Inhibition of alveolarization and altered pulmonary mechanics in mice expressing GATA-6

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GATA-6 is the only known GATA family member expressed in respiratory epithelial cells during lung morphogenesis. During the pseudoglandular and canalicular stages, GATA-6 is expressed at high levels in the epithelial cells lining the terminal saccules of the lung. The levels of GATA-6 mRNA decrease after embryonic day 18.5 and decrease further postnatally (32). Although GATA-6 null embryos fail to survive beyond implantation, chimeric GATA-6-deficient mice were generated (18, 33). In the chimeric mice, GATA-6 null cells were not detected in the bronchial epithelium, suggesting that GATA-6 is required for the specification or survival of respiratory epithelial cells in vivo. Inhibition of GATA-6 utilizing an engrafted GATA-6 transgene inhibited branching morphogenesis and blocked terminal differentiation of respiratory epithelial cells in mouse lung (26, 41). The potential roles of GATA-6 in postnatal lung formation or function are unknown.

Transcriptional programs regulating cell proliferation and differentiation are critical for function of the embryonic and postnatal lung. GATA-6 and thyroid transcription factor (TTF)-1, a homeodomain containing transcription factor, both play important and interactive roles in the regulation of gene expression in the lung. Expression of GATA-6 and TTF-1 is subject to precise cell-specific and developmental regulation during lung morphogenesis. TTF-1 is required for formation of the peripheral lung and for expression of a number of respiratory epithelial-specific genes, including the surfactant proteins (SP-A, SP-B, SP-C) and Clara cell secretory protein (CCSP) (1, 5, 20, 29, 42–44). Levels of TTF-1 and GATA-6 decrease coordinately during lung development. Furthermore, GATA-6 and TTF-1 physically interact and synergistically regulate expression of surfactant proteins, including SP-A and SP-C (3, 25, 35).

To further discern the role of GATA-6 in lung morphogenesis, we generated transgenic mice in which mouse GATA-6 mRNA was expressed in the respiratory epithelium of the developing lung. Increased expression of GATA-6 inhibited postnatal alveolarization, demonstrating that the precise temporal-spatial
control of GATA-6 expression is required for postnatal lung morphogenesis.

**MATERIALS AND METHODS**

**Generation of human SP-C-GATA-6 transgenic mice.** A 2.23-kb fragment containing the entire coding region of mouse GATA-6 cDNA (from SalI site to NdeI site, the kind gift of Dr. Jeffrey Molkentin, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH) was subcloned into a PUC-18 plasmid vector containing the human (h) 3.7-kb SP-C promoter and SV40 t intron-polyA sequences (Fig. 1). The 6.5-kb transgene was excised with NdeI and microinjected into the pronucleus of fertilized eggs from FVB/N mice. Transgenic mice were identified by PCR and Southern blot analyses. PCR primers used to identify the transgene were the following: 5’- primer in GATA-6 cDNA, 5’-GGT CTA TCC TGG AGG CGG AG-3’, 3’ primer in SV40 t intron-polyA region, 5’-AGG TTC AGG GGG TGG TGT GG-3’. PCR product identity was confirmed by sequencing. Amplification of the PCR product encoding hSP-C-GATA-6 transgene was performed after denaturation at 94°C for 10 min followed by 30 cycles of amplification at 94°C for 30 s, 60°C for 30 s, and 72°C for 42 s, and a 7-min extension at 72°C. PCR primers used to identify β-actin were the following: 5’ primer, 5’-GTC CCT GTA TGC CTC TGG TC-3’; 3’ primer, 5’-TGC TAC TCC TGC TGG AT-3’.

**Animal husbandry.** Animals were maintained in a pathogen-free vivarium in filtered cages under Institutional Animal Care and Use Committee-approved procedures. There was no serological evidence of viral pathogens or bacterial infections in sentinel mice maintained with the colony. No histological evidence of infections was detected in transgenic mice at necropsy. All mice were maintained in the FVB/N strain. Two distinct lines of transgenic mice bearing the hSP-C-GATA-6 transgene were generated.

**Lung histology and immunohistochemistry.** Fetuses were isolated after injection of ketamine-xylazine-acepromazine to the dam. Lungs were fixed with 4% paraformaldehyde at 4°C. Lungs from postnatal animals were inflation fixed in situ at 25 cmH2O of pressure via a tracheal cannula. We then tied off the trachea with suture before removing the lungs. Tissue was processed as described by Clark et al. (7) and embedded in paraffin. Antibodies used were: histone H3, phosphorylated (Ser10) (1:1000, rabbit polyclonal; United States Biological), M30 CytoDEATH (1:10, monoclonal; Roche), CCSp (1:7500, rabbit polyclonal; kindly provided by Dr. Barry Stripp, University of Pittsburgh), pro-SP-C (1:1000, rabbit polyclonal, AB3428; Chemicon), TFF-1 (1:1000, rabbit polyclonal; kindly provided by Dr. Roberto Di Lauro, Stazione Zoologica, Naples, Italy), VEGF (1:250, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), Flk-1 (VEGF receptor-2) (1:400, rabbit polyclonal; Santa Cruz), and platelet endothelial cell adhesion molecule (PECAM, 1:500, rat polyclonal, clone CD31; Pharmingen). Orycin staining for elastic fibers was performed using the kit and instructions provided by Poly Scientific (R&D).

**Lung morphometry.** Lung tissue sections were stained with hematoxylin and eosin. Sections were visually scanned for position-matching regions compared with controls. At least four representative lobes were selected from each animal. Fields containing lobar bronchi were excluded. Image 1/Metamorph Imaging System version 2 (Universal Imaging) was used to measure terminal air space area as described previously (14). We determined calibration for ×20 images by acquiring images of a standard. The air spaces were distinguished from tissue based on intensity, and the number of pixels acquired for each air space was converted to square micrometers. Percentages of cell proliferation were determined as number of positive cells from all lung cell lineages normalized for the number of positive nuclei for all cell types per field.

**RNA isolation and analysis.** Lung RNA was prepared using TRIzol reagent (Life Technologies, San Francisco, CA)
following the manufacturer’s specifications. RNA was treated with 2 units of DNase at 37°C for 30 min. DNase was removed by DNase inactivation reagent (Ambion, Austin, TX) before cDNA synthesis. DNase (5 μg)-treated total lung RNA was reverse transcribed and analyzed by PCR for hSP-C-GATA-6 and by real-time PCR for total GATA-6 mRNA with the Smart Cycler System (Cepheid, Sunnyvale, CA). The relative concentration of GATA-6 mRNA was normalized to the concentration of β-actin in each sample. The primers used were: for total GATA-6 mRNA, 5′-CTC AAG TAT TCA GGT CAA GAC GGC-3′ and 5′-CAA AGG CAC AGA AAT CAC GCA TC-3′; for β-actin mRNA, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′.

Lung mechanics. Twelve- to thirteen-week-old hSP-C-GATA-6 transgenic and wild-type mice were anesthetized with 0.1 ml/10 g body wt of a mixture containing 40 mg/ml ketamine and 2 mg/ml xylazine ip. The mice were tracheostomized and ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min and positive end-expiratory pressure (PEEP) of 2 cmH2O by a computerized Flexi Vent system (SCIREQ, Montreal, Canada). This machine features accurate measurement of volume by using the position of the ventilator piston and pressure in the cylinder and allows analysis of dynamic lung compliance in mice. The ventilation mode on the Flexi Vent was changed to forced oscillatory mode on the Flexi Vent was changed to forced oscillatory signal (0.5-19.6 Hz), and respiratory impedance was measured. Estimated tissue damping and tissue elastance for mice at 2 cmH2O PEEP were obtained by fitting a model to each impedance spectrum (13). With this system, the calibration procedure removed the impedance of the equipment and tracheal tube.

Western blots. Lung tissues from E18.5 transgenic and wild-type littermate mice were immediately frozen in liquid nitrogen. Total lung cellular protein (15 mg) were rinsed twice with PBS then lysed in 50 mM Tris (pH 7.4), 150 mM sodium chloride, 2 mM EDTA, 25 mM sodium orthovanadate, 0.3% Nonidet P-40, protease inhibitor (1 μg/ml) (Sigma, St. Louis, MO) for 15 min. Tissues were then homogenized with prechilled tissue tearor and centrifuged for 10 min at 12,000 g. Supernatant (25 μl) was aliquoted and subjected to Western blot with caspase-9 antibody (Santa Cruz Biotechnology).

RESULTS

Expression of GATA-6 mRNA in the developing mouse lung. The GATA-6 transgene was expressed selectively in lung epithelial cells under the control of the 3.7-kb hSP-C promoter (Fig. 1A). This promoter directs gene expression as early as E10.5 in peripheral respiratory epithelial cells in transgenic mice (40). By RT-PCR analysis of lung RNA, GATA-6 mRNA was readily detected at E16.5, E18.5, postnatal day (P) 6, and P22 (Fig. 1B). Two separate transgenic lines were generated. Similar air space abnormalities were observed in heterozygous line 6.1 offspring and homozygous line 6.3 offspring, and line 6.1 was subsequently used for these studies. Total GATA-6 mRNA levels were increased ~1.5- and 4-fold in transgenic mice compared with that of wild-type littermates on P6 and E18.5, respectively (Fig. 1C). The hSP-C-GATA-6 transgenic mice survived postnatally, and transmission of the transgenes followed Mendelian inheritance patterns. Endogenous GATA-6 mRNA levels were high in respiratory epithelial cells at E13.5 and E16 and decreased at E18.5 and P5 (Fig. 1D).

GATA-6 perturbs postnatal lung formation. No defects in lung morphology were detected in hSP-C-GATA-6 transgenic mice at E18.5 (Fig. 2, A and B). Postnatal alveolarization was severely disrupted in hSP-C-GATA-6 transgenic mice. Defects in septation were observed as early as P6 and were extensive by P15 (Fig. 2, C–F). Peripheral air spaces were larger in size. Morphometric analysis demonstrates that mean air space area was significantly increased in hSP-C-GATA-6 transgenic mice (Fig. 2G). There was no evidence of cellular infiltrates or fibrosis in hSP-C-GATA-6 transgenic mice, suggesting that the defects in

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**Figure 2.** Effects of GATA-6 expression on lung morphogenesis. Lung histology is normal in hSP-C-GATA-6 mice (B) compared with WT (A) at E18.5. Defects of septation were observed in lung parenchyma of GATA-6 TG mice at P6 compared with WT controls (C, D). At P15, alveolar areas were significantly increased in GATA-6 TG mice compared with WT mice (E, F). Mean air space areas were measured at P6 (6 animals per genotype) and P15 (G) (4 animals per genotype). Values are means ± SE. *P < 0.05 by Student’s t-test. Original magnification ×10, bar = 100 μm for A–F.
alveolarization were caused by developmental rather than inflammatory processes.

Increased lung volumes and abnormal lung mechanics in GATA-6-expressing mice. In adult GATA-6 transgenic mice, airway resistance and elastance, as well as tissue damping and elastance, were significantly decreased, consistent with the histological evidence of enlarged alveoli (Fig. 3, A, B, D, E). Lung compliance was significantly increased in hSP-C-GATA-6 transgenic mice (Fig. 3C). Maximum lung volumes were markedly increased, consistent with decreases in resistance and elastance. Pressure-volume curves also indicate increased lung volumes compared with controls (Fig. 3F).

Effects of GATA-6 on cell proliferation and apoptosis. Cell proliferation in GATA-6 transgenic mice was estimated by immunostaining for phosphohistone 3 at E18.5, P6, and P16 (38). At P6, a slight but not statistically significant decrease in the number of stained cells was observed in the transgenic mice. A significant increase in the number of cells staining for phosphohistone 3 was detected at P15, suggesting increased cell turnover (Fig. 4). Nuclear fragmentation was not observed at the light microscopic level. M30, a monoclonal antibody specific to the caspase 3-cleaved fragment of cytokeratin 18 (9), showed no difference in staining between wild-type and transgenic mice (Fig. 5A, B). No changes in the protein levels of caspase-9 were observed in GATA-6 transgenic mice compared with wild type (Fig. 5C).

Effects of GATA-6 on respiratory epithelial differentiation. To evaluate the distribution of respiratory epithelial cells in hSP-C-GATA-6 transgenic mice, we performed staining for pro-SP-C, a marker for nonciliated bronchiolar cells. No differences in the proximal-peripheral distribution of CCSP and pro-SP-C staining was observed. Numbers of type II cells per field were decreased in hSP-C-GATA-6 transgenic mice at P6, which is consistent with decreased surface area (Fig. 6). TTF-1 protein was detected in bronchiolar and type II epithelial cells of hSP-C-GATA-6 transgenic and wild-type littermates at P6. Abnormal clusters of TTF-1-positive cells were observed in enlarged alveolar regions in GATA-6 transgenic mice (Fig. 7, A and B). Although alveolar septae were short, the structure and distribution of elastic fibers in the enlarged alveoli were not perturbed in the hSP-C-GATA-6 transgenic mice at P17 (Fig. 7, C and D). Fragmentation of elastic fibers, typically seen in inflammatory remodeling, was not observed.

Effects of GATA-6 on pulmonary vascularization. Normal patterns of VEGF-A and VEGF receptor VEGFR-2 (Flk-1) were observed in the lungs of hSP-C-GATA-6 transgenic mice (Fig. 8, A–D). Quantitative PCR indicates that there was no difference in the level of expression of VEGF-A mRNA (data not shown). Intensity and sites of PECAM staining in the pulmonary vascular bed were not perturbed in GATA-6 transgenic mice, indicating maintenance of the alveolar capillary network in spite of the increase in alveolar size (Fig. 8, E and F).

**DISCUSSION**

In the present study, increased expression of GATA-6 did not perturb lung morphogenesis before birth but altered alveolarization in the early postnatal period. Increased lung volumes and lung mechanics consistent with air space enlargement persisted into adulthood. These findings support the concept that increased expression of GATA-6 in respiratory epithelial cells inhibited alveolarization and perturbed lung function.

Alveolarization of the mouse lung occurs between P5 and P21, in a process that completes the formation of the gas-exchange region of the lung. During this period, type II epithelial cells proliferate rapidly and differentiate into type I epithelial cells. Alveolar septae divide terminal respiratory sacculles, increasing the
number of alveoli and surface area required for gas exchange. The septae are supported by extracellular matrix consisting of an extensive elastin network (21, 38). The growth in the respiratory epithelium is paralleled by a coordinate expansion of the pulmonary capillaries. Reciprocal communication between epithelial and endothelial cells mediates the alveolarization process. For example, VEGF produced primarily by epithelial cells directs vascularization, activating its receptors on endothelial cells and their precursors (6, 16, 17, 24, 34). Complex autocrine, paracrine, and juxtacrine interactions mediate the complex process of alveolarization (28).

Expression of GATA-6 did not perturb lung morphogenesis before birth, a finding distinct from that of Keijzer et al. (18). This discrepancy between these two studies may be related to the differences in levels of GATA-6 expression, being increased only two- to fourfold in the present study. Alternatively, the isoform of GATA-6 expressed in the two studies may influence the

Fig. 4. Effects of GATA-6 expression on phosphohistone 3 staining. Cell proliferation in hSP-C-GATA-6 mice was evaluated at E18.5 (3 animals per group, A and D), P6 (3 animals per group, B and E), and P15 (4 animals per group, C and F). G: phosphohistone 3-stained cells were normalized to total number of nuclei (indicated by nuclear red staining) for all cell types per field. The percentage of histone 3-positive cells is presented as mean ± SE, *P < 0.05, by Student’s t-test.

Fig. 5. Effects of GATA-6 on apoptosis. M30 antibody was used to stain caspase-3-cleaved cytokeratin (an indicator of apoptosis) in lung tissue from WT (A) and GATA-6 TG mice (B) at E18.5. No difference in the intensity or pattern of M30 staining was observed. C: Western blot analysis was used to detect caspase-9 in lung homogenates. Content of caspase-9 was similar in lung tissue from hSP-C-GATA-6 TG and WT littermates at E18.5.
effects on lung formation and function. In the previous study by Koutsourakis et al. (22), expression of a long GATA-6 isoform inhibited peripheral lung differentiation, causing severe lung malformation at birth. Because the GATA-6 isoforms expressed differ on a translational basis, it is, at present, unclear which form of GATA-6 protein is normally synthesized in the developing lung (2). Although no morphological abnormalities were observed at E18.5, cellular alterations were readily apparent in GATA-6-expressing cells before

Fig. 6. Immunostaining for pro-SP-C and Clara cell secretory protein (CCSP). Pro-SP-C (C and D) and CCSP staining (A and B) was performed at P6. CCSP was detected in conducting airways of WT (A) and hSP-C-GATA-6 mice (B). Fewer pro-SP-C-positive cells were observed in GATA-6 TG mice (D). Original magnification ×20, bar = 100 µm for A–D.

Fig. 7. Immunohistochemical staining for thyroid transcription factor (TTF)-1 and orcein staining for elastic fibers. TTF-1 was detected in the nuclei of bronchial and alveolar type II cells in WT and hSP-C-GATA-6 TG mice on P6. Abnormal clusters of TTF-1-positive cells were found in the TG mice (A, B). Orcein staining was performed to detect the elastic fiber structures in lungs from hSP-C-GATA-6 TG and WT mice. On P15, elastin fibers were stained along the alveolar walls and regions of septation in TG and control mice. Although septa were short and air spaces enlarged, the intensity of staining and fiber morphology were not perturbed by GATA-6 (C, D).
birth. Thus cellular abnormalities occurring late in gestation, predating the morphological abnormality seen in alveolarization, may influence subsequent processes that became apparent from P5 to P15 in the mouse (8). In a similar manner, prenatal, but not postnatal, inhibition of FGF signaling caused emphysema in the postnatal period, suggesting that loss or alterations in progenitor cells in the embryonic lung may influence subsequent alveolarization after birth (15). In the present study, however, no changes in cell proliferation were detected at P6. Thus we speculate that GATA-6 perturbed survival or differentiation of progenitor cells in the perinatal and early postnatal period, in turn altering later stages of alveolarization.

Further supporting this concept, the expression of GATA-6 transgene decreased postnatally during a time in which the SP-C promoter is highly active. This finding may reflect a loss of cells expressing the GATA-6 transgene. Although epithelial cell abnormalities were readily apparent at E18.5, ultrastructural changes did not persist. Neither caspase-9 nor M30 staining indicated increased apoptosis, and fragmented nuclei were not seen (36). Thus we did not find clear evidence that expression of GATA-6 activated typical apoptotic pathways.

In the present study, a significant increase in cell proliferation was observed at P15 in the GATA-6 transgenic mice, a time during which air space size has increased and the fraction of the lung parenchyma to air space had decreased. This finding suggests that cell injury caused by expression of GATA-6 may have enhanced mitotic activity in the lung periphery. In the GATA-6 transgenic mice, changes in cell proliferation were not altered at E18.5 despite cell injury at the ultrastructural level. However, loss of even a small number of progenitor cells might inhibit alveolarization.

In the present study, increased expression of GATA-6 inhibited alveolarization, a finding similar to that seen in transgenic mice in which TTF-1 was ex-
pressed in respiratory epithelial cells in the postnatal lung (39). As seen in SP-C-TTF-1 transgenic mice, abnormal clusters of peripheral respiratory epithelial cells that were TTF-1 positive were observed in the SP-C-GATA-6 transgenic mice (39). In contrast to the findings in the GATA-6 transgenic mice that lacked inflammation, increased expression of TTF-1 in respiratory epithelial cells was associated with both enlarged alveoli and pulmonary inflammation. Because TTF-1 and GATA-6 directly interact (25), it is possible that similarities in histological findings in these models are mediated by similar or overlapping pathways that lead to the loss of peripheral respiratory epithelial cells.

Lung morphogenesis requires tightly regulated GATA-6 expression. Deletion of GATA-6 restricted survival of cells, forming the respiratory tract in vivo (18). Likewise, expression of a dominant-negative engrafted GATA-6 fusion protein blocked terminal differentiation of the alveolar type I cells in vivo, demonstrating that GATA-6 is required for perinatal lung formation (26, 41). The present study demonstrates that increased expression of GATA-6, during a time in which its expression is normally decreased, causes epithelial cell injury with resultant disruption of alveolarization. In the present study, increased expression of GATA-6 impaired alveolarization, limiting septation, and resulted in permanently altered lung function.

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DISCLOSURES

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