Loss of Cystic Fibrosis Transmembrane Conductance Regulator Function Produces Abnormalities in Tracheal Development in Neonatal Pigs and Young Children

David K. Meyerholz¹, David A. Stoltz², Eman Namati^{2,3}, Shyam Ramachandran⁴, Alejandro A. Pezzulo², Amanda R. Smith², Michael V. Rector², Melissa J. Suter³, Simon Kao⁵, Geoffrey McLennan^{2,5,6}, Guillermo J. Tearney³, Joseph Zabner², Paul B. McCray, Jr.⁴, and Michael J. Welsh^{2,7,8}

¹Department of Pathology ²Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa; ³Wellman Center for Photomedicine, Harvard Medical School, Boston, Massachusetts; ⁴Department of Pediatrics, ⁵Department of Radiology, ⁶Department of Biomedical Engineering, ⁷Department of Molecular Physiology and Biophysics, and ⁸Howard Hughes Medical Institute, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa

Rationale: Although airway abnormalities are common in patients with cystic fibrosis (CF), it is unknown whether they are all secondary to postnatal infection and inflammation, which characterize the disease.

Objectives: To learn whether loss of the cystic fibrosis transmembrane conductance regulator (CFTR) might affect major airways early in life, before the onset of inflammation and infection.

Methods: We studied newborn *CFTR*^{-/-} pig trachea, using computed tomography (CT) scans, pathology, and morphometry. We retrospectively analyzed trachea CT scans in young children with CF and also previously published data of infants with CF.

Measurements and Main Results: We discovered three abnormalities in the porcine CF trachea. First, the trachea and mainstem bronchi had a uniformly small caliber and cross-sections of trachea were less circular than in controls. Second, trachealis smooth muscle had an altered bundle orientation and increased transcripts in a smooth muscle gene set. Third, submucosal gland units occurred with similar frequency in the mucosa of CF and control airways, but CF submucosal glands were hypoplastic and had global reductions in tissue-specific transcripts. To learn whether any of these changes occurred in young patients with CF, we examined CT scans from children 2 years of age and younger, and found that CF tracheas were less circular in cross-section, but lacked differences in lumen area. However, analysis of previously published morphometric data showed reduced tracheal lumen area in neonates with CF.

Conclusions: Our findings in newborn CF pigs and young patients with CF suggest that airway changes begin during fetal life and may contribute to CF pathogenesis and clinical disease during postnatal life.

Keywords: airway development; cystic fibrosis; mucin; submucosal gland; smooth muscle

Mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) cause cystic fibrosis (1–3). Although CF is characterized by disease in many organs, respiratory disease is currently the leading cause of morbidity

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Early pathology studies of cystic fibrosis (CF) disease concluded that the trachea and large airways were likely normal at birth, but with exposure to postnatal stimuli (e.g., infection) would demonstrate progressive tissue alterations or remodeling changes. Validation of this principle has been limited by the inability to study newborns before infection and inflammation, and by the lack of CF animal models that develop spontaneous lung disease.

What This Study Adds to the Field

We show that neonatal CF pigs have reduced tracheal caliber and circularity, prominent smooth muscle, and, surprisingly, submucosal glands that are small and hypoplastic. Furthermore, we provide evidence that children and infants with CF have tracheal changes of reduced lumen caliber and circularity, similar to the CF pig model. Our data suggest that loss of the cystic fibrosis transmembrane conductance regulator produces tracheal abnormalities that begin as early as fetal life.

and mortality (2, 4). In the CF lung, airway obstruction is a common finding. Both pulmonary function tests and chest X-ray computed tomography (CT) have revealed evidence of air trapping and impaired airway function early in the course of the disease (5, 6). With disease progression, reduced airflow rates become almost universal (7, 8). These abnormalities in airway function plus the defects in airway epithelium and submucosal glands (9–11) have focused attention on airways in investigating CF pathogenesis.

Histopathologic examination has been a useful means to identify airway changes early in the course of disease. Studies on autopsy trachea from infants have suggested that CF airways lack overt histologic lesions soon after birth (12, 13). After exposure to postnatal stimuli including inflammation and infection, airways develop hyperplastic, hypertrophic, and inflammatory changes that can culminate in mucocellular obstruction and airway remodeling (12–16). Although these autopsy studies provided useful information, they were often confounded by variable patient age, infection status, and nutrition status that limited interpretations relative to early CF pathogenesis. Notably, many of these variables can be controlled by using animal models.

We developed a porcine model of CF (17). *CFTR*^{-/-} pigs had meconium ileus, pancreatic destruction, focal biliary cir-

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Correspondence and requests for reprints should be addressed to David K. Meyerholz, DVM, Ph.D., Department of Pathology, Roy J. and Lucille A. Carver College of Medicine, 1165 ML, Iowa City, IA 52242. E-mail: david-meyerholz@uiowa.edu

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rhosis, and gallbladder disease that replicated CF disease in humans. Furthermore, the gastrointestinal disease was accelerated in *CFTR*^{-/-} pigs compared with humans with CF (18). In contrast, the lungs of newborn CF pigs lacked inflammation. However, within months of birth, they spontaneously developed inflammation, remodeling, mucus accumulation, and infection (19), which are characteristic features seen in the lungs of older patients with CF.

In this study, we asked whether the airways of CF pigs show any abnormalities at birth. By studying newborns, we controlled variability in postnatal age and we could avoid the secondary manifestations of disease that often confound tissue studies from humans with CF. We adopted a morphometric approach (12, 15) to determine whether *CFTR*^{-/-} pig trachea and mainstem airways differ from those of wild-type littermates. We then examined CT data from young children with CF, to see whether similar trachea abnormalities could be detected.

METHODS

Animals

Animal studies were reviewed and approved by the University of Iowa (Iowa City, IA) Animal Care and Use Committee. Neonatal pig littermates were obtained from Exemplar Genetics (Sioux Center, IA). Animals were killed (Euthasol; Virbac, Fort Worth, TX) at 8–24 hours of age for study.

Histopathology

Tracheas were dissected, photographed (if required), and placed into 10% neutral buffered formalin for fixation (usually between 48 and 96 h). Whole mount trachea staining was performed with alcian blue (20). Tracheas were consistently cross-sectioned and routinely processed, embedded, sectioned (4 μ m), and stained with hematoxylin and eosin for routine examination. Special stains included periodic acid-Schiff and Masson's trichrome stains.

Immunohistochemistry

Ki-67. Tissues sections were routinely sectioned (4 μ m), deparaffinized, and hydrated. Antigen unmasking was performed with citrate buffer (pH 6.0, 8 min) and a pressure cooker (1,000-W microwave, 4 min). Endogenous peroxidase was quenched with hydrogen peroxide (3%, 8 min) and background was reduced with Background Buster (30 min; Innovex Biosciences, Richmond, CA). Primary antibody (rabbit anti-Ki-67 monoclonal antibody, diluted 1:800, overnight at 4°C) (cat. no. 4203-1; Epitomics Co, Burlingame, CA) was applied and tissue sections were rinsed, followed by application of a commercial secondary antibody kit (rabbit Envision horseradish peroxidase [HRP] system; Dako, Carpinteria, CA). The chromogen diaminobenzidine (DAB) (DAB Plus [5 min] and enhancer [3 min]; Dako) was applied, after which the sections were rinsed and counterstained with hematoxylin (1 min; Surgipath, Richmond, IL) and routinely dehydrated through a series of alcohol baths and coverslipped.

MUC5AC. Tissue sections were routinely sectioned (4 μ m), deparaffinized, and hydrated. Antigen unmasking was performed with citrate buffer (pH 6.0, 4 min) and a pressure cooker (1,000-W microwave, 3 min). Endogenous peroxidase was quenched with hydrogen peroxide (3%, 8 min). Primary antibody (mouse anti-MUC5AC monoclonal antibody, diluted 1:75, 30 min at room temperature) (cat. no. NB120-3649; Novus Biologicals, Littleton, CO) was applied and tissue sections were rinsed, followed by application of a commercial secondary antibody kit (mouse Envision HRP system; Dako). The chromogen DAB (DAB Plus [5 min] and enhancer [3 min]; Dako) was applied, after which the sections were rinsed, counterstained with hematoxylin (1 min; Surgipath), and routinely dehydrated through a series of alcohol baths and coverslipped.

MUC5B. Tissues sections were routinely sectioned (4 μ m), deparaffinized, and hydrated. Antigen unmasking was performed with citrate buffer (pH 6.0, 40 min in a steamer). Endogenous peroxidase was quenched with hydrogen peroxide (3%, 8 min). Primary antibody (rabbit anti-MUC5B polyclonal antibody, diluted 1:50, 120 min at room

temperature) (cat. no. HPA008246; Sigma, St. Louis, MO) was applied and tissue sections were rinsed, followed by application of a commercial secondary antibody kit (rabbit Envision HRP system; Dako). The chromogen DAB (DAB Plus [5 min] and Enhancer [3 min]; Dako) was applied, after which the sections were rinsed, counterstained with hematoxylin (1 min; Surgipath), and routinely dehydrated through a series of alcohol baths and coverslipped.

Cytokeratin-18. Tissues sections were routinely sectioned (4 μ m), deparaffinized, and hydrated. Antigen unmasking was performed with protease K (5 min), after which sections were rinsed. Endogenous peroxidase was quenched with hydrogen peroxide (3%, 8 min). Primary antibody (mouse anti-cytokeratin-18 [CK18] monoclonal antibody, diluted 1:2,000, 120 min at room temperature) (cat. no. C8541; Sigma) was applied and tissue sections were rinsed, followed by application of a commercial secondary antibody kit (mouse Envision HRP system; Dako). The chromogen DAB (DAB Plus [5 min] and Enhancer [3 min], DAKO) was applied, after which the sections were rinsed, counterstained with hematoxylin (1 min; Surgipath), and routinely dehydrated through a series of alcohol baths and coverslipped.

Morphometry of Fixed Pig Tissues

All tissues were examined with a high-resolution microscope (BX51; Olympus America, Center Valley, PA), and digital images were collected (DP71; Olympus) and analyzed (MicroSuite pathology software [Olympus] or ImageJ [National Institutes of Health, Bethesda, MD]). "Whole tracheas" (one cross-section per animal) were analyzed for tissue parameters, using landmarks outlined in Figure 3. Using these landmarks, trachea circumference, area, cartilage, inner wall, and trachealis muscle were assessed (Table 1). Some parameters (e.g., submucosal glands in Figure 6) were further standardized as a percentage of lumen area or to lumen circumference for normalization. Cross-sectional area was analyzed from these sections and stereologically interpreted as "volume" for the selected tissue (15, 21).

Trachea lumen circularity (Figures 1E, 4B, and 8B) was calculated by the formula $4\pi(\text{area}/\text{perimeter}^2)$, with a value of 1.0 representing a perfect circle. Trachea cartilage along the anterior border was assessed as continuous or discontinuous in a manner blinded to treatment groups.

Submucosal gland ducts (Figures 6A and 6B) were identified by epithelial tissue in the submucosa contiguous with surface epithelium, or by epithelial tissue in the submucosa within 20 μ m of the surface epithelium and having a perpendicular trajectory to the surface epithelium. Absolute numbers of ducts were recorded and also then normalized per lumen circumference.

Submucosal gland area included all aspects of the gland adjacent to the surface epithelium interface including acini, tubules, lumen, and ducts. For the study of submucosal gland distribution (Figure 6F), submucosal glands were identified, traced, and enumerated from digital images (magnification, $\times 200$; $n = 2$ samples per wall position) and normalized per millimeter of lumen wall.

We examined submucosal gland (Table 1) structure and focused on the "whole acinus" defined as an acinus with a detectable lumen. Here, whole acinus diameter (perpendicular to the longest axis) was measured along with the lumen subcomponent. The cellular subcomponent of acini was calculated as whole acinus diameter minus lumen subcomponent diameter. Lumen fraction was calculated by lumen divided by whole acinus diameters.

Trachea mucosa (Table 1) images (magnification, $\times 600$) were consistently collected along the lateral wall. Areas in which the epithelium was thinnest were selected for measurement so as to prevent sectioning artifact. Epithelial height and height of cilia were then measured ($n = 4$ measurements) and averaged.

Mucus (Figures 7A–7D) stainings and immunostainings were assessed in submucosal glands and surface epithelium. The respective tissues were outlined to define total area, and the area that was stained for each parameter (periodic acid-Schiff, MUC5AC, MUC5B, and CK18) was quantified (ImageJ software; NIH) and expressed as a percentage of tissue area. Using CK18-stained sections, the area of submucosal gland lumens was assessed and expressed as a percentage of the total submucosal gland area (Figure 7E). Ki-67 staining (Figures 7F and 7G) in surface epithelium or submucosal gland nuclei was consistently determined as stained (brown) or unstained (blue). Stained nuclei were expressed as a percentage of total nuclei.

Optical Frequency Domain Imaging

Optical frequency domain imaging (OFDI) is a second-generation Fourier domain optical coherence tomography technique that provides high-resolution cross-sectional images of tissue microstructure with penetration depths approaching 2 mm (22). The technical details of the OFDI system used in this study have been described elsewhere (23, 24). In short, the OFDI system generates axial depth profiles by measuring the delay of the source signal as it is reflected by subsurface structures, using interferometric techniques. Spiral cross-sectional OFDI was performed on formalin-fixed tracheal segments, using a 0.8-mm diameter (2.4 Fr) catheter. The catheter was rotated at 10 revolutions/second (frame size, $2,048 \times 4,096$) and translated at 0.5 mm/second, enabling volumetric imaging of the tracheal segments with resolutions in cylindrical coordinates of $20 \mu\text{m}$ (ϕ) $\times$ $7 \mu\text{m}$ (r) $\times$ $50 \mu\text{m}$ (z).

Cartilage ring three-dimensional analysis—OFDI: Volumetric OFDI data sets were imported into the Amira visualization software platform (Mercury Computer Systems Inc., Chelmsford, MA) for airway cartilage analysis. Data sets were inverted and a circumferential region surrounding the airway cross-section was extracted, removing both the catheter and fixed pattern noise from the outer edge of the image. The OFDI data were visualized in all three orthogonal orientations (axial, coronal, and sagittal) and individual cartilage rings were manually segmented and labeled. Three-dimensional visualization was performed with a surface generation algorithm based on the segmented cartilage ring masks.

Microarray Analysis

To investigate possible molecular cues underlying the altered tracheal morphology in newborn CF pigs, custom gene sets were generated and used to query a porcine trachea microarray data set (19), using gene set enrichment analysis (GSEA) (25, 26).

We first generated two custom gene sets from a large-scale microarray analysis of transcripts expressed in human bronchus surface and submucosal gland epithelia as reported by Fischer and colleagues (27). Analysis of variance was used to generate lists of significantly differentially expressed transcripts in each of the two tissues (27). We used these transcript lists to create custom gene sets (28) for submucosal gland and surface epithelia. We hypothesized that these sets would serve as markers of “normal” global gene expression in these tissue compartments. To generate unbiased gene sets, all transcripts up-regulated in either tissue by greater than twofold were considered. To obtain gene sets of optimal size for GSEA analysis (<http://www.broadinstitute.org/gsea/>), a further P value cutoff of less than 0.001 was set for surface epithelium cells (174 annotated pig genes; see Table E1 in the online supplement) and less than 0.01 for submucosal glands (110 annotated pig genes; Table E2). To query the trachea microarray data for changes in transcript levels relevant to smooth muscle and cartilage biology, custom gene sets were made, listing genes known to be expressed in these tissues. For this purpose, we put together a gene set of 49 annotated porcine genes involved in smooth muscle biology (Table E3), and a gene set of 20 annotated porcine genes associated with cartilage biology (Table E4).

Human CT Scans

This study was approved by the University of Iowa Institutional Review Board. We studied 6 non-CF subjects and 16 subjects with CF. Their characteristics are shown in Table E5. Nine of the subjects with CF were homozygous for the F508del mutation and the remainder had one F508del mutation and an additional CF disease-causing mutation on the other allele. All subjects in this study were scanned with a SOMATOM Sensation 16- or 64-slice scanner (Siemens, Erlangen, Germany). Data were taken only from nonintubated individuals. Because of the retrospective nature of this comparison and the difficulty of obtaining scans from patients of such a young age, scan protocols could not be controlled between groups. However, limitations were imposed to increase scan homogeneity within each group. Nearly all patients were scanned at 120 kVp (kilovolt peak), although in each group one case was included at 110 kVp. The scans from the CF group were reconstructed with either a Siemens b70f or b70s kernel, whereas control scans were reconstructed with either a b30f, b30s, or b31f kernel; all other kernels were excluded. Scans were required to

have been acquired at a slice thickness of no more than 5 mm. The slice thickness for the CF group ranged from 1 to 2 mm, whereas for the control group it ranged from 2 to 5 mm. Before measurement, overall scan quality was reviewed; scans showing significant motion artifact in the tracheal region were excluded.

CT DICOM (digital imaging and communications in medicine) data sets were imported into a custom, in-house–developed Pulmonary Analysis Software Suite for airway metric analysis. The CT data were visualized in two dimensions in the axial orientation. A tracheal slice that was representative of the mid-trachea was chosen for analysis. Orthogonal ray paths ($n = 25$) were cast from the centroid of the airway toward the luminal wall along which the full-width half-maximum was calculated and used to define the airway boundary. For each defined region, airway metrics including the average inner diameter and luminal area were calculated.

Pig CT Scans

CT data acquisition. Volumetric CT data sets were acquired with a 64 SOMATOM Sensation multidetector CT scanner (Siemens Medical Solutions). The following settings were used for scanning: 120-kVp X-ray source voltage, 250 mA-second effective current, and 0.75-mm slice thickness. Animals were sedated with ketamine (20 mg/kg, intramuscular) and xylazine (1.5 mg/kg, intramuscular) and allowed to breath spontaneously during imaging.

Lung volume. CT DICOM data sets were imported into the Amira visualization software platform (Mercury Computer Systems Inc.) for lung volume analysis. The lung volume was extracted, using a region-growing algorithm in which a seed was first selected inside the trachea and voxels (three-dimensional pixels) connected to this seed and bound between $-1,024$ and -250 Hounsfield units (HU) were automatically selected. The total number of voxels was then cumulated to give the digital lung volume.

Airway Diameter–Pulmonary Analysis Software Suite. CT DICOM data sets were imported into a custom in-house–developed Pulmonary Analysis Software Suite for airway metric analysis. The CT data were visualized in two dimensions in the axial orientation and a manual centroid was selected within each airway. Multiple orthogonal ray paths were projected out from each centroid toward the luminal wall. Airway metrics including the inner diameter and luminal area were calculated on the basis of the average major and minor diameter values from 100 projected lines. Centroids were selected every five transverse slices (2.5 mm) for the length of each airway branch.

Analysis from Published Trachea Data

Features of infants with CF and non-CF infants were analyzed from trachea data published by Sturgess and Imrie (15). Criteria for inclusion in this analysis included airway lumen area, body length for normalization, and postnatal age under 2 weeks to obtain a young cohort similar to the neonatal pigs in this study. We normalized the trachea data to body length to minimize variability associated with gestational length and compared data by Mann-Whitney test.

Statistics

Data were initially analyzed through calculation of group means and SEM for each group. Analysis of morphometric data from histologic samples and CT scans was performed either by Mann-Whitney test or Fisher's exact test as deemed appropriate and $P < 0.05$ was defined as statistically significant.

RESULTS

CT and Gross Examination

We examined the airways of newborn pigs by CT imaging. Because these piglets were sedated and breathing spontaneously, we chose to focus our analysis on the trachea and mainstem bronchi. The trachea and mainstem bronchi in CF pigs were smaller than those of littermate controls (Figures 1A–1C), whereas lung volume, normalized to femur length, did not significantly differ from that of non-CF pigs (Figure 1D).

Figure 1. Computed tomography (CT) scan and morphometry of neonatal pig trachea. (A) Single slice images of trachea (arrows) from chest X-ray CT of non-CF ($CFTR^{+/+}$; body weight, 1.42 kg) and CF ($CFTR^{-/-}$; body weight, 1.47 kg) newborn pig littermates. (B) Airway lumen inner diameter and (C) lumen area measurements of trachea (T), right mainstem bronchus (RB), and left mainstem bronchus (LB) obtained from CT images of non-CF ($CFTR^{+/+}$) and CF ($CFTR^{-/-}$) newborn piglets (mean body weights, 1.4 ± 0.05 and 1.27 ± 0.1 kg, respectively; $n = 4$ per group; $*P < 0.05$ vs. non-CF). (D) Lung volume normalized to femur length was similar between groups. (E) Calculated circularity of cross-sectional trachea was reduced in CF pigs ($P < 0.05$, Mann-Whitney test).

We further noticed that CF pig tracheas were less circular on CT scans (Figure 1A) and this proved true by morphometric analysis (Figure 1E). For circularity calculations, a score of 1 represents a perfect circle. We therefore proceeded to examine the lungs at necropsy.

On gross inspection, CF pigs had a smaller caliber trachea than that of littermate controls (Figure 2A). The small caliber extended through the mainstem bronchi. In addition, the cartilage in CF pig trachea appeared as irregular rings (Figure 2B). Imaging by *ex vivo* optical coherence tomography revealed tracheal cartilage rings with an irregular pattern and variable width (Figure 2C; see also the three-dimensional reconstruction in (Figure E1) in the online supplement).

Morphometry of Trachea in Cross-Section

To perform a quantitative analysis, we prepared cross-sections of fixed trachea and identified morphologic landmarks (Figures 3A and 3B). In cross-sections, CF pig trachea had smaller lumens than those of non-CF littermates, with significantly reduced circumference and area measurements (Figure 4A and Table 1). Similar to CT images, fixed cross-sections of CF trachea had reduced circularity compared with non-CF tracheas (Figure 4B). Rings of trachea cartilage were discontinuous along the anterior midline more often in CF tracheas (Figures 4A and 4C). However, the size and extent of cartilage in the trachea wall did not significantly differ between groups (Table 1).

Figure 2. Gross appearance and three-dimensional volume-rendered optical frequency domain imaging of trachea. (A) Trachea and mainstem bronchi from newborn non-CF ($CFTR^{+/+}$) and CF ($CFTR^{-/-}$) littermates (body weight, 1.45 and 1.69 kg, respectively). CF pig tracheas were of small caliber (scale bar, 1.5 cm). (B) CF pig tracheas had irregular rings; alcian blue stain and illumination to show tracheal cartilage. (C) Three-dimensional volume-rendered optical frequency domain imaging of a tracheal segment from newborn non-CF ($CFTR^{+/+}$) and CF ($CFTR^{-/-}$) piglets. Individual cartilage rings (represented by different colors) in CF pigs appear to be more irregular and have variable width compared with controls.

Thickening of the inner wall (defined as tissue between the inner edge of cartilage and airway lumen) of CF airways is an early marker of disease (29). Although CF pigs had a reduced total inner wall area, there was no detectable difference when normalized to tracheal size (Table 1). The thickness of the inner wall was similar between genotypes along the anterior and lateral walls; however, the mid-posterior wall was significantly thickened in CF pig tracheas (Table 1). The posterior inner wall thickening was due to trachealis smooth muscle that often appeared in cross-section as prominent oblique to longitudinal bundles (Figure 5). These prominent bundles were often detected in CF trachea and extended into bronchi, but were absent in non-CF controls (Figure 5). The total area of trachealis smooth muscle was not significantly different between genotypes; however, when normalized to inner wall area or lumen circumference it tended to be higher in CF tracheas, although the difference was not statistically significant (Table 1).

We consistently examined the anterolateral mucosa for changes in surface epithelium, including cell height and height of cilia. These parameters were similar in CF and non-CF groups (Table 1).

Submucosal Gland Morphometry

We then studied submucosal glands, as there is substantial interest in their potential contribution to the pathogenesis of CF airway disease. In fact, submucosal gland hypertrophy is a characteristic lesion in advanced CF lung disease (12, 14). We counted the submucosal gland ducts in cross-sections of whole trachea to determine the number of individual submucosal gland units (21). In cross-sections, CF pig tracheas

Figure 3. Morphologic landmarks for quantitative analysis of neonatal pig trachea. (A) Cross-sections of “whole trachea” were digitally captured at $\times 20$ magnification and parameters were assessed for each trachea section. Anterior (A), posterior (P) and lateral (L) tracheal walls were identified. (B) Posterior trachea wall is highlighted by the borders of the lumen (yellow arrow), inner cartilage (red arrow), and outer cartilage (green arrow). Note also in this image the (a) surface epithelium, (b) submucosal glands, (c) trachealis muscle, and (d) the cartilage rings. Masson’s trichrome stain. Scale bars: (A) 1 mm; (B) 160 μ m.

had a reduced number of submucosal gland units per trachea (Figure 6A). However, when normalized for trachea lumen circumference, the number of gland ducts was similar for both genotypes (Figure 6B). These results suggest that loss of CFTR did not reduce the frequency of submucosal gland units within the mucosa.

In contrast, the volume of submucosal glands was significantly reduced in CF pigs, even when standardized for tracheal size (Figures 6C–6E). We also examined the preferential distribution of submucosal gland tissues along the anterior, posterior, and lateral tracheal walls (Figure 6F). Submucosal glands were most abundant in the posterior wall, followed by the anterior and then lateral walls in each genotype. This distribution and frequency is similar to that described by Choi and colleagues (21), who found that the pig is unique among experimental laboratory animals in that the preferential distribution (posterior > anterior) of submucosal glands is similar to that in humans. However, Choi and colleagues (21) also found that pig submucosal glands are evenly dispersed over and between cartilage rings, whereas human submucosal glands are located principally between

Figure 4. Cross-sections and morphometry of tracheas in newborn pig littermates. (A) CF ($CFTR^{-/-}$) trachea lumens were smaller in cross-section compared with non-CF ($CFTR^{+/+}$) tracheas, and sometimes had discontinuous gaps (arrow) in the cartilage rings on the anterior border (body weight, 1.6 kg, each), Masson’s trichrome stain; scale bar, 1 mm. (B) CF pigs ($n = 6$) had reduced circularity in tracheal cross-sections compared with non-CF pigs ($n = 6$); $P < 0.05$, Mann-Whitney test. (C) Discontinuous cartilage was more frequently detected in CF ($CFTR^{-/-}$, $n = 15$) versus non-CF ($CFTR^{+/+}$, $n = 13$) tracheas. $P < 0.05$, Fisher’s exact test.

rings. In each of the wall locations, CF submucosal glands had reduced volume compared with controls (Figure 6F). When we measured dimensions of submucosal glands (12, 15), we found that the diameter of whole acini was reduced in CF submucosal glands. However, there were no significant differences in cellular or luminal subcomponents, or in lumen fraction (Table 1).

Mucus can be produced by the surface epithelium and submucosal glands (16, 30, 31). Airway gel-forming mucins include MUC5AC, which is distributed in surface goblet cells with rare submucosal gland duct staining; and MUC5B, which predominates in mucous cells of the submucosal glands and is also detected in surface epithelial goblet cells (31, 32). We examined epithelial cellular mucus by staining with periodic acid–Schiff (PAS), which preferentially stains neutral mucins (33, 34), and MUC5AC and MUC5B immunostaining (Figure 7A). In the surface epithelium (Figure 7B), PAS staining and MUC5AC and MUC5B immunostaining were similar for both genotypes. Because high-quality sections were required for morphometry, sufficient serial sections of close proximity were not available to ascertain whether MUC5AC and MUC5B staining was colocalized to the same goblet cells (Figure 7A). However, most goblet cells observed by the hematoxylin counterstain (i.e., pale cytoplasm devoid of stain), during either

TABLE 1. MORPHOMETRIC PARAMETERS OF WHOLE TRACHEA SECTIONS FROM NEONATAL PIGS

Parameter		Description	CFTR ^{+/+} (n = 10) Mean ± SEM	CFTR ^{-/-} (n = 14) Mean ± SEM	Significance
Trachea circumference		Exterior cartilage (mm)	19.45 ± 0.62	14.37 ± 0.44	<i>P</i> < 0.001
		Interior cartilage (mm)	16.68 ± 0.41	11.65 ± 0.41	<i>P</i> < 0.001
		Lumen (mm)	15.08 ± 0.37	10.04 ± 0.46	<i>P</i> < 0.001
Trachea area		Exterior cartilage (mm ²)	25.96 ± 1.58	13.89 ± 3.09	<i>P</i> < 0.001
		Interior cartilage (mm ²)	18.79 ± 0.86	8.80 ± 0.60	<i>P</i> < 0.001
		Lumen (mm ²)	14.94 ± 0.79	6.19 ± 0.59	<i>P</i> < 0.001
Trachea cartilage		Area (mm ²)	5.71 ± 0.58	4.26 ± 0.29	NS
		Area standardized to lumen circumference (μm ² /μm)	373.4 ± 29.6	423.7 ± 21.1	NS
		Maximal cartilage ring thickness (μm)	519.6 ± 74.8	548.4 ± 26.9	NS
Trachea inner wall		Area (mm ²)	3.84 ± 0.27	2.62 ± 0.11	<i>P</i> < 0.001
		Area standardized to lumen circumference (μm ² /μm)	254.9 ± 16.9	265.8 ± 12.71	NS
		Anterolateral wall diameter, mean of four samples (μm)	184.8 ± 11.2	181.2 ± 8.3	NS
Trachealis muscle		Mid-posterior wall diameter, mean of two samples (μm)	382.6 ± 24.4	557.8 ± 59.7	<i>P</i> < 0.05
		Area (mm ²)	0.50 ± 0.05	0.45 ± 0.06	NS
		Area, portion of inner wall area (%)	13.18 ± 1.26	17.23 ± 2.48	NS
Trachea mucosa		Area standardized to lumen circumference (μm ² /μm)	33.27 ± 3.53	45.88 ± 7.02	NS
		Epithelium height, lateral wall, mean of four samples (μm)	24.28 ± 0.82	25.53 ± 0.95	NS
		Cilia height, lateral wall, mean of four samples (μm)	5.41 ± 0.14	5.45 ± 0.17	NS
Submucosal gland acini		Acinus, diameter (μm)	44.35 ± 0.75	39.29 ± 1.57	<i>P</i> < 0.05
		Acinus, lumen subcomponent (μm)	12.74 ± 0.65	11.94 ± 0.61	NS
		Acinus, cellular subcomponent (μm)	31.61 ± 1.08	27.35 ± 1.53	NS
		Lumen fraction (% of acinus diameter)	28.96 ± 1.55	30.69 ± 1.72	NS

Definition of abbreviations: CFTR = cystic fibrosis transmembrane conductance regulator; NS = not significant.

MUC5AC or MUC5B immunostaining, were positive for each respective mucin, suggesting they were coexpressed in most of the surface epithelial goblet cells. Furthermore, the similar frequency of mucus staining in surface epithelium between PAS and mucins (Figure 7B) would also suggest redundancy of cellular expression by MUC5AC and MUC5B in goblet cells. In contrast, *CFTR*^{-/-} pig submucosal glands (Figure 7C) had reduced PAS staining and MUC5B immunostaining as a proportion of submucosal gland volume. These submucosal gland changes suggest that mucous cell development is reduced in *CFTR*^{-/-} pigs.

To further test whether mucous cells were reduced in *CFTR*^{-/-} submucosal glands, we immunostained for cytokeratin-18 (CK18). Submucosal glands exhibit CK18 immunostaining of ductal and serous cells, but immunostaining is absent in mucous cells and basal cells (35). Given the obvious size differences between mucous and basal cells, the majority of the submucosal gland epithelium that does not immunostain for CK18 represents mucous cell area. The proportion of submucosal gland tissue lacking CK18 staining was significantly reduced in CF pigs (Figure 7D), consistent with reduced mucous cell differentiation. To determine whether lumen volume influenced submucosal gland parameters, we assessed submucosal gland volume in CK18-immunostained sections and found that there was no difference in lumen size as a proportion of submucosal gland volume (Figure 7E).

Small, immature submucosal glands might be due to reduced growth. Therefore, we immunostained tissues for the proliferation marker Ki-67. In the surface epithelium (Figure 7F), CF and control groups showed similar frequencies of Ki-67 staining, whereas CF pig submucosal glands (Figure 7G) had reduced Ki-67 staining compared with non-CF littermates.

Gene Set Enrichment Analysis

As a way to further test the hypothesis that porcine CF trachea has altered amounts of submucosal glands, airway smooth muscle, and/or cartilage, we used data from our microarray analysis of porcine tracheal transcripts (19). To determine whether transcripts from each of these parts of the trachea

showed a global increase or decrease in CF, we used gene set enrichment analysis (GSEA) (25, 26). As a control, we also analyzed transcripts from surface epithelium, which did not appear to vary by genotype in our histopathological studies. For the test, we generated custom gene sets for surface epithelia and submucosal glands, using data from a microarray analysis of transcripts expressed in these human tissues (27). Analysis of variance was used to identify genes differentially expressed in the two tissues. This approach generated unbiased gene sets for surface epithelia and submucosal glands (Table E1 and Table E2, respectively). To generate gene sets for airway smooth muscle and cartilage, we compiled a list of genes known to be expressed in those tissues (Table E3 and Table E4, respectively).

GSEA showed no difference between the two genotypes in the global gene expression profile for surface epithelia or cartilage (Table 2). In contrast, CF trachea showed a reduced submucosal gland global transcript profile and an increased airway smooth muscle transcript profile. The difference between CF and non-CF submucosal gland data could be explained by a reduced number of submucosal gland cells, each with a normal expression profile. This interpretation would be consistent with our finding of decreased submucosal gland area in CF trachea. However, these data cannot exclude the possibility of a normal number of submucosal gland cells, each with decreased expression of submucosal gland transcripts. A similar line of reasoning applies to the data from CF airway smooth muscle, which is consistent with our histopathological findings suggesting increased airway smooth muscle in CF.

Trachea and Airway Abnormalities in Human CF

The data from *CFTR*^{-/-} pig trachea raised questions concerning whether similar features were present in young children with CF. To test this, we retrospectively examined chest CT scans from non-CF children and children with CF, 2 years of age and younger (Table E5), to evaluate the trachea. We found that the lumen area was not significantly different between non-CF and CF epithelia (Figure 8A). However, CF tracheas were distinguished from controls by having less circular walls in cross-

Figure 5. Trachea and bronchus wall from non-CF ($CFTR^{+/+}$) and CF ($CFTR^{-/-}$) neonatal pigs. Tracheal (top) and bronchial (bottom) smooth muscle (asterisks) was often accentuated as distinctive bundles (arrows). The CF panels represent a severe case, Masson's trichrome and hematoxylin–eosin stains. Scale bars: Top: 231 μm ; bottom: 116 μm .

sections (Figures 8B and 8C), similar to that seen in neonatal CF pigs (see also Figures 1A and 1E).

DISCUSSION

We observed significant decreases in size and circularity of trachea lumens and in submucosal glands, and increases in smooth muscle, in newborn CF trachea. In addition, we found evidence of a similar decrease in tracheal circularity in young children with CF. These observations raise interesting questions about how the absence of CFTR during development might cause these changes. They also raise questions about how these changes might influence the pathogenesis of CF lung disease.

Tracheal Caliber Is Reduced in $CFTR^{-/-}$ Pigs and Human Infants with CF

The trachea of neonatal $CFTR^{-/-}$ pigs showed a consistent reduction in caliber. Interestingly, there is a previous report that $CFTR^{-/-}$ and $CFTR\Delta F508/\Delta F508$ mice have tracheal narrowing and a high incidence of disrupted or incomplete tracheal rings (36). But have related abnormalities been seen in humans? One study of adult patients with CF with substantial lung disease found that tracheal lumen area did not differ from non-CF control subjects on maximal inspiration, but did differ with cough or forced expiration; the difference was attributed to tracheomalacia in CF (37). In another study, high-resolution CT

Figure 6. Submucosal gland morphometry in non-CF ($CFTR^{+/+}$) and CF ($CFTR^{-/-}$) neonatal pig littermates. (A and B) CF tracheas ($n = 10$) had a reduced number of submucosal gland ducts (A) in whole trachea cross-sections compared with non-CF ($n = 10$) tracheas, but had similar frequency (B) when normalized to trachea lumen circumference ($P < 0.01$ and not significant [NS], respectively, Mann-Whitney test). (C–E) Submucosal gland tissue area was reduced in CF ($n = 14$) versus non-CF ($n = 10$) whole trachea sections (C) and also when normalized to inner wall area (D) or lumen circumference (E) ($P < 0.001$, $P < 0.05$, and $P < 0.05$, respectively, Mann-Whitney test). (F) Posterior (P), anterior (A), and lateral (L) wall of trachea from non-CF ($n = 9$) and CF ($n = 13$) neonatal pigs. Representative images were consistently collected along the respective walls and the submucosal gland (SMG) area and normalized to the length of the lumen–wall interface. Submucosal glands had similar preferential distribution in both genotypes; however, CF tracheas had reduced submucosal gland area in each wall segment ($P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively, Mann-Whitney test). Columns and error bars represent means $\pm$ SEM.

imaging of 8- to 33-month-old children with CF found a reduced size of CF airway lumens, but thickened airway walls or mucus lining the airways was speculated to be the cause of the reduction (5).

To our knowledge, a reduced tracheal diameter in infants with CF has not previously been reported. We therefore examined an article by Sturgess and Imrie (15) that presented morphometric data from individual CF and non-CF infant tracheas, but the authors determined there was no difference in trachea lumen area based on estimated postconceptional age. Because fetal and perinatal trachea growth is correlated with developmental age and body length (38), we used these parameters for normalization of data. We examined data only from infants with a postnatal age of less than 2 weeks, which provided a sample as close as possible to the newborn $CFTR^{+/+}$ and $CFTR^{-/-}$ pig littermates we studied (Table 3). Despite being slightly older and larger, the average tracheal lumen was 21% smaller ($P < 0.05$) in infants with CF, and 24% smaller ($P < 0.05$) when normalized to body length. This finding further supports the conclusion that the reduced tracheal caliber in $CFTR^{-/-}$ pigs also occurs in young infants with CF.

Figure 7. Histochemical and immunohistochemical staining and morphometry of tracheal tissues in neonatal pigs. (A) MUC5AC and MUC5B immunostaining of CF and non-CF trachea; scale bar, 58 μ m. (B and C) Periodic acid–Schiff (PAS), MUC5AC, and MUC5B staining in trachea from non-CF ($CFTR^{+/+}$, n = 9, 6, and 6, respectively) and CF ($CFTR^{-/-}$, n = 13, 9, and 9, respectively) neonatal pigs was similar in surface epithelium (SE) (B), but in submucosal glands (SMG) (C) both PAS and MUC5B staining were reduced as a percentage of total submucosal gland area ($P < 0.01$ and $P < 0.05$, respectively, all others not significant [NS], Mann-Whitney test; columns and error bars, means $\pm$ SEM). (D and E) Cytokeratin-18 (CK18) staining and lumen area in submucosal glands from non-CF ($CFTR^{+/+}$, n = 10) and CF ($CFTR^{-/-}$, n = 12) neonatal pigs. CF pigs had reduced CK18 staining (D), but submucosal gland lumen area (E) was similar as a percentage of total submucosal gland area ($P < 0.05$ and NS, Mann-Whitney test; columns and error bars, means $\pm$ SEM). (F and G) Ki-67 immunohistochemical staining of trachea in non-CF ($CFTR^{+/+}$, n = 16) and CF ($CFTR^{-/-}$, n = 18) neonatal pigs was similar in the surface epithelium (SE) (F), but stained nuclei were significantly decreased in the submucosal glands (SMG) (G) as a percentage of total nuclei, (NS and $P < 0.05$, respectively, Mann-Whitney test; columns and error bars, means $\pm$ SEM).

The mechanisms responsible for the reduced tracheal area are not certain, but our study offers some insight. First, the lack of inflammation and infection in the airways of newborn CF pigs indicate that these factors are not responsible for the changes and further suggest that the changes are attributable to the loss

TABLE 2. GENE SET ENRICHMENT ANALYSIS—NORMALIZED ENRICHMENT SCORES FOR $CFTR^{-/-}$ VERSUS $CFTR^{+/+}$ TRACHEA

Tissue	Enrichment Score	FDR q Value
Surface epithelium	1.02	0.53
Cartilage	0.78	0.74
Submucosal gland	−1.37	0.06*
Airway smooth muscle	1.27	0.18*

Definition of abbreviation: FDR = false discovery rate.
A positive enrichment score indicates an increase in the global expression profile in cystic fibrosis, and a negative score indicates the converse.
* An FDR q value less than 0.25 was considered statistically significant.

of CFTR. Second, the absence of CFTR can reduce submucosal gland secretion (11, 39, 40), which might produce a more viscous mucus, and perhaps that somehow prevents airway growth and expansion (7). This possibility seems unlikely because newborn CF pigs do not demonstrate airway mucus accumulation (17, 19). Third, cartilage abnormalities, especially along the anterior border, might be responsible and have also been detected in CF mice (36). However, the absence of a difference in the cross-sectional area of CF and non-CF tracheal cartilage, the lack of differences in cartilage-associated transcripts in the gene set enrichment analysis, and the absence of abnormalities in other tissues supported by hyaline cartilage (e.g., ears) suggest that the cartilage abnormalities might be a secondary versus primary response. Fourth, absence of CFTR might alter contraction of airway smooth muscle, producing a hypercontractive state and persistent reduction in airway caliber. The selective enrichment of smooth muscle gene set profiles, smooth muscle bundle remodeling, and the trend toward increased smooth muscle volume would support this interpretation.

Smooth Muscle Shows Remodeling in $CFTR^{-/-}$ Pigs

How might smooth muscle be influenced by the loss of CFTR during development? CFTR has been localized to smooth muscle and activation of the CFTR channel in rat trachealis muscle results in airway dilation (41). In the fetus, airway smooth muscle is active, producing peristalsis-like contractions of airways (42). In the absence of CFTR, fetal smooth muscle might produce (1) dysregulated peristalsis of airways similar to that seen in the $CFTR^{-/-}$ mouse intestine (43), and/or (2) a hypercontracted state (44). Increased contraction of the trachealis muscle might decrease tracheal caliber and simultaneously increase biomechanical stress on the cartilage rings and airway wall, which with sufficient stimulus could promote tissue remodeling.

Interestingly, we observed significant enrichment for the smooth muscle gene set in the CF pig trachea. This finding suggests that the observed trend toward an increase in tracheal smooth muscle volume in the CF pig trachea might be associated with changes in gene expression in the developing airway smooth muscle.

Altered smooth muscle has also been reported in patients with CF (5, 45, 46) and in CF mice (36, 47). In $CFTR^{-/-}$ mice, altered smooth muscle contraction is restricted to the site of narrowing in the proximal trachea (36), suggesting they are functionally related. We can also compare the $CFTR^{-/-}$ pig with mouse models of TMEM16A, a Ca^{2+} -activated Cl^{-} channel (48–50). Mice deficient for TMEM16A have 100% mortality by 1 month of age because of developmental tracheal lesions resembling tracheomalacia (20). Fetal mice deficient for TMEM16A lack the expected transverse orientation of the trachealis muscle, which is replaced by clusters of smooth muscle (20). These circular clusters of smooth muscle are

Figure 8. Tracheal morphometry from computed tomography (CT) images of trachea of children with CF ($n = 16$; mean age, 1.7 yr) and non-CF children ($n = 6$; mean age, 1.3 yr) 2 years of age and younger. (A) Trachea lumen area was not significantly different between groups. (B) CF tracheas were less circular than non-CF controls ($P < 0.05$, Mann-Whitney test; horizontal bars, means). (C) Representative CT scans of tracheas (arrows) of a non-CF infant and an infant with CF (0.6 and 0.8 yr, respectively) demonstrated less circular structure of the CF trachea.

reminiscent of the increased oblique to longitudinal bundles seen in cross-sections of $CFTR^{-/-}$ pig airways. The commonality of smooth muscle changes in CF pigs, mice, and humans and in $TMEM16A^{-/-}$ mice could indicate that (1) Cl^{-} channels play an important physiologic role in smooth muscle activity during normal airway development; (2) smooth muscle is a common target tissue for Cl^{-} channels during development; and (3) altered smooth muscle function may contribute to trachea narrowing.

Submucosal Gland Volume Is Reduced in $CFTR^{-/-}$ Pigs

We found that neonatal $CFTR^{-/-}$ pigs had significant submucosal gland changes compared with controls. Submucosal glands develop when buds of undifferentiated epithelium invade the airway submucosa (51, 52). Mucous cells first differentiate in the proximal gland, but with gland expansion and maturation, mucous cells and then serous cells are increasingly detected distally (52). In the current study, we observed altered immunostaining patterns of MUC5B, PAS, and CK18 in CF pig submucosal glands, suggesting reduced mucous cell differentiation. This, along with decreased submucosal gland volume and a reduced proliferation rate, are consistent with immature, hypoplastic submucosal glands. In addition, the decreased expression of submucosal gland-associated genes in CF trachea supports the idea that a developmental program governing gland development is altered in the airways of CF pigs. This conclusion is strengthened by the observation of a lack of significant difference in the GSEA analysis for transcripts enriched in surface airway epithelia.

We were surprised to find hypoplastic submucosal glands in neonatal CF pigs. Decades ago, pathology studies on the tracheas of infants with CF suggested that the submucosal glands were normal with no mention of hypoplasia. However, all those studies were performed on patients who ranged in age from a few days to many months (12–16, 53). We speculate that if hypoplastic submucosal glands were present at birth in infants with CF, sufficient postnatal stimuli might have quickly returned submucosal glands to “normal” size before pathologic examination. Consistent with this speculation, previous studies have shown that daily isoprenaline administration in both rats and pigs produced significant expansion in submucosal gland volume within 1 week, indicating the potential for dynamic regulation of submucosal gland size (54, 55). Thus, whether submucosal glands in humans with CF are “normal” or hypoplastic at birth remains an open question.

Implications for Humans with CF and for Understanding CF Pathogenesis

Finding a reduced tracheal caliber, abnormal airway smooth muscle, and hypoplastic submucosal glands in newborn $CFTR^{-/-}$ pigs may have implications for humans with CF. In considering relationships between humans with CF and CF pigs, we note that the CF pigs carry a *null* allele, whereas most humans with CF have at least one $\Delta F508$ allele. Whether this difference influences our speculations remains unknown.

Airflow obstruction is an early clinical finding in patients with CF (6, 16). We speculate that the reduced caliber of CF airways might contribute to this clinical observation in patients.

TABLE 3. INFANT TRACHEA MEASUREMENTS FROM PUBLISHED DATA

Group	Gestational Age (wk)	Postnatal Age (d)	Body Length (cm)	Airway Lumen (mm ²)	Lumen Area/Body Length
Non-CF					
Mean	36.8	3.1	46.8	9.7	0.21
SEM	1.0	0.8	1.1	0.6	0.01
n	15	15	15	15	15
CF					
Mean	38.1	7.6	49.1	7.6	0.16
SEM	1.1	1.0	1.5	0.7	0.02
n	7	7	7	7	7
P value	0.414	0.003*	0.244	0.035*	0.023*

Data from Reference 15.

* All children less than 2 weeks postnatal age with trachea lumen area and body length measurements. Data were assessed by Mann-Whitney test and significance determined at $P < 0.05$.

In addition, small-caliber airways might influence progression of CF respiratory disease in postnatal life. It is also interesting to speculate about whether the observed structural changes in the trachea (i.e., reduced area or circularity) functionally overlap with or contribute to the diagnostic features of tracheomalacia, which is increasingly recognized in patients with CF (37).

Through its contractile properties, airway smooth muscle serves as a regulator of airway size (56). Patients with CF have increased smooth muscle mass detected on airway biopsies (45, 46, 57). Furthermore, approximately 40–60% of patients with CF have increased bronchial reactivity (58). Altered smooth muscle in patients with CF may contribute to airway reactivity that exacerbates clinical disease (45, 59), but it may also regulate mediators of remodeling and inflammation, as suggested to occur in patients with asthma (60).

Submucosal glands are a major source of airway secretions (61) and CFTR has a key role in this process (9, 62, 63). CFTR-mediated submucosal gland secretions are an important source for antimicrobials and for fluid and mucus critical for effective mucociliary clearance (9, 27, 63, 64). Because these secretions contribute to multiple host defense mechanisms, we speculate that hypoplastic submucosal glands might increase susceptibility to infection. This is consistent with our observation that CF pigs have a host defense defect at birth with a reduced ability to eradicate bacteria (19).

The trachea abnormalities we detected in neonatal *CFTR*^{−/−} pigs and young patients with CF are surprising aspects of early CF disease. Studies in this porcine model may be useful for defining the causes of these changes *in utero*, and for determining how they ultimately affect the pathogenesis of CF lung disease.

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