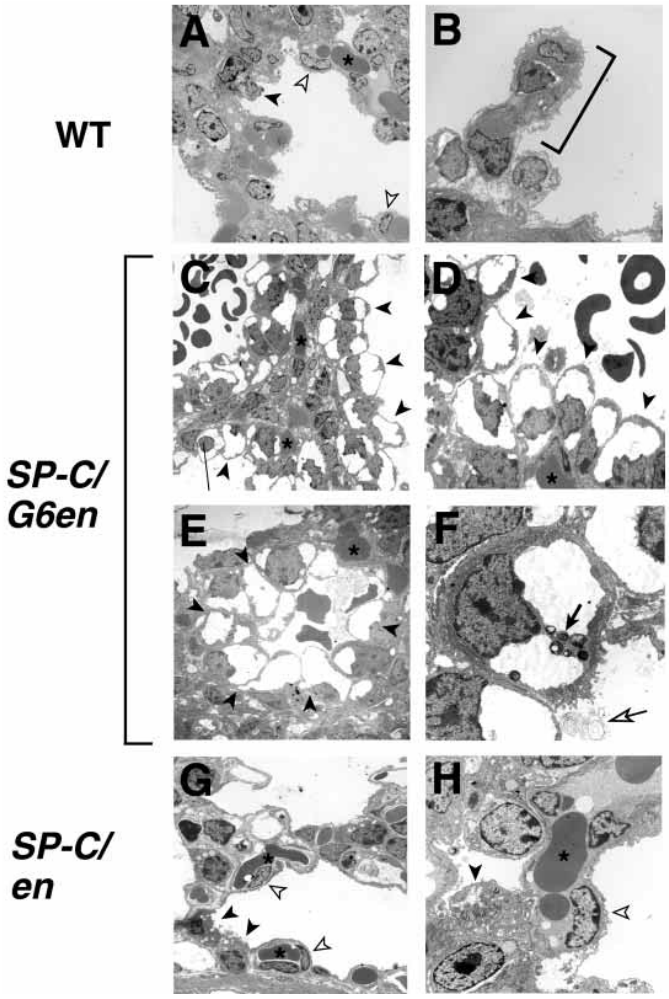

Fig. 4. Abnormal ultrastructure of distal airway epithelium in *SP-C/G6en* mice. Transmission electron microscopy was used to determine the ultrastructural morphology of the distal airway epithelial cells in *SP-C/G6en* and *SP-C/en* mice at E19.5. Wild-type littermates show normal distal airway structure including AEC-1 (A, white arrowheads) and AEC-2 (A, black arrowhead) cells lining the airways, with small blood vessels closely adjoining the thin epithelial barrier (A, asterisk). In addition, numerous interalveolar septa were observed (B, bracket). In contrast, *SP-C/G6en* mice had AEC-2 cells lining all of their distal airways (C-E, black arrowheads). These AEC-2 cells contained large vacuoles reminiscent of the glycogen storage vesicles observed in immature AEC-2 cells in early lung development (Ten Have-Opbroek, 1991). However, many of these cells still contained lamellar bodies (F, black arrow) and secreted surfactant was observed in the airways (F, white arrow) showing that these cells were not arrested at an earlier stage of AEC-2 differentiation. Also, *SP-C/G6en* mice contained normal blood vessel development underlying the distal airway epithelium suggesting that pulmonary vascular development was not perturbed (C,D, asterisks).

Expression of *SP-A* in *SP-C/G6en* embryos

Ultrastructural analysis suggested that distal airway epithelium in *SP-C/G6en* embryos exhibit some characteristics of late epithelial cell differentiation such as lamellar body formation and secreted surfactant. However, attenuated endogenous *SP-C* expression also suggests that early AEC-2 marker gene expression had been disrupted in *SP-C/G6en* embryos. To further delineate how far distal airway epithelial cell differentiation had progressed, in situ hybridization was performed with a gene-specific riboprobe for *SP-A*. *SP-A* expression is initiated in the early canalicular stage of lung development (E16.0) and as such is an excellent marker gene to detect whether *SP-C/G6en* embryonic distal airway epithelium had progressed to the later stages of lung development (Mendelson, 2000). At E17.5, *SP-A* expression is observed in a punctate pattern in the distal airway epithelium of both wild-type and *SP-C/G6en* embryos where both the level and pattern of expression were the same (Fig. 7). These data, combined with the ultrastructural analysis showing lamellar body formation, indicate that distal airway epithelium in *SP-C/G6en* embryos had differentiated to the later stages (E16.0 and later) of lung epithelial development.

Expression of proximal airway epithelial marker genes in *SP-C/G6en* transgenic mice

Although expression of GATA6 is not observed in proximal airway epithelium during development, previous studies with chimeric mice generated using *Gata6*$^{-/-}$ ES cells suggested that proximal cell gene expression is altered upon disruption of GATA6 expression (Keijzer et al., 2001). To determine whether *SP-C/G6en* mice exhibited defects in proximal epithelial cell development and gene expression, in situ hybridization was performed on sections from E17.5 and E19.5 transgenic embryos using probes specific for the mouse *Foxj1* and *CC10* mRNAs. Foxj1 is a winged-helix transcription factor expressed specifically by ciliated epithelium of the upper respiratory tract during lung development in mice, whereas CC10 is a secreted glycoprotein expressed specifically in non-ciliated Clara epithelial cells of the upper respiratory tract (Tichelaar et al., 1999b; Zhou et al., 1996b). Thus, their expression can be used to identify two specific proximal airway epithelial cell lineages. As has been previously reported, *Foxj1* and *CC10*

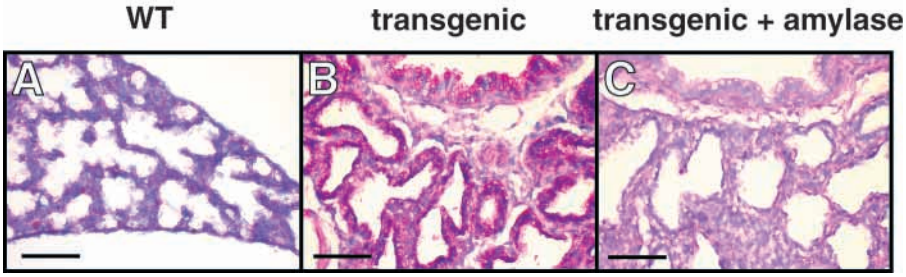

Fig. 5. *SP-C/G6en* mice have increased glycogen content in the distal airway epithelium. PAS staining was performed on histological sections of E19.5 wild-type (A) and *SP-C/G6en* mice (B,C) with (C) or without (A,B) prior treatment with amylase. At E19.5, wild-type littermates contain little glycogen in the distal airway epithelium (A), while *SP-C/G6en* mice show a significant increase in glycogen as determined by PAS staining (B). Pre-treatment with amylase (which digests the glycogen and eliminates PAS staining) demonstrates the specificity of the PAS staining (C). Scale bar: 80 μm.

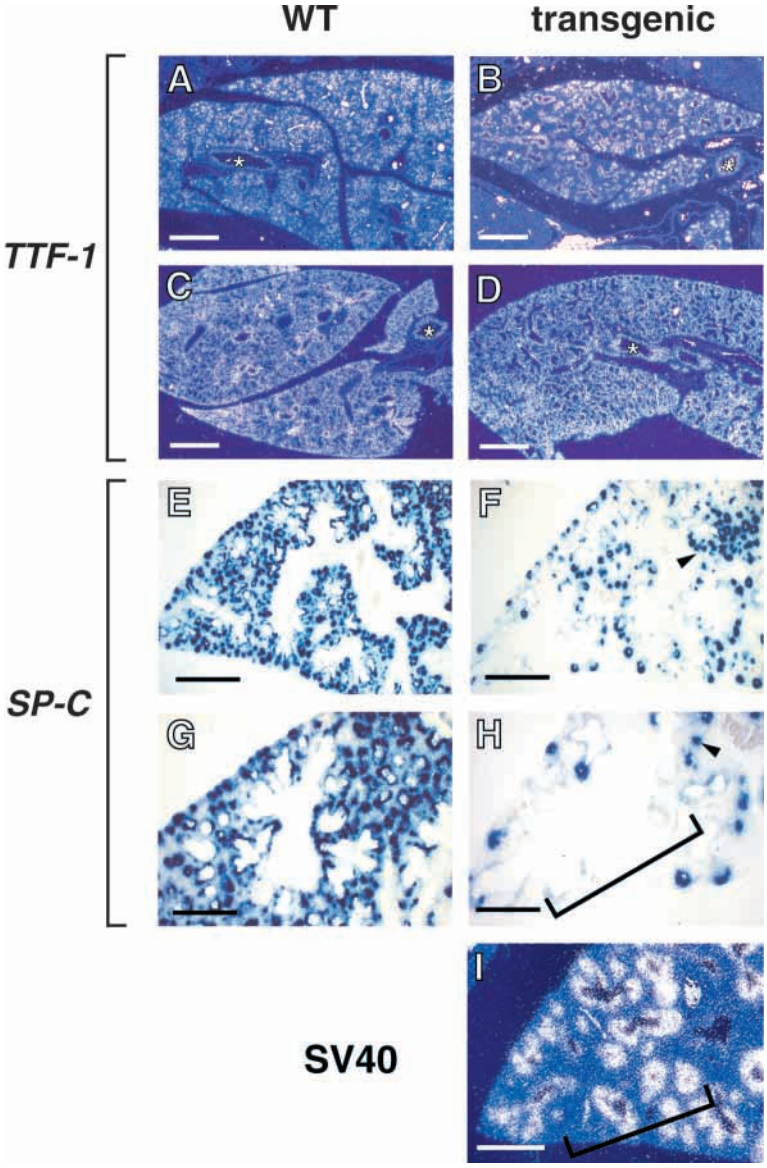

Fig. 6. AEC-2 cell marker gene expression in *SP-C/G6en* mice. In situ hybridization using specific probes for *TTF-1* (A-D) and *SP-C* (E-H) was performed on wild-type (A,C,E,G) and *SP-C/G6en* (B,D,F,H,I) sections from E17.5 (A,B,E-I) and E19.5 (C,D) embryos. Non-radioactive in situ hybridization was utilized for the *SP-C* probe to better analyze the specific expression pattern, whereas radioactive in situ hybridization was used for *TTF-1*. *TTF-1* expression is observed throughout the airway epithelium in both wild-type (A,C) and *SP-C/G6en* embryos (B,D). *SP-C* expression, however, was reduced in *SP-C/G6en* mice and in some regions almost completely absent from the distal airway epithelium expressing the transgene as demarcated by the SV40 probe (compare adjacent sections in H and I). However, *SP-C* expression was observed in regions outside the area of transgene expression (F,H, arrowheads). There was no detectable hybridization with the sense probes for *TTF-1* and *SP-C* (data not shown). Asterisks denote proximal airways in A-D. Scale bar: 250 μm (A-D), 125 μm (E,F), 67.5 μm (G,H,I).

expression is observed in the epithelium of the major proximal airways in the lungs of non-transgenic mice (Fig. 8A,C). Although the levels of *Foxj1* and *CC10* expression appeared normal in *SP-C/G6en* mice, the number of individual airways positive for *Foxj1* and *CC10* expression was reduced in all of the independent *SP-C/G6en* founders at E17.5 and E19.5 (Fig. 8B,D,F). This defect is most obvious in the significant reduction in the number of smaller *CC10*- and *Foxj1*-positive airways whereas the number of the largest most proximal *CC10*- and *Foxj1*-positive airways remains relatively the same (Fig. 8G,H). Quantification of the number of *CC10*-positive airways shows a 55%±7.2% decrease at E17.5 and a 39%±4.1% decrease at E19.5 in *SP-C/G6en* mice.

Examination of H+E stained slides from E17.5 wild-type and *SP-C/G6en* embryos confirms that the reduced number of airways expressing *CC10* and *Foxj1* correlates with a decrease in proximal airways containing convoluted and columnar epithelium in *SP-C/G6en* mice (Fig. 8G,H). Together, these data suggest that expression of the *SP-C/G6en* transgene results in a reduction in proximal airway development.

Expression of *aquaporin 5* and *Foxp2* in the lungs of *SP-C/G6en* embryos

Aquaporin 5 (*Aqp5*) is a water channel gene whose expression is restricted to AEC-1 cells during lung development and is one of the few AEC-1 marker genes known (Krane et al., 1999; Kreda et al., 2001; Lee et al., 1997). *Foxp2* is a winged-helix/forkhead transcription factor gene that is expressed in the distal airway epithelium of the developing lung (Shu et al., 2001). To investigate whether expression of either of these genes was disrupted in the lungs of *SP-C/G6en* embryos, in situ hybridization was performed using gene-specific riboprobes for *Aqp5* and *Foxp2*.

At E17.5, *Aqp5* gene expression was observed at low levels in the distal airways of wild-type lungs as has been previously demonstrated (Fig. 9A) (Kreda et al., 2001). However, *Aqp5* expression was not observed in the distal airways in any of the E17.5 *SP-C/G6en* embryos (Fig. 9B). By E19.5, expression was very robust in the distal airways of wild-type embryos but severely attenuated in all E19.5 *SP-C/G6en* embryos (Fig.

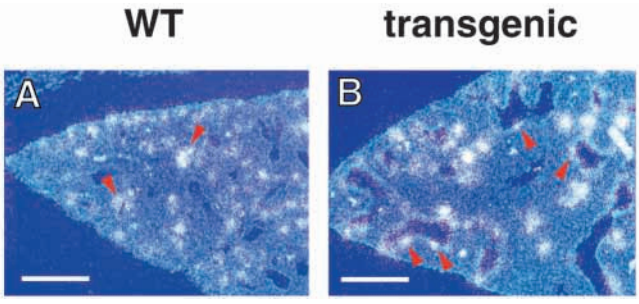

Fig. 7. *SP-A* gene expression in *SP-C/G6en* mice. Radioactive in situ hybridization was performed on E17.5 wild-type (A) and *SP-C/G6en* (B) embryos using a specific antisense riboprobe for *SP-A*. Note hybridization to distal airway epithelium in both wild-type and transgenic embryos (red arrowheads). Sense probe did not produce a detectable hybridization signal (data not shown). Scale bar: 67.5 μm.

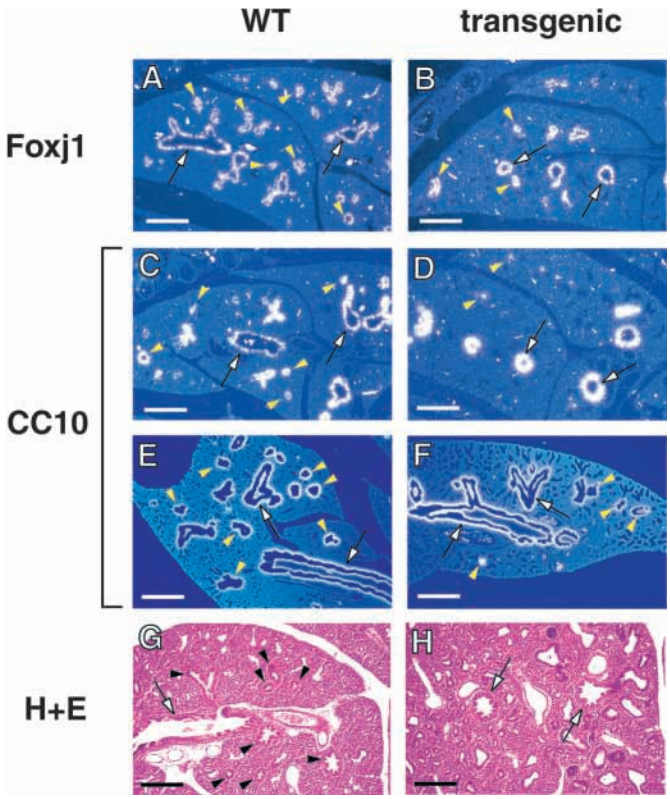

Fig. 8. Proximal airway development and gene expression in *SP-C/G6en* mice. In situ hybridization analysis was performed on wild-type (A,C,E) and *SP-C/G6en* (B,D,F) embryos at E17.5 (A-D) and E19.5 (E,F) using gene-specific riboprobes for *Foxj1* (A,B) and *CC10* (C-F). *Foxj1* and *CC10* are expressed at similar levels in the proximal airways of wild-type and *SP-C/G6en* mice (A-F). However, the number of individual airways positive for *Foxj1* and *CC10* hybridization was significantly reduced at both E17.5 and E19.5 (A-F, yellow arrowheads). This decrease was most extreme in the number of smaller *CC10*- and *Foxj1*-positive proximal airways while the number of larger, more central airways was similar (A-F, arrows). These data correlated with a decrease in the number of peripheral airways containing thick convoluted epithelium (G, black arrowheads) which is characteristic of proximal airway epithelium while the number of large centralized proximal airways was similar in H+E stained sections from of wild-type and *SP-C/G6en* E17.5 embryos (G,H, arrows). Scale bar: 250 μm (A-F), 150 μm (G,H).

9C,D). *SP-C/en* embryos showed no difference in *Aqp5* gene expression from their wild-type littermates (Fig. 9E,F). These data suggest that aquaporin-5 gene expression is significantly down-regulated in *SP-C/G6en* embryos.

At E17.5 and E19.5, *Foxp2* is expressed in the distal airway epithelium of wild-type embryos in a pattern similar to that observed for *SP-C* (Fig. 9G,I,K). In addition, *Foxp2* expression is down-regulated in wild-type embryos from E17.5 to E19.5 (Fig. 9G,K). However, in *SP-C/G6en* transgenic mice, *Foxp2* expression is not down-regulated at E19.5 but instead remains robust and is greater than in E19.5 wild-type littermates (Fig. 9K,L). This difference was observed in all of the E19.5 *SP-C/G6en* embryos analyzed. Of note, the pattern of *Foxp2* expression in *SP-C/G6en* embryos closely resembles that of the transgene as determined by the SV40 poly(A)+ in situ probe, clearly showing the dilated nature of the distal airways at E17.5 and E19.5 in *SP-C/G6en* embryos (compare Fig. 9H,J with Fig. 2B).

To confirm the in situ hybridization results showing a decreased level of *Aqp5* expression and an increased level of *Foxp2* expression in the airways of *SP-C/G6en* embryos, equal amounts of total RNA from the lungs of two E19.5 *SP-C/G6en* transgenic embryos and two wild-type littermates was analyzed by northern blotting to quantitate the changes in *Foxp2* and *Aqp5* expression. Both of the independent F0 *SP-C/G6en* founder embryos expressed high levels of the *SP-C/G6en* transgene (Fig. 10). Phosphoimager analysis shows that *Aqp5* gene expression is decreased an average of 7.8-fold while *Foxp2* gene expression is increased by 3.5-fold in *SP-C/G6en* transgenic embryos at E19.5 (Fig. 10). These data support the in situ hybridization analysis showing a significant decrease in *Aqp5* and increase in *Foxp2* expression in the lungs of *SP-C/G6en* embryos.

GATA6 activates the mouse *aquaporin 5* promoter

Previous reports have implicated GATA6, as well as other tissue restricted transcription factors such as TTF-1 and Foxa2, in the regulation of AEC-2 cell-specific gene expression (Bohinski et al., 1994; Bruno et al., 1995; Bruno et al., 2000; Margana and Boggaram, 1997; Shaw-White et al., 1999). In contrast, AEC-1 cell-specific gene expression has not been extensively studied and few cell-specific marker genes or transcriptional regulators have been characterized. The disruption of AEC-1 cell differentiation in *SP-C/G6en* mice, as demonstrated by ultrastructural analysis and reduced *Aqp5* gene expression, suggested that GATA6 may play a role not only in the differentiation of AEC-1 cells from AEC-2 cells but also in the regulation of AEC-1 cell-specific gene expression. In addition, expression of *Aqp5* is induced in AEC-2 cells in response to lung injury or remodeling (Tichelaar et al., 2000). These data suggest that AEC-2 and AEC-1 cells may share some transcriptional regulators and mechanisms for cell-specific gene expression. Isolation and sequencing of the 1.4 kb promoter for the AEC-1 restricted mouse *Aqp5* revealed four putative GATA6 binding sites, two of which are conserved in the rat *Aqp5* promoter (Fig. 11A) (Krane et al., 1999). To determine whether GATA6 could activate the mouse *Aqp5* promoter, co-transfection studies were performed using two different regions of this promoter and an expression plasmid containing the GATA cDNA. The pGL3/*Aqp5*/–1.4 plasmid contains all four of the identified GATA6 DNA binding sites while the truncated pGL3/*Aqp5*/–0.5 plasmid lacks these four sites (Fig. 11A). Co-transfection of the GATA6 expression plasmid dramatically stimulated the pGL3/*Aqp5*/–1.4 plasmid by more than 25-fold, while deletion of the four GATA6 binding sites in the pGL3/*Aqp5*/–0.5 plasmid resulted in a 68% decrease in this GATA6-dependent trans-activation (Fig. 11B). The residual trans-activation of the pGL3/*Aqp5*/–0.5 plasmid is likely due to GATA6 interactions with ubiquitous transcription factors such as Sp-1 or binding of GATA6 to cryptic GATA DNA binding sites within the backbone of the pGL3basic reporter plasmid (Merika and Orkin, 1995). To verify that the GATA6/Engrailed fusion protein could specifically repress the *Aqp5* promoter, the pGL3/*Aqp5*/–1.4 plasmid was transfected into NIH-3T3 cells along with a constant amount of the GATA6 expression plasmid and with increasing amounts of either a GATA6/Engrailed fusion protein

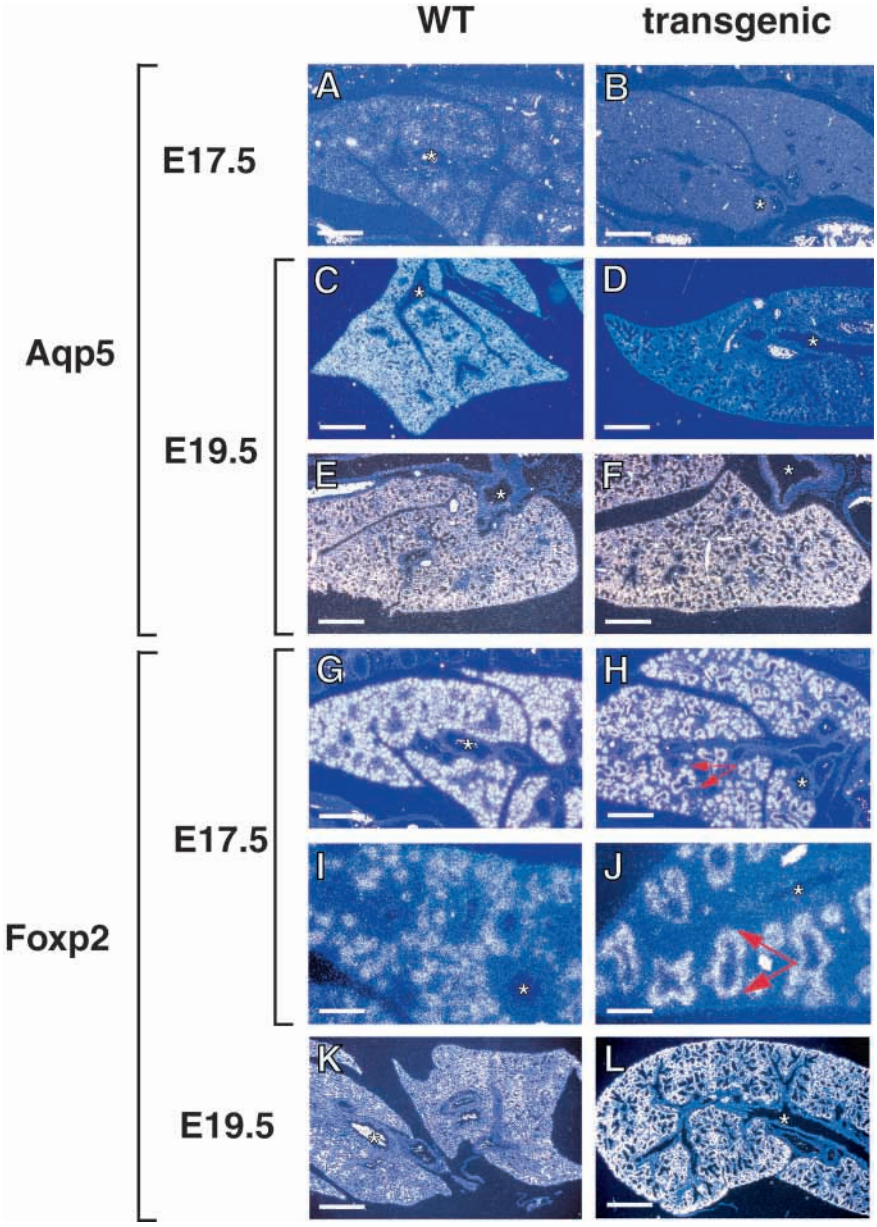

Fig. 9. Disrupted *Aqp5* and *Foxp2* gene expression in *SP-C/G6en* embryos. Radioactive in situ hybridization analysis was performed on wild-type (A,C,E,G,I,K), *SP-C/G6en* (B,D,H,J,L), and *SP-C/en* (F) embryos at E17.5 (A,B,G-J) and E19.5 (C-F,K,L). *Aqp5* is expressed at low levels primarily in distal airway epithelium at E17.5 in wild-type embryos (A). However, *Aqp5* expression is not observed at appreciable levels in the lungs of E17.5 *SP-C/G6en* embryos (B). By E19.5, *Aqp5* is expressed at high levels in the airways of wild-type (C,E) embryos but its expression is significantly reduced in the airways of *SP-C/G6en* embryos (D). *SP-C/en* mice express normal levels of *Aqp5* as compared to wild-type littermates (F). At E17.5, *Foxp2* is expressed at similar levels in both wild-type (G and I) and *SP-C/G6en* (H,J) embryos in the distal airway epithelium. By E19.5, *Foxp2* gene expression has decreased in wild-type (K) embryos but remains high in the distal airways of *SP-C/G6en* (L) embryos. Note the dilated nature of the distal airways in *SP-C/G6en* embryos as highlighted by *Foxp2* gene expression at E17.5 (H,J, red arrows). Sense probes for *Aqp5* and *Foxp2* did not produce a detectable hybridization signal (data not shown). Asterisks denote proximal airways in A-L. Scale bar: 250 μm (A-H,K,L), 67.5 μm (G,H).

expression plasmid, an expression plasmid encoding a mutant GATA6/Engrailed fusion protein with point mutations in the conserved carboxy-terminal zinc-finger that abrogate *Gata6* DNA binding, or an expression plasmid encoding the Engrailed repression domain used in the *SP-C/en* transgenic mice. As shown in Fig. 11C, only the GATA6/Engrailed fusion protein expression plasmid was capable of repressing GATA6-dependent trans-activation of the *Aqp5* promoter. These data show that GATA6 can directly activate mouse *Aqp5* gene expression in vitro.

DISCUSSION

GATA6 regulates AEC-1 cell differentiation and gene expression

From the data presented above, we propose a model wherein GATA6 regulates late distal epithelial differentiation processes in the lung that lead to the generation of AEC-1 cells (Fig. 12). These cells are required to generate a thin, diffusible membrane for efficient gas exchange between the airway lumen and the pulmonary vasculature. Little is known

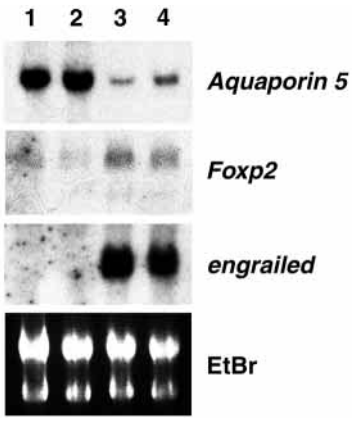

Fig. 10. Northern blot analysis of *Aqp5* and *Foxp2* gene expression in the lungs of *SP-C/G6en* mice. Total RNA (10 μg) from E19.5 wild-type (lanes 1 and 2) and *SP-C/G6en* (lanes 3 and 4) dissected lung tissue was analyzed by northern blot analysis using radiolabeled probes specific for *Aqp5*, *Foxp2*, and the *engrailed* repression domain (to detect transgene expression). Bottom panel represents the ethidium bromide-stained gel before transfer showing equal loading of RNA in all the lanes. All RNAs represent individual and separate F0 E19.5 wild-type or *SP-C/G6en* transgenic littermates.

about the differentiation of AEC-2 to AEC-1 cells in the lung. Previous reports have shown that AEC-2 cells have the ability to self-renew in addition to trans-differentiating into AEC-1 cells (Evans et al., 1975). However, most of these data have been derived from in vitro culture systems and therefore may not accurately reflect in vivo developmental processes. Although our data suggest a necessary role for GATA6 in the differentiation of AEC-1 cells, a recent study suggests that over-expression of GATA6 in distal airway epithelium does not result in increased AEC-1 cell differentiation (Koutsourakis et al., 2001). Thus, GATA6 may be necessary, but is not sufficient to promote AEC-1 cell differentiation.

SP-C/G6en embryos are distinct from other lung transgenic phenotypes in that they exhibit a specific loss of AEC-1 cells, as shown by ultrastructural analysis and attenuated *Aqp5* gene expression. The study of AEC-1 cell differentiation has been hampered by knowledge of only a few lineage-restricted marker genes. The best analyzed of these are *Aqp5*, a water channel gene expressed in lung AEC-1 cells as well as in tracheal and nasal epithelium, and a gene of unknown function called *T1α* (Lee et al., 1997; Nielsen et al., 1997; Ramirez et al., 1999; Ramirez et al., 1997). Both of these genes have been reported to be expressed exclusively in AEC-1 cells in the lung (Lee et al., 1997; Nielsen et al., 1997; Ramirez et al., 1999; Ramirez et al., 1997). However, the expression of *T1α* in early lung epithelium (E11.5) suggests that it is not specific to AEC-1 cells since these cells do not appear until E17.5 (Ramirez et al., 1999). Also, *Aqp5* expression can be induced in AEC-2 cells upon remodeling of the epithelium in adult mice (Tichelaar et al., 2000). In light of these data, *Aqp5*, which is first expressed in lung development at approximately E17.5 , is an excellent AEC-1 marker gene because its expression pattern and timing are identical to the appearance of AEC-1 cells during lung development (Lee et al., 1997). Thus, attenuated *Aqp5* gene expression supports the concept that AEC-1 differentiation is blocked in *SP-C/G6en* embryos.

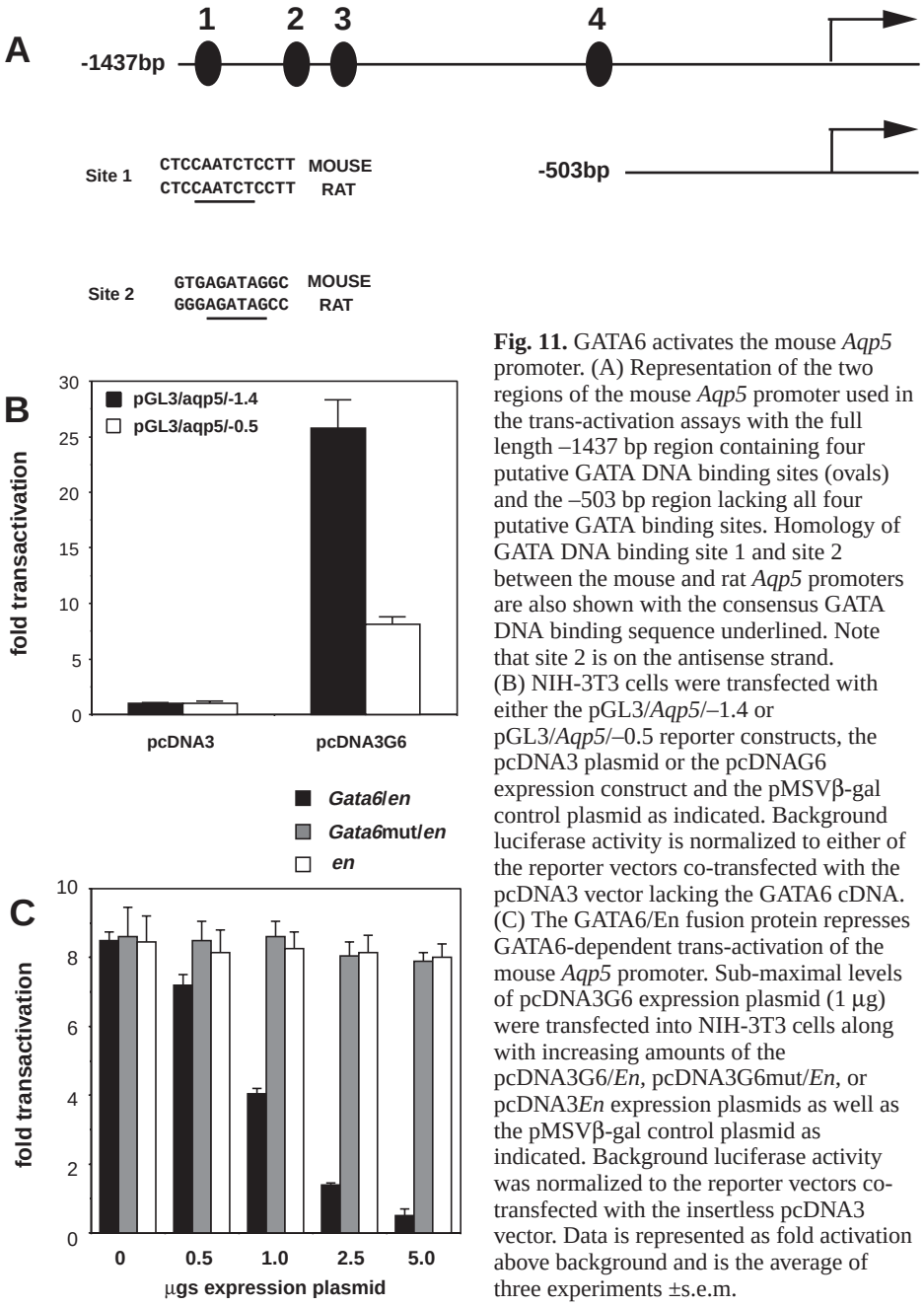

Fig. 11. GATA6 activates the mouse *Aqp5* promoter. (A) Representation of the two regions of the mouse *Aqp5* promoter used in the trans-activation assays with the full length –1437 bp region containing four putative GATA DNA binding sites (ovals) and the –503 bp region lacking all four putative GATA binding sites. Homology of GATA DNA binding site 1 and site 2 between the mouse and rat *Aqp5* promoters are also shown with the consensus GATA DNA binding sequence underlined. Note that site 2 is on the antisense strand. (B) NIH-3T3 cells were transfected with either the pGL3/*Aqp5*/–1.4 or pGL3/*Aqp5*/–0.5 reporter constructs, the pcDNA3 plasmid or the pcDNAG6 expression construct and the pMSVβ-gal control plasmid as indicated. Background luciferase activity is normalized to either of the reporter vectors co-transfected with the pcDNA3 vector lacking the GATA6 cDNA. (C) The GATA6/En fusion protein represses GATA6-dependent trans-activation of the mouse *Aqp5* promoter. Sub-maximal levels of pcDNA3G6 expression plasmid (1 μg) were transfected into NIH-3T3 cells along with increasing amounts of the pcDNA3G6/*En*, pcDNA3G6mut/*En*, or pcDNA3*En* expression plasmids as well as the pMSVβ-gal control plasmid as indicated. Background luciferase activity was normalized to the reporter vectors co-transfected with the insertless pcDNA3 vector. Data is represented as fold activation above background and is the average of three experiments ±s.e.m.

Other loss-of-function experiments have revealed phenotypes that are related but distinct to that observed in the lungs of *SP-C/G6en* embryos. Parathyroid hormone-related peptide (PTHrP) mice also lack *Aqp5* expression and die at birth from respiratory failure (Karaplis et al., 1994; Ramirez et al., 2000). However, these mice appear to contain squamous epithelium in their lungs (Karaplis et al., 1994; Ramirez et al., 2000). In addition, since mice homozygous for an *Aqp5* null allele do not exhibit a postnatal lung phenotype, the lack of *Aqp5* in the PTHrP mice would not account for perinatal lethality (Ma et al., 1999). Mouse lungs containing a null mutation in the tumor necrosis factor-α converting enzyme (TACE) also exhibit attenuated SP-C and *Aqp5* gene expression and display dilated airways similar to those observed in *SP-C/G6en* embryos (Zhao et al., 2001). As with *SP-C/G6en* embryos, TACE mice die soon after birth as a result of respiratory failure (Zhao et al., 2001). However, *Tace*$^{-/-}$ mice have defects in lung vascular development which were not observed in *SP-C/G6en* embryos (Zhao et al., 2001). Therefore, the *SP-C/G6en* transgenic embryos have a unique phenotype that correlates well with a

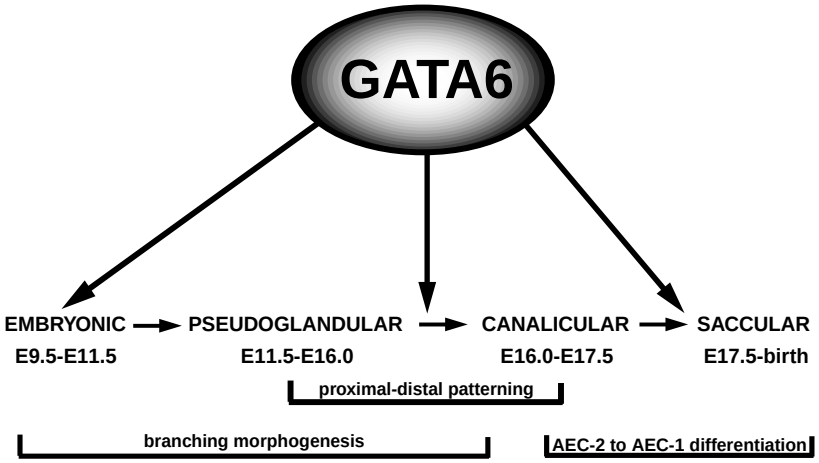

Fig. 12. Model of GATA6 function during lung development. A model for GATA6 function in AEC-2 differentiation (i.e. SP-C expression), AEC-1 differentiation (loss of squamous epithelium and *Aqp5* gene expression), and proximal airway development (reduced number of proximal airways) during lung development. Lack of SP-C expression in both the *SP-C/G6en* embryos and in chimeric lung tissue generated from *Gata6*$^{-/-}$ ES cells shows that GATA6 is crucial for *SP-C* gene expression and thus for certain aspects of AEC-2 differentiation (Keijzer et al., 2001). Lack of AEC-1 cells and direct activation of the mouse *Aqp5* proximal promoter in *SP-C/G6en* embryos indicate that GATA6 plays an important role in AEC-1 cell differentiation and gene expression (this report). Reduced number of proximal airway tubules indicates that disruption of distal airway differentiation in *SP-C/G6en* embryos leads to disrupted proximal airway development which correlates with a recent model of lung epithelial differentiation and development (Weaver et al., 1999). GATA6 may affect all of these processes through stage-specific requirements or by disrupting early lung epithelial differentiation, leading to affects observed later in development.

specific loss in AEC-1 cell differentiation during late lung development. This may contribute to the lethality observed in the transgenic mice because of the increased diffusion distance for gas exchange caused by the thicker AEC-2 cells lining the airways. Although AEC-2 differentiation was disrupted as shown by reduced *SP-C* expression, high levels of transgene expression are not observed with the human *SP-C* promoter until after E15.5 (Wert et al., 1993). Thus, the *SP-C/G6en* transgene is not likely to have affected GATA6 function until late lung development, further supporting a defect in the AEC-2 to AEC-1 cell differentiation process.

GATA6 is the only GATA factor expressed in distal lung epithelium and its ability to trans-activate the mouse aquaporin-5 promoter through GATA factor DNA binding sites conserved in the mouse and rat promoters, further reinforces a model wherein GATA6 regulates AEC-1 differentiation and gene expression at the transcriptional level. These data suggest that GATA6 can regulate AEC-1 cell-specific gene expression in addition to the differentiation process leading to AEC-1 cell formation. Indeed, one of the hallmark functions of GATA factors is their ability to regulate cell-specific gene expression programs crucial for tissue-specific differentiation processes. For instance, GATA1 is absolutely required for terminal erythrocyte differentiation while GATA2 and GATA3 are essential for hematopoeitic stem cell and T-cell differentiation, respectively (Pevny et al., 1991; Ting et al., 1996; Tsai et al., 1994). Our data suggests that the AEC-2 to AEC-1 cell differentiation process and AEC-1 cell-specific gene expression may be added to the repertoire of the roles performed by GATA6 during development.

Disruption of distal lung epithelial differentiation leads to attenuated proximal airway development

Histological and gene expression data using the proximal airway epithelial marker genes *Foxj1* and *CC10* suggests a defect in late stage proximal airway development in *SP-C/G6en* mice as exhibited by reduced numbers of proximal airway tubules. A previously reported transgenic model using the *SP-C* promoter to over-express a constitutively active form of the signaling protein TGFβ1 resulted in decreased expression of the proximal epithelial gene *CC10* (Zhou et al., 1996a). This result led the authors to conclude that the lung epithelium of *SP-C/Tgfβ1* mice was arrested in the pseudoglandular stage of development. In addition, over-expression of *Foxa2* (HNF3β) in the distal airway epithelium led to decreased levels of *CC10* expression in lung epithelium (Zhou et al., 1997). Because the *SP-C* promoter drives expression in the distal and not proximal airway epithelium, these reports suggest that disruption of distal airway development and differentiation results in aberrant proximal airway development. In *SP-C/G6en* mice, normal expression levels of *CC10* and *Foxj1* in the upper airways indicates that lung development has not been arrested in the pseudoglandular stage of development. In contrast, the number of proximal airways is decreased in these embryos compared to wild-type littermates. In light of these results, it is interesting to note that a previous model of lung development proposed that proximal airway epithelium develops from more distal epithelial cell types through combined proliferation and differentiation events (Weaver et al., 1999). Because we have not observed any differences in epithelial cell proliferation or programmed cell death in *SP-C/G6en* mice as compared to wild-type or *SP-C/en* mice (data not shown), the decreased numbers of *CC10*-positive airways probably result from a disruption of distal epithelial cell differentiation in *SP-C/G6en* mice. Low levels of transgene expression in some part of the proximal airways may also confer this phenotype. However, endogenous GATA6 is not expressed in proximal airway epithelium and the SP-C promoter does not drive expression in these cells (Morrisey et al., 1997; Weaver et al., 1999; Wert et al., 1993). Therefore, our results correlate with those observed in a previous study showing that CC10 expression was attenuated in chimeric lung tissue made from *Gata6*$^{-/-}$ ES cells (Keijzer et al., 2001). However, in contrast to that report, our data suggests that reduced CC10 expression is due to a reduction in proximal airway development and not to direct control of CC10 expression by GATA6, indicating a role for GATA6 in distal-proximal airway epithelial differentiation.

Regulation of AEC-2 cell-specific gene expression and branching morphogenesis by GATA6

The relatively normal expression of *SP-A* suggests that *SP-*

C/G6en embryos are not arrested at the pseudoglandular stage of development as has been observed in other transgenic models of disrupted lung epithelial differentiation (Zhou et al., 1996a; Zhou et al., 1997). In contrast, *Foxp2* expression was elevated during late gestation and *SP-C* expression was attenuated, indicating aberrant AEC-2 development. These data suggest a model in which GATA6 plays an important role in certain aspects of AEC-2 development, in particular the regulation of *SP-C* at the transcriptional level (Fig. 12). These results also support a hypothesis wherein inhibition of GATA6 function leads to a broader and earlier inhibition of lung epithelial cell differentiation that results in the later observed defects in proximal airway development and AEC-1 cell differentiation. Although the human *SP-C* promoter does not produce high levels of transgene expression until E15.5 and later (Wert et al., 1993), tissue-restricted inactivation of the mouse *Gata6* gene will be required to discern the difference between an early and broad role and/or a later and more specific role for GATA6 in lung epithelial differentiation as is suggested by our results.

SP-C expression is initiated as early as E11.0 in the mouse where it is restricted to the distal tips of developing airway epithelium (Wert et al., 1993; Zhou et al., 1996b). Later in development SP-C expression is restricted to AEC-2 cells in the lung (Zhou et al., 1996b). The transcriptional regulation of SP-C has been previously examined and several lung restricted transcription factors have been implicated in its regulation including TTF-1 and Foxa2 (Glasser et al., 2000; Glasser et al., 1991; Wert et al., 1993). A recent study has shown that *SP-C* expression was down-regulated in chimeric lung tissue derived from *Gata6*$^{-/-}$ ES cells (Keijzer et al., 2001) and our results corroborate this finding. These observations are supported by the presence of conserved GATA factor DNA binding sites in the proximal promoter region of both mouse and human *SP-C* genes, which can bind GATA6 and mediate GATA6-dependent trans-activation of these promoters (data not shown). Another study showed that over-expression of GATA6 in distal lung epithelium resulted in decreased levels of SP-C expression (Koutsourakis et al., 2001). Together, these data indicate that wild-type GATA6 activity is essential for SP-C expression.

Another transcription factor that has been shown to be essential for SP-C expression in a loss-of-function experiment is TTF-1 (Minoo et al., 1999). TTF-1 null mice exhibit arrested lung development and lack expression of SP-C (Kimura et al., 1996; Minoo et al., 1999). Interestingly, TTF-1 (Nkx2.1) belongs to the same family of homeodomain proteins as Nkx2.5, an important regulator of cardiac gene expression, which has been shown to interact and synergize with GATA4 to activate cardiac-specific gene expression (Durocher et al., 1997; Sepulveda et al., 1998). It will be interesting to determine whether GATA6 and TTF-1 are also capable of physically interacting and synergistically regulating lung epithelial gene expression.

SP-C/G6en embryos also exhibited a reduction in airway branching morphogenesis as shown by the dilated distal airways containing fewer interalveolar septa at E17.5 and E19.5. Branching morphogenesis is a complex process that is regulated by both autocrine/paracrine signaling pathways and regulation of gene expression at the transcriptional level (for reviews, see Hogan et al., 1997; Metzger and Krasnow, 1999). Thus, this process is extremely sensitive to both cell intrinsic and extrinsic perturbations. Many in vitro and in vivo loss-of-function models of lung development involve some level of disrupted branching morphogenesis (Arman et al., 1999; Bellusci et al., 1997; Miettinen et al., 1997; Pepicelli et al., 1998; Serra et al., 1994; Volpe et al., 2000). These findings are not surprising since airway branching and epithelial cell differentiation and development are closely linked (Warburton et al., 2000). Therefore, GATA6 may regulate lung branching morphogenesis directly through pertinent signaling pathways, but it is equally possible that disrupted branching observed in *SP-C/G6en* embryos is a secondary effect of disrupted distal and proximal airway differentiation.

In summary, *SP-C/G6en* transgenic embryos exhibit a unique phenotype during late lung epithelial development, leading to a failure of AEC-1 cell differentiation and disruption in proximal airway development. The *SP-C/G6en* transgenic model provides key information on the role of GATA6 during lung development, both during late processes (AEC-1 differentiation) and earlier epithelial development (*SP-C* gene regulation). Disrupted proximal airway development in these embryos also suggests that GATA6 plays an important role in the distal-proximal differentiation of lung airway epithelium.

The authors wish to thank Jon Epstein, Phil Ballard and Celeste Simon for their helpful comments on the manuscript. This work was supported, in part, by a NIH-NHLBI grant (HL-64632) to E. E. M.

REFERENCES

Arman, E., Haffner-Krausz, R., Gorivodsky, M. and Lonai, P. (1999). Fgfr2 is required for limb outgrowth and lung-branching morphogenesis. *Proc. Natl. Acad. Sci. USA* **96**, 11895-11899.

Badiani, P., Corbella, P., Kioussis, D., Marvel, J. and Weston, K. (1994). Dominant interfering alleles define a role for c-Myb in T-cell development. *Genes Dev.* **8**, 770-782.

Bellusci, S., Grindley, J., Emoto, H., Itoh, N. and Hogan, B. L. (1997). Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* **124**, 4867-4878.

Bohinski, R. J., Di Lauro, R. and Whitsett, J. A. (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.

Braun, H. and Suske, G. (1998). Combinatorial action of HNF3 and Sp family transcription factors in the activation of the rabbit uteroglobin/CC10 promoter. *J. Biol. Chem.* **273**, 9821-9828.

Bruno, M. D., Bohinski, R. J., Huelsman, K. M., Whitsett, J. A. and Korfhagen, T. R. (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.

Bruno, M. D., Korfhagen, T. R., Liu, C., Morrisey, E. E. and Whitsett, J. A. (2000). GATA-6 activates transcription of surfactant protein A. *J. Biol. Chem.* **275**, 1043-1049.

Clevidence, D. E., Overdier, D. G., Peterson, R. S., Porcella, A., Ye, H., Paulson, K. E. and Costa, R. H. (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.

Costa, R. H., Kalinichenko, V. V. and Lim, L. (2001). Transcription factors in mouse lung development and function. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **280**, L823-838.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J. and Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J.* **16**, 5687-5696.

Evans, M. J., Cabral, L. J., Stephens, R. J. and Freeman, G. (1975).

Transformation of alveolar type 2 cells to type 1 cells following exposure to NO2. *Exp. Mol. Pathol.* **22**, 142-150.

Fu, Y., Yan, W., Mohun, T. J. and Evans, S. M. (1998). Vertebrate tinman homologues XNkx2-3 and XNkx2-5 are required for heart formation in a functionally redundant manner. *Development* **125**, 4439-4449.

Glasser, S. W., Burhans, M. S., Eszterhas, S. K., Bruno, M. D. and Korfhagen, T. R. (2000). Human SP-C gene sequences that confer lung epithelium-specific expression in transgenic mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **278**, L933-945.

Glasser, S. W., Korfhagen, T. R., Wert, S. E., Bruno, M. D., McWilliams, K. M., Vorbroker, D. K. and Whitsett, J. A. (1991). Genetic element from human surfactant protein SP-C gene confers bronchiolar-alveolar cell specificity in transgenic mice. *Am. J. Physiol.* **261**, L349-356.

Hogan, B. L., Grindley, J., Bellusci, S., Dunn, N. R., Emoto, H. and Itoh, N. (1997). Branching morphogenesis of the lung: new models for a classical problem. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 249-256.

Ikeda, K., Shaw-White, J. R., Wert, S. E. and Whitsett, J. A. (1996). Hepatocyte nuclear factor 3 activates transcription of thyroid transcription factor 1 in respiratory epithelial cells. *Mol. Cell. Biol.* **16**, 3626-3636.

Kaestner, K. H., Hiemisch, H., Luckow, B. and Schutz, G. (1994). The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. *Genomics* **20**, 377-385.

Kalinichenko, V. V., Lim, L., Stolz, D. B., Shin, B., Rausa, F. M., Clark, J., Whitsett, J. A., Watkins, S. C. and Costa, R. H. (2001). Defects in pulmonary vasculature and perinatal lung hemorrhage in mice heterozygous null for the forkhead box f1 transcription factor. *Dev. Biol.* **235**, 489-506.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M. and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* **8**, 277-289.

Keijzer, R., van Tuyl, M., Meijers, C., Post, M., Tibboel, D., Grosveld, F. and Koutsourakis, M. (2001). The transcription factor GATA6 is essential for branching morphogenesis and epithelial cell differentiation during fetal pulmonary development. *Development* **128**, 503-511.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzalez, F. J. (1996). 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.

Koutsourakis, M., Keijzer, R., Visser, P., Post, M., Tibboel, D. and Grosveld, F. (2001). Branching and differentiation defects in pulmonary epithelium with elevated Gata6 expression. *Mech. Dev.* **105**, 105-114.

Krane, C. M., Towne, J. E. and Menon, A. G. (1999). Cloning and characterization of murine Aqp5: evidence for a conserved aquaporin gene cluster. *Mamm. Genome* **10**, 498-505.

Kreda, S. M., Gynn, M. C., Fenstermacher, D. A., Boucher, R. C. and Gabriel, S. E. (2001). Expression and localization of epithelial aquaporins in the adult human lung. *Am. J. Respir. Cell. Mol. Biol.* **24**, 224-234.

Lee, M. D., King, L. S., Nielsen, S. and Agre, P. (1997). Genomic organization and developmental expression of aquaporin-5 in lung. *Chest* **111**, 111S-113S.

Liang, Q., De Windt, L. J., Witt, S. A., Kimball, T. R., Markham, B. E. and Molkentin, J. D. (2001). The transcription factors GATA4 and GATA6 regulate cardiomyocyte hypertrophy in vitro and in vivo. *J. Biol. Chem.* **276**, 30245-30253.

Lu, M. M., Yang, H., Zhang, L., Shu, W., Blair, D. G. and Morrisey, E. E. (2001). The BMP antagonist gremlin regulates proximal-distal patterning of the lung. *Dev. Dyn.* **222**, 667-680.

Ma, T., Song, Y., Gillespie, A., Carlson, E. J., Epstein, C. J. and Verkman, A. S. (1999). Defective secretion of saliva in transgenic mice lacking aquaporin-5 water channels. *J. Biol. Chem.* **274**, 20071-20074.

Mahlapuu, M., Enerback, S. and Carlsson, P. (2001). Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. *Development* **128**, 2397-2406.

Mahlapuu, M., Pelto-Huikko, M., Aitola, M., Enerback, S. and Carlsson, P. (1998). FREAC-1 contains a cell-type-specific transcriptional activation domain and is expressed in epithelial-mesenchymal interfaces. *Dev. Biol.* **202**, 183-195.

Margana, R. K. and Boggaram, V. (1997). Functional analysis of surfactant protein B (SP-B) promoter. Sp1, Sp3, TTF-1, and HNF-3alpha transcription factors are necessary for lung cell- specific activation of SP-B gene transcription. *J. Biol. Chem.* **272**, 3083-3090.

Mendelson, C. R. (2000). Role of transcription factors in fetal lung development and surfactant protein gene expression. *Annu. Rev. Physiol.* **62**, 875-915.

Merika, M. and Orkin, S. H. (1995). Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. *Mol. Cell Biol.* **15**, 2437-2447.

Metzger, R. J. and Krasnow, M. A. (1999). Genetic control of branching morphogenesis. *Science* **284**, 1635-1639.

Miettinen, P. J., Warburton, D., Bu, D., Zhao, J. S., Berger, J. E., Minoo, P., Koivisto, T., Allen, L., Dobbs, L., Werb, Z. et al. (1997). Impaired lung branching morphogenesis in the absence of functional EGF receptor. *Dev. Biol.* **186**, 224-236.

Minoo, P., Su, G., Drum, H., Bringas, P. and Kimura, S. (1999). Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(–/–) mouse embryos. *Dev. Biol.* **209**, 60-71.

Miura, N., Kakinuma, H., Sato, M., Aiba, N., Terada, K. and Sugiyama, T. (1998). Mouse forkhead (winged helix) gene LUN encodes a transactivator that acts in the lung. *Genomics* **50**, 346-356.

Morrisey, E. E., Ip, H. S., Lu, M. M. and Parmacek, M. S. (1996). GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev. Biol.* **177**, 309-322.

Morrisey, E. E., Ip, H. S., Tang, Z., Lu, M. M. and Parmacek, M. S. (1997). GATA-5: a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development. *Dev. Biol.* **183**, 21-36.

Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S. and Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12**, 3579-3590.

Nielsen, S., King, L. S., Christensen, B. M. and Agre, P. (1997). Aquaporins in complex tissues. II. Subcellular distribution in respiratory and glandular tissues of rat. *Am. J. Physiol.* **273**, C1549-1561.

Pepicelli, C. V., Lewis, P. M. and McMahon, A. P. (1998). Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Curr. Biol.* **8**, 1083-1086.

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., Orkin, S. H. and Costantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**, 257-260.

Ramirez, M. I., Cao, Y. X. and Williams, M. C. (1999). 1.3 kilobases of the lung type I cell T1alpha gene promoter mimics endogenous gene expression patterns during development but lacks sequences to enhance expression in perinatal and adult lung. *Dev. Dyn.* **215**, 319-331.

Ramirez, M. I., Chung, U. I. and Williams, M. C. (2000). Aquaporin-5 expression, but not other peripheral lung marker genes, is reduced in PTH/PTHrP receptor null mutant fetal mice. *Am. J. Respir. Cell. Mol. Biol.* **22**, 367-372.

Ramirez, M. I., Rishi, A. K., Cao, Y. X. and Williams, M. C. (1997). TGT3, thyroid transcription factor I, and Sp1 elements regulate transcriptional activity of the 1.3-kilobase pair promoter of T1alpha, a lung alveolar type I cell gene. *J. Biol. Chem.* **272**, 26285-26294.

Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M. and Schwartz, R. J. (1998). GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol. Cell. Biol.* **18**, 3405-3415.

Serra, R., Pelton, R. W. and Moses, H. L. (1994). TGF beta 1 inhibits branching morphogenesis and N-myc expression in lung bud organ cultures. *Development* **120**, 2153-2161.

Shaw-White, J. R., Bruno, M. D. and Whitsett, J. A. (1999). GATA-6 activates transcription of thyroid transcription factor-1. *J. Biol. Chem.* **274**, 2658-2664.

Shu, W., Yang, H., Zhang, L., Lu, M. M. and Morrisey, E. E. (2001). Characterization of a new subfamily of winged-helix/forkhead (Fox) genes which are expressed in the lung and act as transcriptional repressors. *J. Biol. Chem.* **276**, 27488-27497.

Simon, M. C. (1995). Gotta have GATA. *Nat. Genet.* **11**, 9-11.

Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., Simon, M. C., Leiden, J. M. and Wilson, D. B. (1995). Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. *Development* **121**, 3877-3888.

Ten Have-Opbroek, A. A. (1991). Lung development in the mouse embryo. *Exp. Lung Res.* **17**, 111-130.

Tichelaar, J. W., Lim, L., Costa, R. H. and Whitsett, J. A. (1999a). HNF-3/forkhead homologue-4 influences lung morphogenesis and respiratory epithelial cell differentiation in vivo. *Dev. Biol.* **213**, 405-417.

Tichelaar, J. W., Lu, W. and Whitsett, J. A. (2000). Conditional expression

of fibroblast growth factor-7 in the developing and mature lung. *J. Biol. Chem.* **275**, 11858-11864.

Tichelaar, J. W., Wert, S. E., Costa, R. H., Kimura, S. and Whitsett, J. A. (1999b). HNF-3/forkhead homologue-4 (HFH-4) is expressed in ciliated epithelial cells in the developing mouse lung. *J. Histochem. Cytochem.* **47**, 823-832.

Ting, C. N., Olson, M. C., Barton, K. P. and Leiden, J. M. (1996). Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* **384**, 474-478.

Toonen, R. F., Gowan, S. and Bingle, C. D. (1996). The lung enriched transcription factor TTF-1 and the ubiquitously expressed proteins Sp1 and Sp3 interact with elements located in the minimal promoter of the rat Clara cell secretory protein gene. *Biochem. J.* **316**, 467-473.

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-226.

Volpe, M. V., Vosatka, R. J. and Nielsen, H. C. (2000). Hoxb-5 control of early airway formation during branching morphogenesis in the developing mouse lung. *Biochim. Biophys. Acta* **1475**, 337-345.

Warburton, D., Schwarz, M., Tefft, D., Flores-Delgado, G., Anderson, K. D. and Cardoso, W. V. (2000). The molecular basis of lung morphogenesis. *Mech. Dev.* **92**, 55-81.

Weaver, M., Yingling, J. M., Dunn, N. R., Bellusci, S. and Hogan, B. L. (1999). Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development* **126**, 4005-4015.

Wert, S. E., Glasser, S. W., Korfhagen, T. R. and Whitsett, J. A. (1993). Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev. Biol.* **156**, 426-443.

Zhao, J., Chen, H., Peschon, J. J., Shi, W., Zhang, Y., Frank, S. J. and Warburton, D. (2001). Pulmonary hypoplasia in mice lacking tumor necrosis factor-alpha converting enzyme indicates an indispensable role for cell surface protein shedding during embryonic lung branching morphogenesis. *Dev. Biol.* **232**, 204-218.

Zhou, L., Dey, C. R., Wert, S. E. and Whitsett, J. A. (1996a). Arrested lung morphogenesis in transgenic mice bearing an SP-C-TGF-beta 1 chimeric gene. *Dev. Biol.* **175**, 227-238.

Zhou, L., Dey, C. R., Wert, S. E., Yan, C., Costa, R. H. and Whitsett, J. A. (1997). Hepatocyte nuclear factor-3beta limits cellular diversity in the developing respiratory epithelium and alters lung morphogenesis in vivo. *Dev. Dyn.* **210**, 305-314.

Zhou, L., Lim, L., Costa, R. H. and Whitsett, J. A. (1996b). Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. *J. Histochem. Cytochem.* **44**, 1183-1193.