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Branching and differentiation defects in pulmonary epithelium with elevated *Gata6* expression

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Abstract

The transcription factor GATA6 is expressed in the fetal pulmonary epithelium of the developing mouse lung and loss of function studies strongly suggested that it is required for proper branching morphogenesis and epithelial differentiation. We have further investigated the role of GATA6 in this process by utilizing a pulmonary epithelium specific promoter to maintain high levels of GATA6 protein during fetal lung development. Transgenic mice expressing *Gata6* cDNA under the control of the human *Surfactant Protein-C* (*SP-C*) promoter were generated and their lungs were analyzed during fetal stages. Transgenic lungs exhibit branching defects as early as embryonic day (E) 14.5 and molecular analysis just before birth (E18.5) shows a lack of distal epithelium differentiation whereas proximal epithelium is unaffected. Electron microscopic analysis and glycogen staining confirm the lack of differentiation to mature Type II cells. Thus, elevated levels of GATA6 protein affect early lung development and in analogy to other GATA factors in other tissues, GATA6 also plays a crucial role in the terminal differentiation in this case of the distal pulmonary epithelium.

Keywords: Gata6; Overexpression; Differentiation; Branching; Distal pulmonary epithelium

1. Introduction

The complexity of the lung tissue with respect to morphogenesis and cell type constitution reflects the complexity in the control of its development. Early specification (around embryonic day (E) 9.5 in the mouse) is far from understood, and molecules like HNF3β that is thought to be involved has a more general role in foregut and midgut formation (Ang and Rossant, 1994; Dufort et al., 1998). For the initial lung bud outgrowth mesenchymal–epithelial interactions are crucial since genetic studies have shown that molecules like FGF10, Gli2 and Gli3, all expressed in the lung mesenchyme, are key factors (Min et al., 1998; Motoyama et al., 1998). Mesenchymal–epithelial interactions are also important throughout branching morphogenesis and in the current concept of lung budding, localized high expression of *Fgf10* induces the endoderm to bud (Hogan, 1999; Weaver et al., 2000). This pre-programmed process is reiterated and leads to the establishment of an extensive respiratory tree which, by the end of the pseudoglandular stage of lung development (E16.5), consists of proximal and distal airways. At this stage, epithelial cells undergo extensive differentiation to give rise to a number of distinct cell types (ciliated, non-ciliated secretory, goblet, basal, alveolar Type I and Type II cells), which will establish a functional lung. Although some studies describe these different cell types morphologically and in terms of surfactant protein expression (Ten Have-Opbroek, 1991, 1988, 1990) their differentiation program remains obscure at the molecular level.

Bmp4 expression in the endoderm is thought to contribute to the acquisition of cell fate with distal epithelium resulting from exposure to high growth factor concentration (Weaver et al., 1999). A number of other molecules including FGFs, glucocorticoids and some transcription factors like MASH1, HES1, TTF1, HFH4 and GATA6 have been implicated in epithelial cell differentiation and surfactant protein production (Perl and Whitsett, 1999; Warburton et al., 2000; Whitsett and Tichelaar, 1999). MASH1 and HES1 regulate the differentiation of pulmonary neuroendocrine cells (Ito et al., 2000). TTF1 was shown to positively influence BMP4

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levels and its absence results in a lack of distal structures and the lack of surfactant proteins expression (Minoo et al., 1999). The HNF3/forkhead homologue 4 (HFH4) has been associated with induction of proximal fate and specifically the appearance of ciliated cells (Tichelaar et al., 1999). GATA6, a member of the GATA DNA binding family of zinc finger transcription factors, is expressed in the early branching endoderm and it was thought to be essential for bronchial endoderm specification (Morrisey et al., 1998). However, recent studies have shown that the protein is not required for early specification but for normal branching morphogenesis and epithelial differentiation. Lung endoderm depleted of GATA6 fails to branch normally and to acquire either distal or proximal fate (Keijzer et al., 2001). Furthermore, GATA binding sites have been identified in the promoters of *Nkx2.1*, *Clara cell marker 10* and *Surfactant Protein A* and *C* (Bruno et al., 2000; Shaw-White et al., 1999; Wert et al., 1993).

GATA proteins have been shown to play key roles in the differentiation of a number of distinct cell lineages and their levels can influence cell choices between differentiation and proliferation (Molkentin, 2000). GATA1 functions in erythrocytes and alteration of its levels results in a block of differentiation (Pevny et al., 1991; Whyatt et al., 1997). Overexpression of GATA2 blocks differentiation of early hematopoietic progenitors (Briegel et al., 1993) and overexpression of GATA3 is involved in the differentiation of T-cells (Hendriks et al., 1999; Zheng and Flavell, 1997). In vivo loss of function studies in mice have shown distinct roles for GATA4, 5 and 6 during development and both GATA4 and 6 primarily function in the extraembryonic visceral endoderm (Koutsourakis et al., 1999; Kuo et al., 1997; Molkentin et al., 1997; Morrisey et al., 1998). In vitro studies have implicated GATA6 in the differentiation program of vascular smooth muscle cells, VSMCs (Mano et al., 1999), and more recently in glomerular mesangial cells, GMCs (Nagata et al., 2000). In *Xenopus* embryos, it has been demonstrated that decreasing *Gata6* expression is associated with differentiation of cardiac precursors. When GATA6 levels were sustained high, the cardiac differentiation program was blocked (Gove et al., 1997).

In this study we utilized a previously characterized promoter/enhancer from the human *Surfactant Protein-C* (*SP-C*) gene (Wert et al., 1993) to keep *Gata6* expression levels elevated in the pulmonary epithelium. Transgenic embryos were generated and their lungs were analyzed morphologically at various stages. Just before birth molecular as well as Electron Microscopic analysis were performed to evaluate the status of differentiation of the lung epithelium. Our results parallel data from other GATA factors and show that elevated *Gata6* expression in the lung epithelium, in vivo, not only affects its normal branching pattern but also impairs the differentiation program in the distal airways.

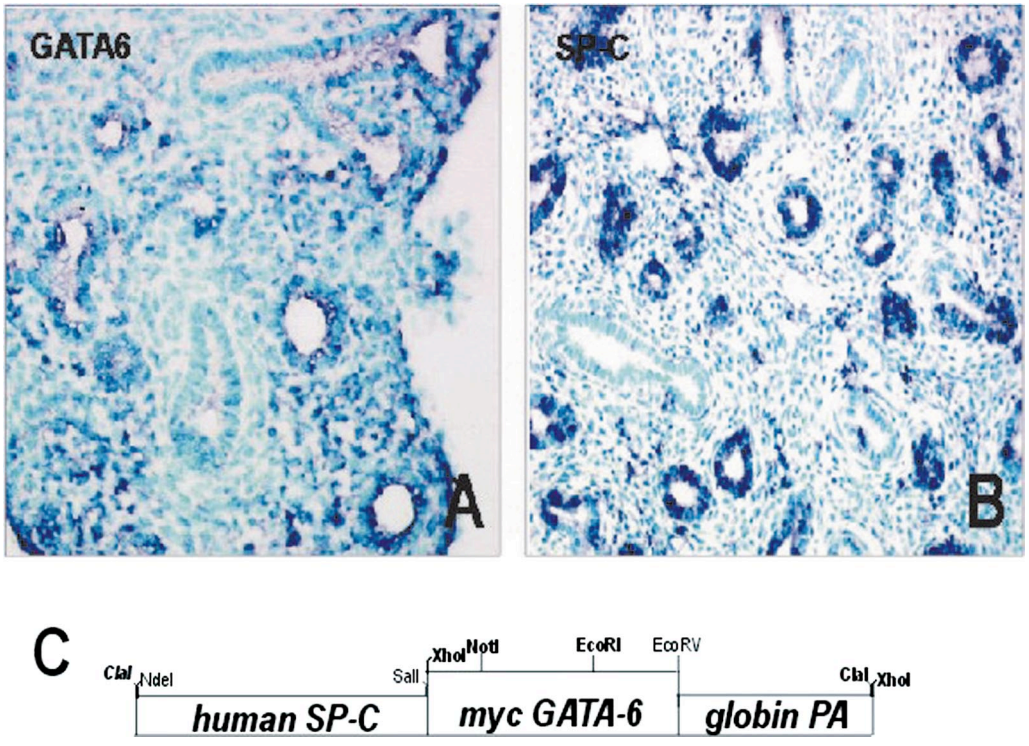

Fig. 1. Distribution of *Gata6* (A) and *SP-C* (B) mRNA in E15.5 lungs. Both genes, although at a different level, are expressed in cells lining the distal epithelium (blue is the detected mRNA and the tissue is counterstained with methyl-green). In C the transgene used to overexpress *Gata6* in the pulmonary epithelium is schematically depicted. A myc-tagged version of the complete *mGata6* cDNA is under the control of the 3.7 kb human *SP-C* promoter and it is followed by the human *β-globin* last intron and polyadenylation signal. For a more detailed description see Section 4.1.

2. Results

2.1. Gata6 expression and over-expression in the pulmonary epithelium

Expression of *Gata6* in the developing embryonic bronchial epithelium has been described previously (Morrisey et al., 1996). In a recent study (Keijzer et al., 2001), a more extensive analysis of *Gata6* expression revealed that initially it is expressed at the tips of the growing buds (E10.5) and by E12.5 it is expressed in the entire endoderm lining the developing buds. By E15.5, *Gata6* mRNA is predominantly expressed in the endoderm lining the growing airways and to a lower extent in parts of the surrounding mesenchyme (Fig. 1A). This expression pattern, although not at a comparable level, is quite reminiscent to that of *SP-C* at the same developmental stage (Fig. 1B). This observation led us to employ the 3.7 kb promoter/enhancer of the human *SP-C* gene (Wert et al., 1993) in order to sustain high *Gata6* expression levels in the developing epithelium in vivo. The complete mouse *Gata6* cDNA was cloned downstream of the *SP-C* promoter and was followed by the human *β-globin* last exon, intron and poly(A) for mRNA stability. Sequences coding for the myc epitope were introduced in frame with the first ATG of the gene (Fig. 1C). The *SP-C–mycGata6* transgene was injected into fertilized eggs and lungs were isolated from embryos at different developmental stages, E14.5, 16.5 and 18.5. Embryos were genotyped by Southern blotting (data not shown), transgenic lungs were identified and expression of the transgene was assessed by immunostaining with anti-myc antibody. At E14.5, one out of the two transgenic lungs was expressing the transgene; at E16.5, two out of the four; at E18.5, three out of five and at P1, two out of seven. All the analyses were performed in F_0 transgenic founder embryos or pups. For every developmental stage, only the transgenic lungs that were expressing the transgene had similar abnormal morphology and they were not significantly different in size from their wild-type littermates (data not shown). Only after careful examination of the whole transgenic lungs was a difference in the texture noticed (similar to a sponge with bigger airspaces), especially in the affected lungs at E18.5 (data not shown). At this stage, the three transgenic founders that were expressing the transgene had a comparable phenotype shown in Figs. 2F and 4H. Sections from the same transgenic E18.5 lung were used for both morphological (Fig. 2F) and transgene expression analysis (Fig. 3B,D). Expression analysis of molecular markers (Fig. 4) and electron microscopy (Fig. 5) were

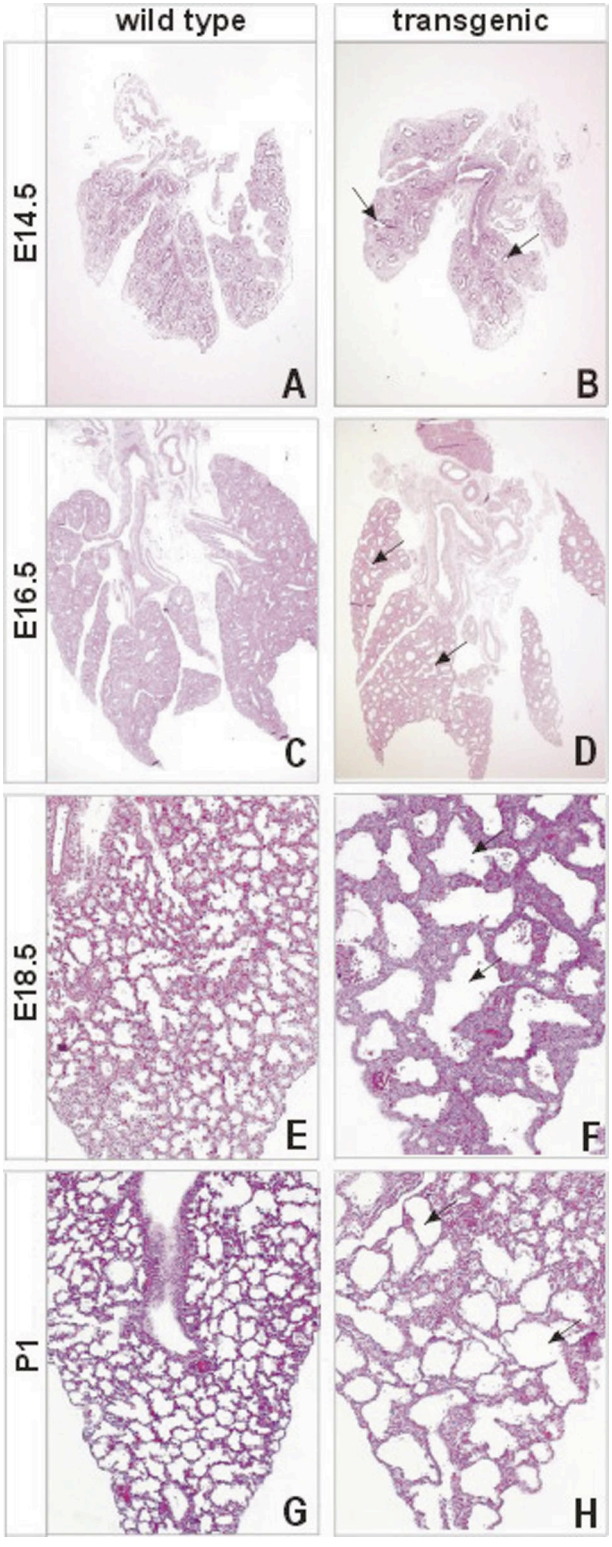

Fig. 2. Histological analysis of *SP-C–Gata6* transgenic lungs during fetal lung development. At E14.5, transgenic lungs (B) are similar in size to wild-type (A), but they have fewer terminal buds (arrows). Two days later, at E16.5 the branching defect is more prominent with big dilated distal buds present in the transgenic lungs (D, arrows) while fine branching is already apparent in wild-type littermates (C). This phenotype is more dramatic just before birth, at E18.5, with the presence of abnormally shaped and sized distal alveoli (F, arrows) in contrast to normal alveolization that can be seen in wild-type lungs (E). A few transgenic pups were born alive and the most severely affected one (H) had a milder branching phenotype when compared to most of the E18.5 transgenic lungs (F). This phenotype (H, arrows point at abnormal alveoli) is similar to the clinical central-acinar aeration pattern. All pups were killed a few hours after birth. For all the panels shown the magnification is the same.

performed on sections from a different transgenic E18.5 lung.

2.2. Branching morphogenesis in the SP-C–Gata6 transgenic fetal lungs

Starting at embryonic day 14.5, the transgenic lungs looked similar in size with the wild-type but they had undergone less extensive branching judged by the fewer terminal buds (Fig. 2B). Two days later, at E16.5, the defect in branching morphogenesis was more pronounced because of the presence of grossly dilated terminal buds separated by excess of mesenchyme (Fig. 2D). Just before birth, at E18.5, the phenotype was comparable to that seen at E16.5 and the overall growth of the tissue was not severely affected (Fig. 2F). The mesenchyme appears to be thickened although it is at present not clear whether this is due to an actual increase in the number of mesenchymal cells or the lack of being intersected by branching endoderm. Postnatally, although the remains of a transgenic pup were found subsequent to its perinatal death, few transgenic pups were born alive and they were breathing normally. These transgenic pups were left to breathe for a few hours before they were killed for genotyping and histologic analysis. In Fig. 2H a section of an abnormal lung among the transgenic pups is shown with a phenotype similar to the clinical centroacinar aeration pattern. The severity of the phenotype was less than that seen in E18.5 lungs (Fig. 2F) most likely due to the lower expression levels of the transgene. Expression of the transgene in the affected E18.5 lungs was confirmed by immunostaining for the myc epitope (Fig. 3B) and *Gata6* mRNA, transgenic and endogenous, was detected by in situ hybridization (Fig. 3C,D).

2.3. Molecular analysis of differentiation in the Gata6 overexpressing epithelium

Since extensive differentiation and the initiation of surfactant production start at the saccular stage of lung development (E17.5), which extends even after birth (to

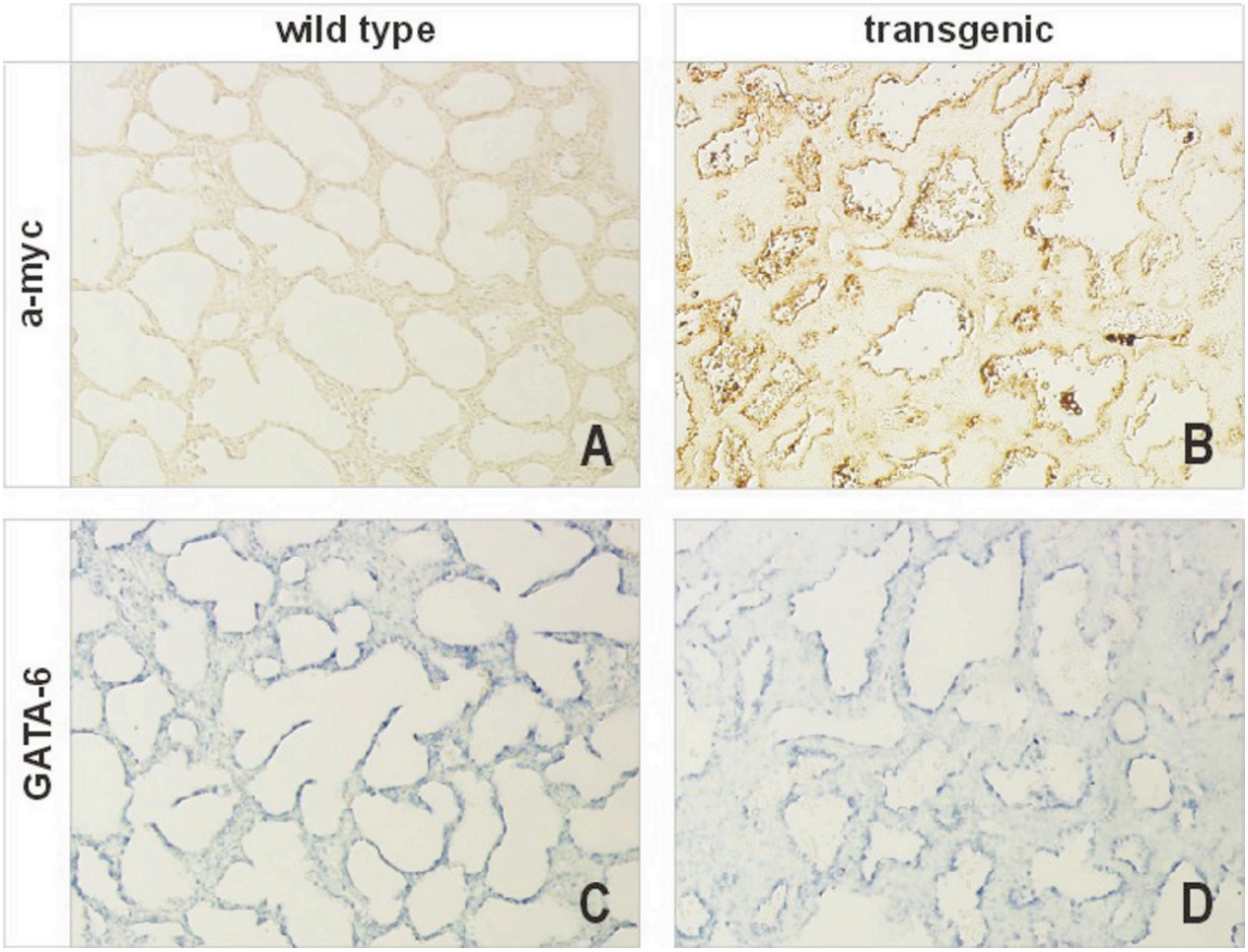

Fig. 3. Expression of the *SP-C–Gata6* transgene in E18.5 lungs. The anti-myc antibody was used to detect chimeric protein in transgenic lungs (B). Intense staining can be seen in the epithelium lining the abnormally dilated alveoli (brown). Wild-type lungs show no staining (A). *Gata6* mRNA was detected in both wild-type and transgenic lungs by in situ hybridization. In C, normal *Gata6* expression in parts of the epithelium and in the surrounding mesenchyme can be seen in wild-type E18.5 lungs (blue). In transgenic lungs (D) the signal for RNA expression (blue) is very intense in the lining of the epithelium as seen with the anti-myc antibody staining (B). For this analysis, sections from the lung that is shown on Fig. 2F were used.

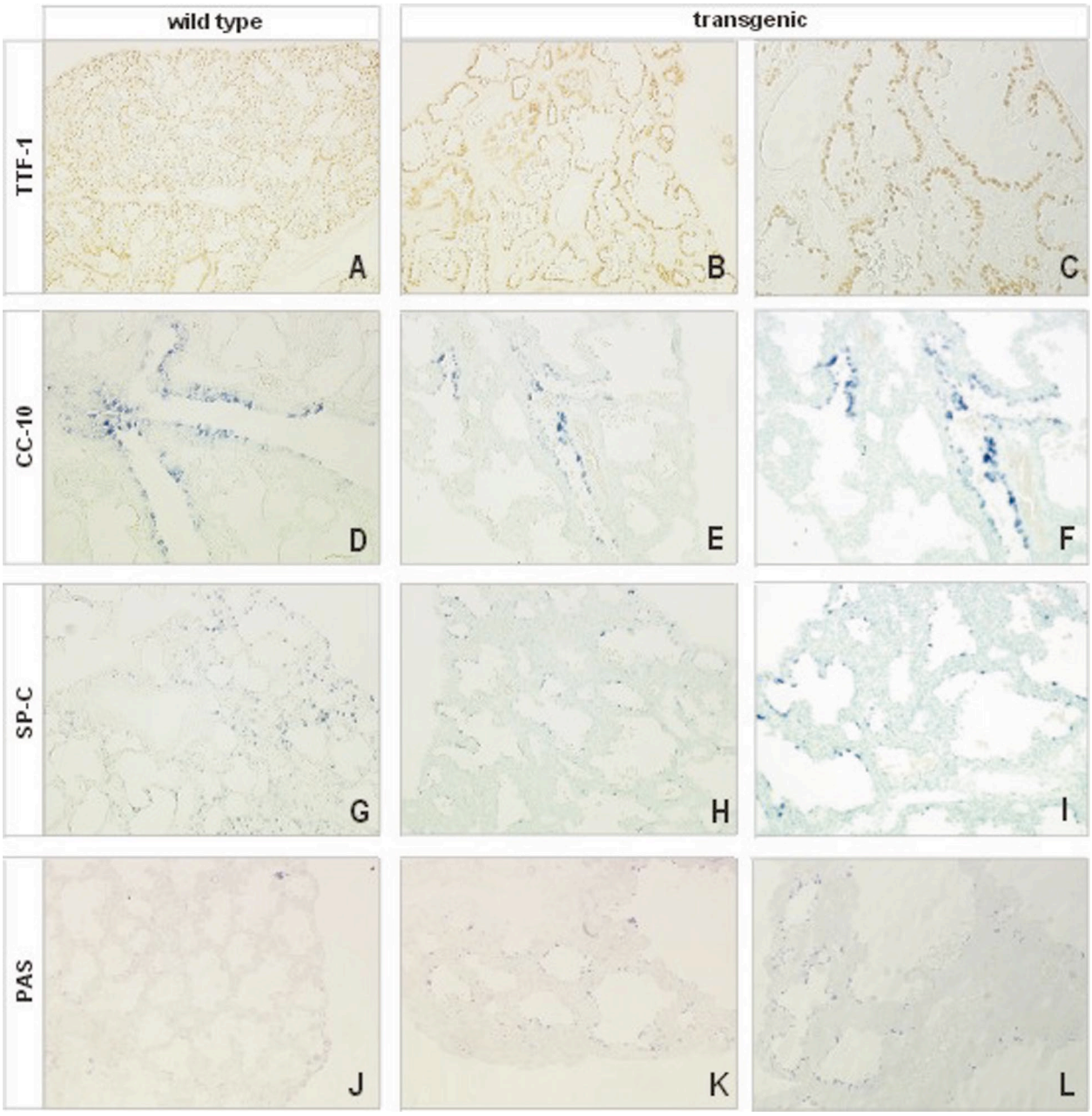

Fig. 4. Molecular analysis of differentiation in the transgenic epithelium at E18.5. TTF1 protein localization in wild-type (A) and transgenic (B,C) epithelium shows the abnormally, for this stage, TTF1-positive epithelium which lines the complete transgenic alveoli (brown). In (D–F) the normal expression of *CC10* mRNA, a marker for proximal non-ciliated cells, is shown in both wild-type and transgenic lungs, respectively (blue). In the distal epithelium, expression of endogenous *SP-C*, a marker for Type II cells, can be seen in a wild-type lung (G) and the decreased levels with the different distribution in the transgenic lung (H,I) (blue). Periodic Acid Schiff staining for glycogen (purple) reveals that in contrast to the wild-type lung (J), the transgenic lung has an alveolar epithelium which is lined almost exclusively by glycogen positive cells (K,L). Panels (A,D,G,J) from wild-type lungs, are the same magnification as panels (B,E,H,K) from transgenic lungs. Only (C,F,I,L) are higher magnification.

P5), we decided to investigate epithelium differentiation in E18.5 transgenic lungs. Thyroid Transcription Factor 1 (TTF1 or Nkx2.1) protein, a marker for specified pulmonary endoderm (Minoo et al., 1999), was abundantly present in transgenic lungs (Fig. 4B,C). However, the staining in all cells lining the dilated terminal buds is more intense than the characteristic staining for this stage of development as seen in wild-type E18.5 lungs (Fig. 4A). *Clara Cell marker 10* mRNA (Ray et al., 1996) was expressed in transgenic lungs at a normal level indicating the presence of non-ciliated secretory cells in the proximal epithelium (Fig. 4D–F). In the distal epithelium, topologically represented by the dilated buds, *Surfactant Protein-C* (Kalina et al., 1992) expressing cells could be detected among the cells lining the epithelium (Fig. 4H,I) although the number of cells and their distribution were very different from that observed in

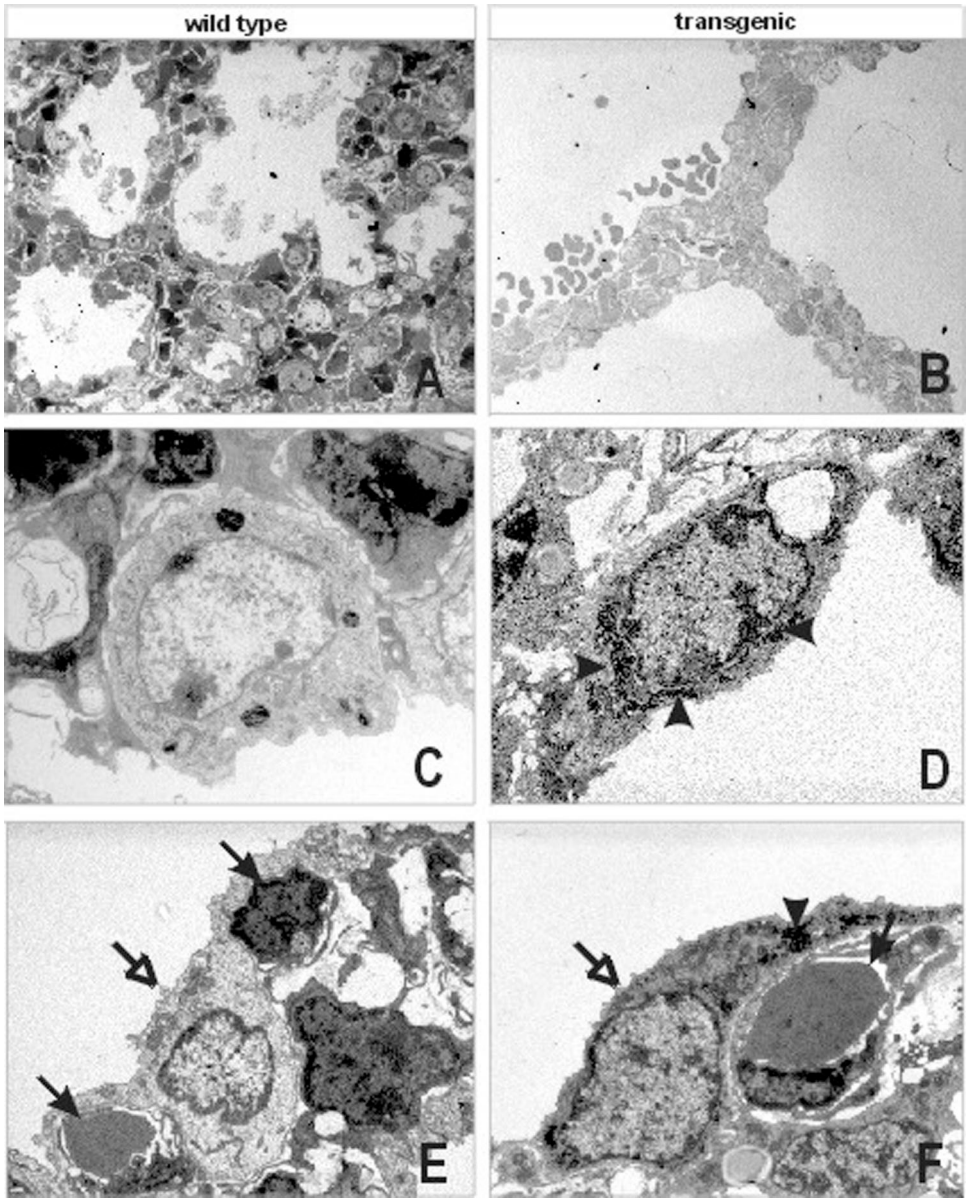

Fig. 5. Electron microscopic analysis of the distal epithelium at E18.5. A low-power overview of a wild-type and a transgenic lung is shown in (A,B), respectively. In the distal epithelium of a wild-type lung both typical Type II cells, with multilamellar bodies in their cytoplasm (C) and flat Type I pneumocytes, around capillaries, can be detected (E). In the transgenic lung atypical cells lining the alveoli are detected and the presence of glycogen in their cytoplasm is a common feature (D). Flat cells are present around the capillaries (F), although they look distinct from wild-type Type I cells and some of them still have glycogen in their cytoplasm (arrowheads point to glycogen fields, arrows indicate capillaries and open arrowhead arrows indicate Type I cells). Magnification: (A,B) ×620; (C–F) ×5800.

wild-type lungs (Fig. 4G). *SP-C* is expressed in the Type II cells which function mainly in surfactant production and they can further differentiate into Type I pneumocytes, the functional cells for gas exchange.

2.4. Morphological analysis of differentiation in the Gata6 over-expressing epithelium

From morphological studies (Ten Have-Opbroek, 1991, 1988) it was shown that before maturation, Type II cells have an excess of glycogen present in their cytoplasm and their nucleus is less round. We therefore stained the epithelium of the transgenic lungs (E18.5) for glycogen (Fig. 4J–L) and used electron microscopy to examine the ultrastructure of the cells lining the dilated buds (Fig. 5). Periodic acid Schiff staining (PAS) for glycogen revealed that a very high percentage of cells lining the distal epithelium of the transgenic lungs is positive for glycogen in their cytoplasm (Fig. 4K,L). Normally at this stage, due to advanced differentiation, glycogen-positive cells are hardly detectable in the lining of the distal epithelium (Fig. 4J). The caudal lobe of a transgenic (Fig. 4) and a wild-type littermate lung

were processed for electron microscopy. In the epithelium of the wild-type lung, normal Type II cells could be easily identified by the cuboidal shape, the almost round nuclei and the presence of several multilamellar bodies (Fig. 5C). Squamous Type I cells were also observed surrounding capillaries (Fig. 5E, arrows indicate capillaries). Both Type II and I cells were represented in every alveoli that was examined. In contrast, examining the epithelial lining in the dilated alveoli in the transgenic lung we were not able to find any typical Type II or I cells. Instead, a number of cells with more irregular nuclei and numerous glycogen fields in their cytoplasm were found (Fig. 5D, glycogen fields are indicated by arrowheads). Although formation and localization of capillaries appeared normal, the squamous cells present around them had different morphology than that of a typical Type I cell and some even had glycogen in their cytoplasm (Fig. 5F, arrow indicates capillary and arrowhead glycogen). Proximal epithelium appeared normal and ciliated cells were observed as in the wild-type epithelium (data not shown).

3. Discussion

Depletion of *Gata6* expression in the pulmonary endoderm, both in vitro and in vivo, resulted in lack of branching morphogenesis and failure of the epithelium to differentiate (Keijzer et al., 2001). To better understand the role of this transcription factor in lung development we used the *SP-C* promoter to artificially maintain high levels of *Gata6* expression in the fetal pulmonary epithelium in vivo. This alteration also resulted in defective branching morphogenesis and epithelial differentiation. However, the branching phenotype was manifested later during development and in a less severe form probably due to the late onset of the transgene expression (Wert et al., 1993). The promoter is active after pulmonary endoderm specification, initial budding and lobe determination but before the differentiation program starts at the pseudoglandular stage (E14.5) of lung development (Perl and Whitsett, 1999; Warburton et al., 2000). Hence, *Gata6* expression in the *SP-C–Gata6* lungs is already elevated in the primordial epithelium before any proximal or distal fate had been acquired. Nevertheless, this overexpression affected only distal epithelium where the gene is normally expressed. Thus, this study shows that GATA6 has a specific regulatory role in the differentiation program of the distal epithelium and also confirms that GATA6 protein levels are crucial during both lung morphogenesis and cell type specification (Keijzer et al., 2001)

3.1. Branching morphogenesis in the SP-C–Gata6 transgenic lungs

The interaction between epithelium and mesenchyme is a determining factor in lung development starting from the initial budding and continuing during branching morphogenesis involving well-studied molecules like FGF10, BMP4 and SHH (Hogan, 1999; Weaver et al., 2000). The localized high expression of *Ffg10* in the mesenchyme promotes the endoderm to bud and to express high levels of *Bmp4*. Subsequently, BMP4 together with SHH downregulates *Fgf10* to prevent new budding at the same position while allowing the newly formed bud to grow. When a certain distance has been reached, the high expression domains of *Fgf10* present on either side or both sides of the growing bud induce new budding resulting in lateral or dichotomous branching, respectively. *Gata6* expression in the endoderm coincides with branching morphogenesis but unlike *Bmp4* expression (Weaver et al., 2000), there are no detailed *Gata6* expression data during the induction and growth of a bud. In E10.5 lungs *Gata6* mRNA has been localized to the growing tips of the initial buds (Keijzer et al., 2001). At that stage, the *SP-C* promoter becomes active resulting in high expression of *Gata6* in all *SP-C* expressing cells throughout the transgenic endoderm. This alteration of *Gata6* expression results in the branching defects in the transgenic lungs which could be explained in two ways: either by distorting a molecular pathway intrinsic to the branching endoderm or by interrupting a pathway involved in processing an inductive signal from the mesenchyme.

In cardiac development, FGFs and BMPs have been placed upstream of GATAs (Schultheiss et al., 1997; Monzen et al., 1999; Reifers et al., 2000). Cardiac induction involves GATA4 and its co-factor Nkx2.5 and they are both necessary to establish cardiac phenotype (Durocher et al., 1997). An analogous regulatory pathway appears to operate in the lung endoderm. TTF1 (or Nkx 2.1), a gene from the same Nkx homeobox family and GATA6 are expressed in the lung endoderm from early pulmonary specification and both are required for branching morphogenesis (Lazzaro et al., 1991; Keijzer et al., 2001). It has been suggested that *Ttf1* expression may be downstream and depends on *Gata6* expression (Shaw-White et al., 1999), however, *Ttf1* expression is normal in *Gata6* $-/-$ endoderm implying that *Ttf1* expression is independent of GATA6 (Keijzer et al., 2001). Unfortunately it is not known whether *Gata6* is still expressed in *Ttf1* $-/-$ embryos (Minoo et al., 1999)

3.2. GATA6 levels and pulmonary epithelium differentiation

Expression of *Gata6* starts in the primordial epithelium and it is restricted to the distal epithelium by E15.5. The levels of its expression are difficult to quantitate by in situ hybridization. By expressing *Gata6* under the control of *SP-C*, a constant high level of the protein was present from the onset of differentiation resulting in a distal epithelium attenuated of any terminal differentiation. Unlike overexpression of *Tgfβ1* (Zhou et al., 1996) and *Hnf3β* (Zhou et al., 1997) that resulted in endodermal arrest at the primordial or pseudoglandular stages, *Gata6* overexpression did not affect initiation of the differentiation program. The pathway of proximal epithelium differentiation was unaffected since both ciliated cells, seen in the electron microscopic analysis,

and non-ciliated secretory cells, visualized by *CC10* expression, were detected as normal. Along the distal epithelium no mature Type II cells were present and all the cells were still expressing *Ttf1*. Normally the presence of numerous glycogen fields in the cells lining the distal airways is indicative of their differentiating status (Sorokin, 1965). This has been described in detail in an extensive morphological study of the features of Type II alveolar epithelial cells by Ten Have-Opbroek et al. (1988). Later during lung development, before birth, a multi-step formation of multilamellar bodies (MLB) is thought to compartmentalize glycogen (Ten Have-Opbroek et al., 1990). Our data show a continued presence of glycogen in the distal epithelial cells, suggesting that GATA6 is involved in a particular stage of this maturation process. When the levels are elevated, the cells do not initiate the final differentiation step which is characterized by multilamellar bodies formation. Instead, most of the cells lining the alveolar epithelium have glycogen in their cytoplasm, as visualized by PAS staining (Fig. 4K,L), indicating a block in the Type II differentiation pathway. The flat Type I cells which function in gas exchange, are thought to originate from further differentiation of Type II cells although it is not clear whether they are derived from a mature Type II cell or from one of the intermediate stages during their maturation. In the *Gata6* overexpressing lungs, flat epithelial cells surrounding the capillaries were observed and their shape and localization suggest that they are Type I cells. However, some of them still had glycogen fields, they were not as flat as normal Type I cells and their nucleus had an irregular shape. At this final step, differentiation is thought to be driven by growth and particularly by the intercalation of the forming capillaries with the epithelium (Ten Have-Opbroek et al., 1988). Thus, the apparent Type I-like cells we see could be the same precursors of Type II cells as discussed above, except that they are in close proximity with the capillaries and have less glycogen fields. This would suggest that elevated levels of GATA6 would prevent the formation of fully differentiated Type I cells even when in contact with capillaries. In conclusion, our data demonstrate that GATA6 plays an important role in lung organogenesis and especially during the multi-step process of maturation of Type II cells. Elevated GATA6 protein levels result in a block of terminal differentiation to mature Type II and Type I pneumocytes.

4. Experimental procedures

4.1. Construction of the Gata6 transgene

The full *Gata6* cDNA was constructed by joining the two *Eco*RI fragments which were isolated from a mouse E11.5 library (Clontech) (Brewer et al., 1999; Koutsourakis et al., 1999). 3′ UTR was eliminated by polymerase chain reaction (PCR) from the unique *Pst*I site to the stop codon (144 bp) and a unique site (*Eco*RV) was introduced in the 3′ PCR primer in order to clone the last non-coding exon, intron and poly(A) (PA) (2.8 kb) from the human *β-globin* gene (Drabek et al., 1997). PCR between the first ATG and the unique *Not*I (520 bp) site was used to eliminate 5′ sequences. In the 5′ PCR primer the myc epitope (EQKLISEEDL) was introduced immediately after the ATG of *Gata6* (Elefanty et al., 1996). The sequence of all PCR-generated clones was confirmed before further cloning. The complete (1.7 kb) *Gata6* cDNA with the myc sequences was transfected in COS-1 cells and the overexpressed protein was immunoreactive in situ with both the anti-myc (9E10) and the anti-GATA6 (SantaCruz) antibodies (data not shown). The human *SP-C* promoter (Wert et al., 1993) was cloned as a 3.7 kb blunted *Nde*I–*Sal*I fragment in blunted *Sal*I of pBS and the *myc-Gata6-globinPA* as an *Xho*I fragment. The transgene was released with *Cla*I between the pBS polylinker and the *Cla*I that was present at the end of the *PA*. The 8.2 kb fragment was gel purified with Concert Matrix (Gibco BRL) and passed through an Elutip-D column (Schleicher and Schuell).

4.2. Generation of transgenic embryos

Transgenic mouse embryos were generated by pronuclear injection of FVB/N oocytes with transgene concentration 2–3 ng/μl according to standard protocols (Hogan et al., 1994). Day of injection and transfer to pseudopregnant females was considered as day 0.5 for the subsequent staging of the dissected embryos and lungs. Part of the tail of the dissected embryos was used for Southern blot genotyping using the human *β-globin PA* as a probe.

4.3. In situ hybridization and immunohistochemistry

Fetal or newborn lungs were dissected out and fixed in 4% PFA overnight at 4°C before processing for paraffin embedding according to routine protocols. Sections (5–7 μm thick) were used for H&E staining, RNA in situ hybridization (Motoyama et al., 1998) and immunohistochemistry (Keijzer et al., 2000). Digoxigenin-labeled RNA probes were made from cDNA fragments of 1.5 kb for *Gata6*, 0.33 kb for *SP-C* and 0.315 kb for *CC10* according to the protocol supplied by Roche Diagnostics. For TTF1, a monoclonal antibody (Neomarkers, CA, USA) was used in 1:100 dilution and the sections were microwave boiled in citrate buffer for 15 min. The same dilution was used for the monoclonal anti-myc antibody (9E10) but for antigen retrieval, trypsin treatment (0.6 mg/ml for 5 min at room temperature) was used.

4.4. Electron microscopy

The caudal lobes of E18.5 fetal lungs were fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.3) at 4°C for a few days until the genotypes were known. One transgenic and one wild-type littermate lobe were washed in 0.1 M cacodylate buffer and postfixed in 1% OsO_4 in 0.1 M

cacodylate buffer, and finally embedded in Epon as has been previously described (De Bruijn and Den Beejen, 1975). Ultrathin sections were contrasted with uranyl acetate and lead citrate prior to electron microscopic analysis (Hanaichi et al., 1986).

4.5. PAS staining

From the Epon-embedded E18.5 lungs, semithin (1 μm) sections were used for periodic acid Schiff staining as previously described (Nevalainen et al., 1972).

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