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Molecular Pathogenesis of Genetic and Inherited Diseases

Pathology of Gastrointestinal Organs in a Porcine Model of Cystic Fibrosis

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Cystic fibrosis (CF), which is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), is characterized by multiorgan pathology that begins early in life. To better understand the initial stages of disease, we studied the gastrointestinal pathology of *CFTR*$^{-/-}$ pigs. By studying newborns, we avoided secondary changes attributable to environmental interactions, infection, or disease progression. Lesions resembling those in humans with CF were detected in intestine, pancreas, liver, gallbladder, and cystic duct. These organs had four common features. First, disease was accelerated compared with that in humans, which could provide a strategy to discover modifying factors. Second, affected organs showed variable hyperplastic, metaplastic, and connective tissue changes, indicating that remodeling was a dynamic component of fetal life. Third, cellular inflammation was often mild to moderate and not always present, which raises new questions as to the role of cellular inflammation in early disease pathogenesis. Fourth, epithelial mucus-producing cells were often increased, producing a striking accumulation of mucus with a layered appearance and resilient structure. Thus, mucus cell hyperplasia and mucus accumulation play prominent roles in early disease. Our findings also have implications for CF lung disease, and they lay the foundation for a better understanding of CF pathogenesis. *(Am J Pathol 2010, 176:1377–1389; DOI: 10.2353/ajpath.2010.090849)*

Early in the twentieth century, pioneering researchers established a framework for understanding the pathology of cystic fibrosis (CF). In 1905, Landsteiner described meconium ileus associated with pancreatic disease in a newborn.[1] By 1938, Andersen documented the common pancreatic pathology in a cohort of CF patients and applied the term "cystic fibrosis of the pancreas" in her description of 49 pediatric cases.[2] Later, Farber recognized significant involvement of multiple organs by a tenacious, thick mucus, which led to his descriptive terminology of "mucoviscidosis."[3,4] Almost a decade later, Bodian published a seminal record of young pediatric CF disease with detailed medical history and images that arguably have not been equaled since.[5] At that time, CF was a pediatric disease with a high mortality, and the study of pathological changes in CF tissues from young infants and children[5–8] generated hypotheses about pathogenesis and therapies. Fortunately, that era has passed with advances in medical management that have improved median lifespan to more than 37 years.[9,10] Hence, early lesions are now rarely accessible for pathological examination and study.

It has been twenty years since the gene responsible for CF was identified and more than fifteen years since the first cystic fibrosis transmembrane conductance regulator (*CFTR*$^{-/-}$) mouse models were developed.[11,12] Although much progress has been made,[9,13,14] there remains a lack of understanding regarding the pathogenic events that orchestrate disease in affected organs. To provide a model in which to investigate CF, we recently developed *CFTR*$^{-/-}$ pigs and found that they display gastrointestinal lesions consistent with CF disease.[15,16] These pigs provide several advantages for pathology studies and avoid limitations of human studies. We are able to study newborn *CFTR*$^{-/-}$ pigs before they develop secondary changes attributable to interactions with their

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environment and infection. We can also avoid changes attributable to severe terminal disease and postmortem alterations. In addition, pathological data from humans can be skewed by many factors, including toward the most severe cases that come to autopsy, whereas with newborn pigs we have the opportunity to assess the spectrum of disease at one point in time. Thus, the goal of this study was to assess the gastrointestinal pathology in newborn $CFTR^{-/-}$ pigs to provide insight into the early changes of the disease.

Materials and Methods

Animals

All studies were approved by the University of Iowa Animal Care and Use Committee. Neonatal pigs (eight to twenty-four hours old) were used for the study to minimize environmental influences on tissues. Littermates from $CFTR^{+/-}$ matings were obtained from Exemplar Genetics (Sioux Center, IA). Piglets included $CFTR^{+/+}$ ($n = 12$), $CFTR^{+/-}$ ($n = 12$), and $CFTR^{-/-}$ ($n = 26$) genotypes. For determining the frequency of fetal perforation/peritonitis, we examined the necropsy records of the 26 $CFTR^{-/-}$ pigs along with the necropsy records of six additional $CFTR^{-/-}$ pigs whose tissues were not available for subsequent histopathology.

Tissues and Pathology Examination

After euthanasia (Euthasol, Virbac, Fort Worth, TX), all tissues were collected and immersed in fixative for 48 to 96 hours depending on tissue size, routinely processed, embedded, sectioned (4 micrometers), and stained with hematoxylin and eosin. Some sections were further stained with periodic acid-Schiff (PAS) stain, which detects neutral mucins, Alcian Blue/Pyronine Y (ABPY) stain, which detects acidic mucins, or Masson trichrome stain for identification of collagen deposition.[16–18] All tissues were examined by a pathologist (D.K.M.) familiar with the species and CF. Human CF tissues samples were identified from autopsy archival material in the Department of Pathology, University of Iowa.

Morphometry

After histopathological examination, high-resolution digital images were collected (DP71 digital camera and BX41 microscope, Olympus, Center Valley, PA) and these images were morphometrically analyzed with MicroSuite Pathology Edition software (Olympus, Center Valley, PA). Thickness of the intestinal smooth muscle layer (ie, tunica muscularis) was consistently defined as the minimal diameter of the layer (to avoid sectioning artifacts) in tissue cross-sections and reported in micrometers. Pancreas parenchyma was assessed by low magnification examination, and the lobular area of pancreatic parenchyma (containing a mixture of exocrine with endocrine tissue) was defined, area quantified, and then reflected as a percentage of the total pancreatic area. For gallbladder diameter assessment, all sections were consistently sectioned in the same plane at the widest portion of the intact gallbladder. To account for "collapsed" gallbladders postsectioning, and to provide consistent/reproducible measurements, the histopathological circumference of the gallbladder lumen was enumerated by software analysis and the diameter calculated (diameter = circumference/π).

Statistics

Analysis for genotypic ($CFTR^{+/+}$, $CFTR^{+/-}$, and $CFTR^{-/-}$) differences was performed using one-way analysis of variance, and intergroup post tests were made with a Bonferroni multiple comparison test. Statistical analysis was performed using Prism software (Graphpad, La Jolla, CA).

Results

Intestinal Disease

Meconium Ileus Obstruction

Meconium ileus involves an obstruction of the intestine that occurs in about 15% of CF neonates and may be detected as early as 17 weeks gestation.[19–22] In all neonatal $CFTR^{-/-}$ pigs, meconium ileus was present (Figure 1, A and B). The site of obstruction ranged from the distal jejunum to the proximal spiral colon (Figure 1, A–C), an elongated and coiled equivalent to the human ascending colon. This distribution is similar to that reported in humans with meconium ileus.[23] In $CFTR^{-/-}$ pigs, the site of obstruction consisted of a dilated meconium-filled proximal bowel segment, a transition zone (abrupt to gradual), and a distal bowel that was diffusely small in caliber (Figure 1A) and variably filled with white to gray-green firm cords of mucus. The small caliber distal bowel was firm and lacked pliability compared with normal bowel and may have contributed to the intestinal obstruction. In addition, $CFTR^{-/-}$ meconium was sticky and adherent to the intestinal wall, and it may have contributed to the formation of the obstruction.

Inflammation was not observed in $CFTR^{-/-}$ intestine compared with controls, except as a secondary feature near sites of perforation and necrosis (Figure 1D); there, neutrophils were the predominant leukocytes in acute lesions. Studies of human CF have reported intestinal perforation, peritonitis, and/or volvulus as *in utero* and postnatal complications of meconium ileus.[24,25] Likewise in $CFTR^{-/-}$ pigs, we observed intestinal perforation and peritonitis that occurred in the fetal and early postnatal period usually in severely dilated small intestine. Fetal perforation was uncommon (2 of 32, 6%) and when present was characterized by fibrin, adhesions, exudative fluid filling the abdomen, and granulomatous peritonitis centered on meconium material.

The extent of mucus production depended on the intestinal location. In the spiral colon distal to the obstruction, the lumen contained mucocellular accumulations (Figure 1E), and the adjacent colonic glands were often

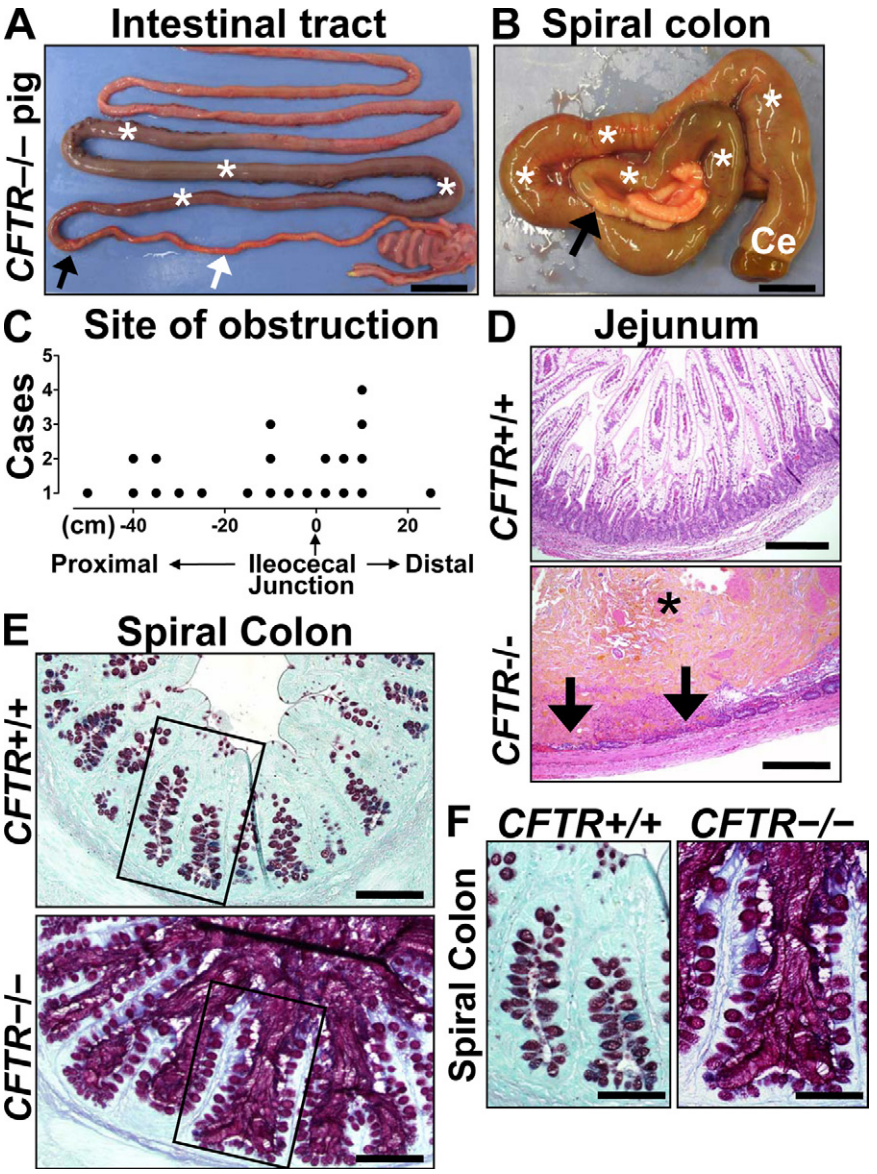

Figure 1. Meconium ileus in *CFTR*$^{-/-}$ intestine. **A:** Meconium-filled bowel (**asterisks**) was proximal to the obstruction interface (**black arrow**); distal to the interface, the bowel was hypoplastic (**white arrow**). Scale bar = 2.45 cm. **B:** Meconium (**white asterisks**) dilated the spiral colon (ventral view) and cecum (Ce) proximal to the obstruction interface (**black arrow**). Microcolon appeared distally. Scale bar = 1.34 cm. **C:** Sites of obstruction in *CFTR*$^{-/-}$ pigs were distributed on either side of the ileocecal junction extending from the small intestine (proximal) into the spiral colon (distal). **D:** *CFTR*$^{-/-}$ jejunum showing luminal meconium (**asterisk**) causing severe distension and mucosal thinning with focal hemorrhage, necrosis (**arrows**), and neutrophilic inflammation, HE stain. Scale bars = 0.29 mm. **E:** *CFTR*$^{-/-}$ spiral colon had luminal mucus accumulation with hyperplastic mucus-producing cells especially noticeable compared with *CFTR*$^{+/+}$ along the surface epithelium (**inset** boxes, see **F**). ABPY stain. Scale bars = 117 μm. **F:** *CFTR*$^{-/-}$ spiral colon glands were distended by stringy mucus that extends from stout mucus-producing cells in the epithelium, ABPY stain. Scale bars = 69 μm.

distended by stringy mucus (Figure 1, E and F). The *CFTR*$^{-/-}$ colonic epithelium showed mild to moderate mucinous hyperplasia, especially toward the surface epithelium, and the cells were relatively stout and distended by mucus (Figure 1F). The presence of luminal mucus and epithelial mucinous change in the colon did not appear to depend on the proximity to the site of intestinal obstruction. The histochemical staining quality of the *CFTR*$^{-/-}$ mucus often showed enhanced production of sulfated mucus (Figure 1, E and F; red coloration) similar to that reported in human CF.[26] The *CFTR*$^{-/-}$ colon with mucinous hyperplasia lacked detectable inflammation.

In the proximal small intestine, focal thin strands of eosinophilic mucus were uncommonly detected within the crypts and between villi with some minor focal luminal accumulation. Mucinous hyperplasia in the small intestine proximal to the obstruction was lacking to focal and mild. Brünner glands of the duodenum were focally ectatic, filled by wispy mucus, and had a thin epithelial border (Figure 2A). In the small intestine distal to the obstruction, luminal mucus was more readily detected and combined with sloughed cells in a layered fashion to form mucocellular cords (pellets; Figure 2B). The presence of these cords did not appear to be dependent on the proximity to the obstruction. Patches of meconium were occasionally intermixed with these mucocellular cords, which sometimes dislodged from the intestinal wall to accumulate distally, especially in the hours after *CFTR*$^{-/-}$ pigs consumed their first meal. The morphological appearance of these dislodged cords (Figure 2B, right panel) was similar to their appearance before dislodgement (Figure 2B, left panel), suggesting resilience in physical structure. The proximal spiral colon was a common site of accumulation for dislodged cords, which in some cases formed large luminal masses (Figure 2, C and D). These large aggregates occasionally caused severe distension, which led to necrosis and perforation in *CFTR*$^{-/-}$ pigs. Although the small intestine is the most

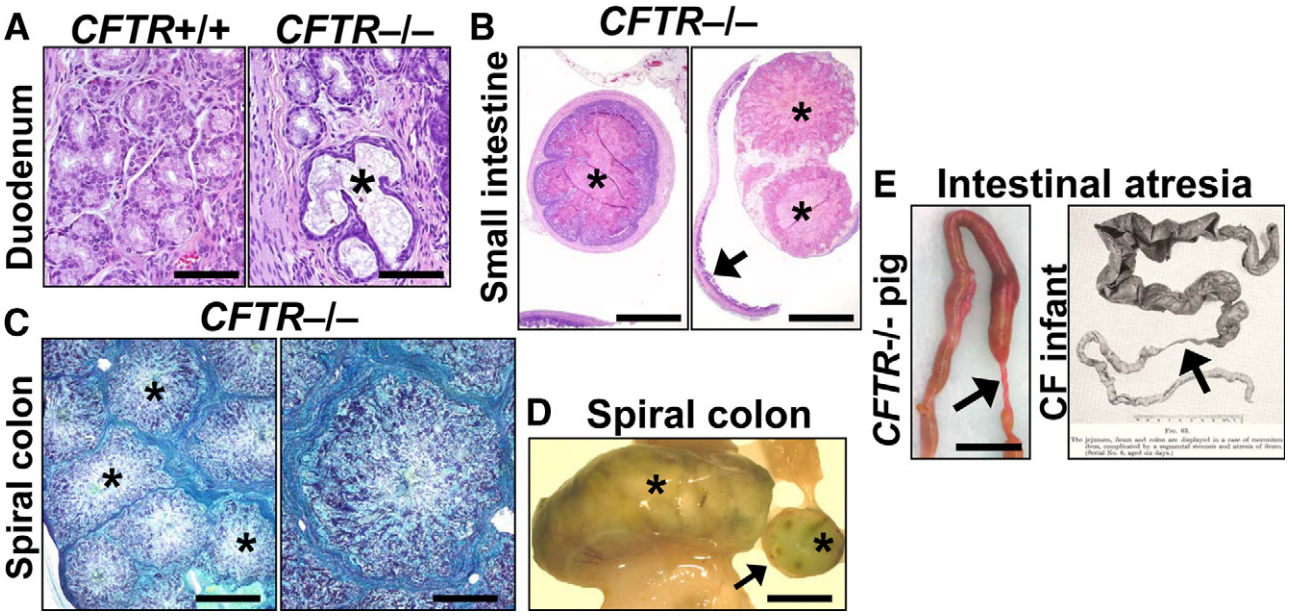

Figure 2. Mucus accumulation and atresia in $CFTR^{-/-}$ intestine. **A:** Duodenal Brünner glands were focally distended by stringy mucus (**asterisk**) with flattening of adjacent epithelium. HE stain. Scale bars = 80 μm. **B:** Distal to the obstruction interface, mucocellular cords (**asterisks**) filled the $CFTR^{-/-}$ intestinal lumen (**left panel**) and sometimes became dislodged from the intestinal wall to aggregate (**right panel**) distally in the bowel (**arrow**). HE stain. Scale bars = 1.2 mm. **C:** Cross section of a distended $CFTR^{-/-}$ spiral colon filled with large aggregates of mucocellular cords (**asterisks**) that retained their morphology from the small intestine (see also **B**, **left panel**). ABPY stain. Scale bars = 1.7 mm (**left panel**) and 0.7 mm (**right panel**). **D:** Aggregates of mucocellular cords (**asterisks**) distended a loop of $CFTR^{-/-}$ spiral colon and were detectable in cross section (**arrow**). Scale bar = 11.1 mm. **E:** Segmental atresia (**arrows**) was present distal to the obstruction interface in the $CFTR^{-/-}$ pig (Scale bar = 11.5 mm) and is similar to what Bodian described in CF infants.[5] Reprinted from Fibrocystic Disease of the Pancreas: a Congenital Disorder of Mucus Production-Mucosis, Martin Bodian, Page 84, Copyright 1953.

common site of perforation in CF infants (and $CFTR^{-/-}$ pigs), colonic perforations have been reported to occur in children due to numerous cords filling the colon and causing direct irritation and injury.[27]

We observed no difference between $CFTR^{+/+}$ and $CFTR^{+/-}$ animals at any site along the intestinal tract or in any of the other organs we describe below. We also observed no differences between lesions in male and female piglets.

Additional Intestinal Lesions

CF infants are prone to intestinal atresia with a >200-fold increased risk compared with the general Caucasian population.[23,28] Atresias were also detected in $CFTR^{-/-}$ pigs (Figure 2E, left panel). Stenotic and atretic segments of bowel were commonly detected distal to the site of meconium obstruction. Most $CFTR^{-/-}$ pig atresias were small gauged (less than 2 mm outer diameter), continuous with adjacent bowel and had rare serosal twisting. The atresia formation was similar to that described for CF infants (Figure 2E, right panel).[5,29]

The mechanisms that produce atresia remain uncertain. Atresias might result from *in utero* vascular compromise caused by lesions such as volvulus.[21,29,30] Although we did not observe overt volvulus in the $CFTR^{-/-}$ pigs, it is possible that obstruction predisposed to secondary vascular compromise, manifested as serosal twisting at birth. It is also possible that intestine distal to the site of obstruction failed to develop normally because of a lack of mechanical distention by luminal contents or because obstruction prevented distal delivery of luminal contents that were required for intestinal growth.[31]

Diverticulosis has been reported in the appendix and colon of CF patients,[32,33] and George[33] found that CF patients had a 7- to 14-fold increased incidence of diverticulosis of the vermiform appendix (median age ≈13 years, including one infant). Histologically, the lesions were classified as acquired pulsion or pressure type diverticula. The pathogenesis was proposed to result from increased pressure/distension by luminal material and concurrent smooth muscle hypertrophy. Increased intraluminal pressure may then lead to herniation of the mucosa through the weakest part of the muscular wall at sites of vascular penetration along the mesenteric border.[33,34] Diverticulosis was a common intestinal lesion in $CFTR^{-/-}$ pigs. It appeared as saccular bulges along the mesenteric border of dilated bowel (Figure 3A). Diverticulosis usually occurred in the meconium-filled jejunum when there was severe obstruction. Histologically, intestinal diverticula were composed of tunica mucosa and submucosa that were herniated through a hypertrophic tunica muscularis (Figure 3A, right panel). In fact, the tunica muscularis was hypertrophied throughout the intestinal tract including the duodenum and the spiral colon (Figure 3A, right panel, and Figure 3B). In pathology studies of CF infants, the tunica muscularis has also been described as "well-formed" to "hypertrophic."[5,8]

A review of the literature did not identify reports of jejunoileal diverticula (excluding Meckel's diverticula) in CF. Although this may reflect the true clinical situation, some have suggested the overall incidence of diverticula may be under-represented because inci-

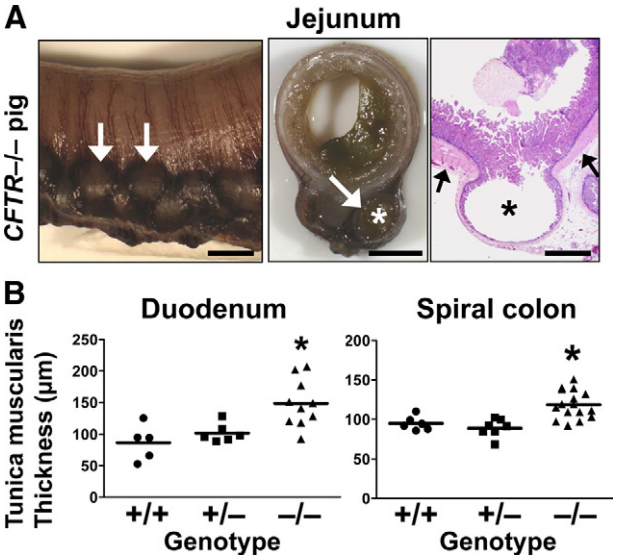

Figure 3. Diverticuli and smooth muscle thickening in $CFTR^{-/-}$ intestine. **A:** Diverticula (**asterisks** and **white arrows**) formed along the mesenteric border and were lined by a thin mucosa and submucosa, which herniated through a thickened tunica muscularis (**black arrows**). Scale bars = 3.8 mm (**left** and **middle panel**) and 1.4 mm (**right panel**, HE stain). **B:** Tunica muscularis of the $CFTR^{-/-}$ pig intestine was generally hypertrophied, including in the duodenum (**left**, $P < 0.01$ versus $CFTR^{+/+}$, $P < 0.05$, versus $CFTR^{+/-}$) and the spiral colon (**right**, $P < 0.01$ versus $CFTR^{+/+}$, $P < 0.001$ versus $CFTR^{+/-}$, one-way analysis of variance with Bonferroni post test).

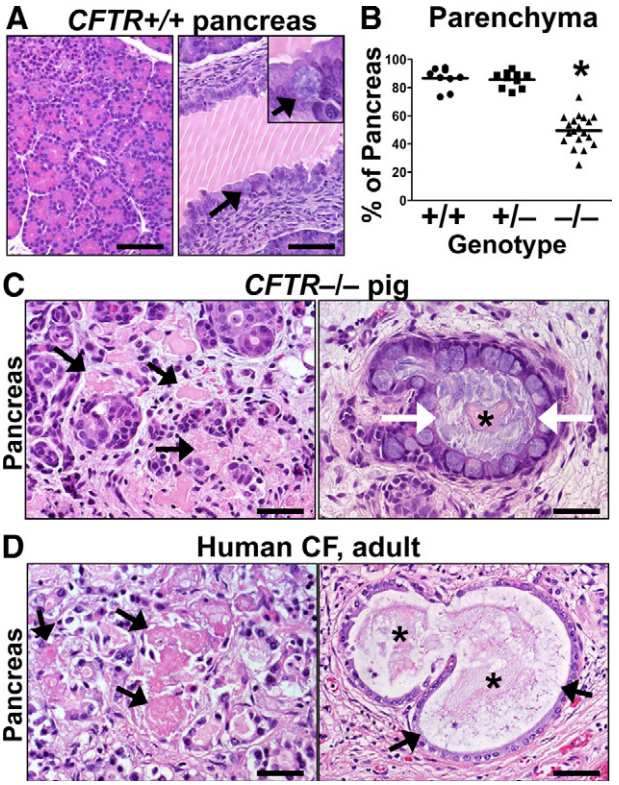

Figure 4. Exocrine pancreatic destruction in $CFTR^{-/-}$ pigs. **A:** $CFTR^{+/+}$ pig pancreas was rich in exocrine tissue (**left panel**) with large ducts (**right panel**) that were partially filled with normal zymogen secretions (white lines are sectioning artifact). Mucus cells were uncommon in the epithelium (**arrows** and **inset**). HE stain. Scale bars = 72 μm. **B:** $CFTR^{-/-}$ pancreata had reduced lobular parenchyma ($P < 0.001$ vs. $CFTR^{+/+}$ and $CFTR^{+/-}$, one-way analysis of variance with Bonferroni post test). Scale bar = mean. **C:** Degenerative pools of eosinophilic zymogen secretions were detected "free" within the interstitium (**black arrows**). Within dilated ducts, centrally oriented zymogen secretions (**asterisk**) were often surrounded by stringy mucus (**white arrows**). HE stain. Scale bars = 37 μm. **D:** Adult human CF pancreas (with autolysis making some features less appreciable) showed similar degenerative pools of interstitial zymogen secretions (**arrows**, **left panel**) and similar secretions (**asterisk**) were detected in dilated ducts surrounded by mucus (**arrows**, **right panel**). HE stain. Scale bars = 35 μm and 53 μm (**left** and **right panel**, respectively).

dental identification often requires high vigilance during examination.[33,34] In people without CF, jejunoileal diverticula are typically observed in elderly patients.[34] Pigs have been reported to have smooth muscle hypertrophy and diverticulosis of the small intestine as incidental findings at necropsy and rarely as a cause of perforation. A genetic predisposition has also been speculated for some pig breeds.[35] Consistent with the proposed pathogenesis in humans, diverticula in $CFTR^{-/-}$ pigs may have occurred in the dilated meconium filled bowel because of the combined increased intraluminal pressure and hypertrophic smooth muscle during fetal life.

Pancreatic Disease

The pancreas is affected in ≈85% to 90% of CF patients,[36,37] and pancreatic lesions have been reported in neonates and fetuses as young as seventeen weeks gestation. These lesions consist of luminal dilation of acini/ducts by eosinophilic zymogen material. With increased luminal material there is progressive thinning of the lining epithelium, and mucus metaplasia may be detected in the larger ducts.[20,38,39] In fact, the classic lesion of the CF pancreas consists of dilated ("cystic") ducts filled by stringy zymogen material and mucus.[2,4,5,40] In neonatal $CFTR^{+/+}$ pig pancreas, the exocrine tissue contained zymogen-rich acini (Figure 4A), and on occasion, we observed ducts filled with normal appearing zymogen secretions. Mucus cells were typically restricted to large ducts (Figure 4A), and small submucosal glands were noted in the terminal duct near the duodenum.

In $CFTR^{-/-}$ pigs, pancreatic lesions were ubiquitous and there was a range in severity of exocrine tissue destruction (Figure 4B). Endocrine tissue was spared from the destruction and appeared morphologically intact.[16] Acinar cells were reduced in number and had decreased amounts of cytoplasmic zymogen granules. Zymogen secretions often filled the acinar and ductular lumens of $CFTR^{-/-}$ pancreata (Figure 4C). In some ducts, stringy zymogen material was surrounded by mild to moderate amounts of mucus (Figure 4C). With increased filling of acinar and ductal lumens by altered zymogen material, the adjacent acinar and ductular epithelium became thinner. Adjacent to the residual exocrine tissue were pools of eosinophilic zymogen material that appeared to be free within the interstitium (Figure 4C). The interstitial zymogen material was morphologically degenerate (eg, hyalinized, fragmented) compared with zymogen material still confined by an epithelial border. Because the pancreas is not easily accessible to biopsy and tissues from young children with CF are no longer common, we examined autopsy tissue from an adult with

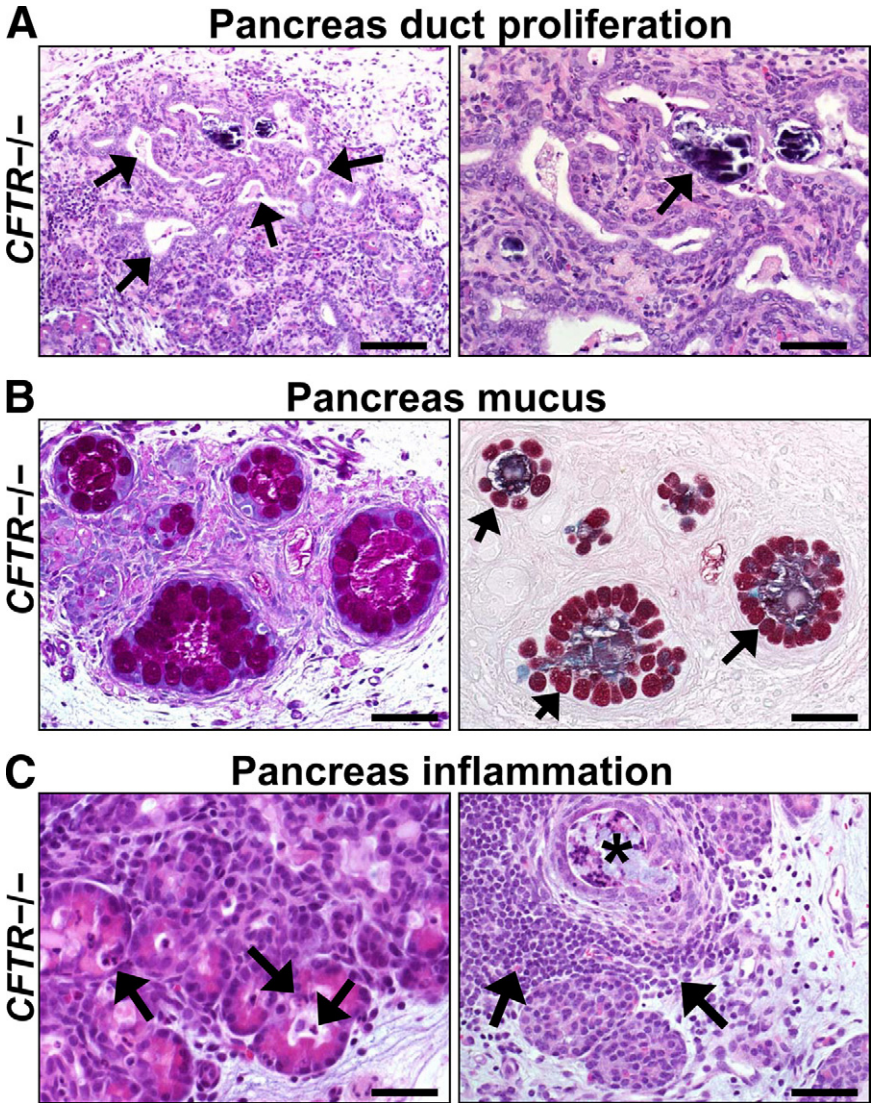

Figure 5. Duct proliferation, mucus accumulation, and inflammation in $CFTR^{-/-}$ pancreas. **A:** Foci of proliferative (**left panel**, **arrows**) and dilated ducts were common in $CFTR^{-/-}$ pancreata. Dystrophic calcification was rarely detected (**right panel**, **arrow**). HE stain. Scale bars = 110 μm and 55 μm (**left** and **right panel**, respectively). **B:** In severely disease pancreata, increased mucinous metaplasia of ducts was detected. Note the large mucus cells (**arrows**) circumferentially lining variably sized ducts that are moderately distended by stringy mucus. PAS (**left panel**) and ABPY (**right panel**) stains. Scale bars = 55 μm. **C:** Acini and ducts dilated by zymogen material had scattered infiltrates of neutrophils (**arrows**, **left panel**) and to a lesser extent, macrophages. Severe exocrine destruction was often associated with interstitial lymphoid aggregates (**arrows**, **right panel**) adjacent to dilated, cyst-like ducts (**black asterisk**). HE stain. Scale bars = 36 μm (**left panel**) and 55 μm (**right panel**).

clinically mild CF disease. We found remnant exocrine tissue that had zymogen material in a similar interstitial distribution with degenerative characteristics as that in $CFTR^{-/-}$ pigs (Figure 4D). In both pigs and humans, the interstitial pools of zymogen material elicited minimal direct cellular inflammatory response even with the appearance of morphological degeneration. The loss of exocrine tissue was replaced by increased connective tissue in the pancreata of CF infants[41,42] and $CFTR^{-/-}$ pigs.

In addition, foci of duct proliferation were detected in exocrine tissue (Figure 5A), especially noted in pancreata with more severe exocrine destruction. In her detailed description of CF pancreas pathology, Sturgess[38] noted that proliferation of ducts was common in CF infant pancreas. Recent evidence suggests that ductular proliferation may be a manifestation of acinar to ductular metaplasia, a reaction to pancreatic injury.[43,44] An additional mechanism of degenerative change seen in a few rare ducts included purple granular material consistent with dystrophic calcification (Figure 5A). In pancreata with mild disease, mucinous metaplasia of ducts was only observed in uncommon large ducts. This is in contrast to pancreata with severe destruction where mucinous metaplasia was especially prominent and seen in large, medium, and small ducts (Figure 5B). The proliferative mucus cells lining ectatic ducts were often plump and full of mucus.

In CF infants, pancreas inflammation was described by Andersen[2] as mild with a small number of neutrophils and macrophages in the acinar tissue and scattered mononuclear aggregates in the interstitium. This was also the case in porcine $CFTR^{-/-}$ pancreas. Although all $CFTR^{-/-}$ pancreata had detectable cellular inflammation, most of this was patchy in distribution with increased detection noted with progressive disease severity. Scattered neutrophils and macrophages were detected principally within dilated acini (Figure 5C, left panel) and ectatic ducts. There, they infrequently formed loose cellular aggregates, which morphologically could be described as a "pancreatitis." Interstitial inflammation usually consisted of scattered lymphocytic aggregates that became more prominent around ectatic obstructed ducts in areas of severe pancreatic destruction (Figure 5C, right panel).

Table 1. Liver and Gallbladder Lesion Parameters according to Pig Genotype

Genotype	Biliary proliferation	Fibrosis	Inflammation	Triad bridging	Extra-medullary hematopoiesis	Steatosis	GB mucinous change
CFTR$^{+/+}$	0% (1/12)	0% (0/12)	0% (0/12)	0% (0/12)	100% (12/12)	17% (2/12) mild	8% (1/12) mild
CFTR$^{+/-}$	0% (0/12)	0% (0/12)	0% (0/12)	0% (0/12)	92% (11/12)	0% (0/12)	0% (0/12)
CFTR$^{-/-}$	58% (15/26)	42% (11/26)	54% (14/26)	8% (2/26)	100% (24/24)	8% (2/26) mild	94% (17/18)

GB indicates gallbladder.

Liver Disease

Liver disease is now the second leading cause of CF mortality.[45–47] Focal biliary cirrhosis (FBC), the hallmark lesion in patients with CF, is characterized by biliary proliferation, fibrosis, and inflammation.[2,5,41,48,49] FBC may progress to bridging and multilobular cirrhosis in up to 17% of patients, with most being diagnosed before age fourteen.[50] In *CFTR*$^{-/-}$ pigs, biliary proliferation was the most readily detected parameter of FBC (Table 1). Fibrosis (fibroplasia) was typically mild in severity and usually adjacent to proliferative ducts. Lymphocytic inflammation, when present, was detected adjacent to proliferative ducts and ranged from scattered cells to moderate cellular aggregates expanding the triad. Bridging of triads by these lesions was rare, but when present was accentuated by biliary proliferation (Figure 6A, top left panel) with fibroplasia and inflammation—all suggestive of early multilobular change (Figure 6B, Table 1). In the pig, peribiliary glands appear principally in larger ducts, thus should mucus obstruction occur here, it could result in proliferation of the smaller bile ducts. Uncommonly, alterations were found in biliary ducts including mucinous change (increased epithelial mucus staining), obstruction by mucocellular plugs (inflammatory cells, cell debris, and variable mucus depending on the size of duct), and concretions of bile (choleliths; Figure 6A).

There were no detectable differences between *CFTR*$^{+/+}$ and *CFTR*$^{-/-}$ livers in steatosis, hemosiderosis, or cholestasis. Likewise, in CF infants, steatosis is generally considered to be secondary to the various chronic metabolic changes in postnatal life.[51] Independent of genotype, neonatal pigs had multifocal cellular aggregates of hematopoietic cells (ie, extramedullary hematopoiesis) in the liver (Figure 6C). Cellular aggregates were often grouped according to lineage, they were localized in the parenchyma and less commonly adjacent to hepatic triads, and they required differentiation from inflammation.

Gallbladder Disease

Gallbladder lesions are reported in ≈20% to 30% of CF infants and typically consist of a small gallbladder (microgallbladder) with proliferative epithelium that sometimes form mucosal folds or "cysts."[2,5,51–53] The CF infant gallbladder can also manifest features such as luminal mucus, thickened bile, choleliths, and/or cholecystitis.[52] Incidentally, most CF patients lack clinical consequences of microgallbladder.[51] All *CFTR*$^{-/-}$ pigs had a microgallbladder, which was readily apparent at necropsy and histopathology (Figure 7, A and B). It resembled that seen in humans with CF (Figure 7A, right panel).[54]

We stained the gallbladder with PAS, which detects neutral mucins and ABPY histochemical stains, which detect sialylated (blue), sulfated (red), or mixed (purple) acidic mucins.[17,18] The *CFTR*$^{+/+}$ gallbladders had minor staining (Figure 8A, top panels), whereas the gallbladder epithelium of *CFTR*$^{-/-}$ pigs showed diffuse epithelial mucinous change in most cases (Table 1), although the extent of luminal mucus accumulation varied. In the *CFTR*$^{-/-}$ gallbladder, the PAS stain accentuated concentrically lamellar striations in the mucus that were parallel to the epithelial surface and that were reminiscent of the growth rings in a tree (Figure 8A, bottom left). *CFTR*$^{-/-}$ gallbladders stained with ABPY showed a heterogeneous cellular production of sialylated, sulfated, and some mixed acidic mucus with a detectable preference toward sulfated mucus. This histochemical stain also highlighted distinct ribbons of mucus extending out perpendicularly from the epithelium. These ribbons often retained their form and structure with minimal amalgamation of adjacent mucus ribbons (Figure 8A, bottom right). It was difficult to obtain serial sections because of frequent tissue folds associated with sectioning gallbladders filled with mucus plugs. When available, serial sections using both stains demonstrated similar morphological features (Figure 8B).

Additional microscopic changes in the *CFTR*$^{-/-}$ gallbladder included luminal obstruction by a mixture of mucus and altered bile with proliferative mucosal folds (Figure 9A). Occasionally, small focal aggregates of neutrophils were detected within the luminal mucus near the epithelium. Mild mononuclear inflammation was sometimes detected in the lamina propria and connective tissue of the gallbladder wall, which also had mild to moderate thickening by increased collagen and smooth muscle. The apparent discrepancy between the severity of the gallbladder disease and the milder liver disease in neonatal *CFTR*$^{-/-}$ pigs could suggest that their pathogenesis is not sequential or directly related. Alternatively, given the focal nature of CF liver lesions (eg, FBC), we cannot rule out that every *CFTR*$^{-/-}$ liver may have some degree of FBC that is not readily detectable in routine tissue sampling.

Cystic Duct Disease

The cystic duct connects the gallbladder to the common bile duct. The occurrence of cystic duct lesions paralleled gallbladder lesions and included mucinous change with

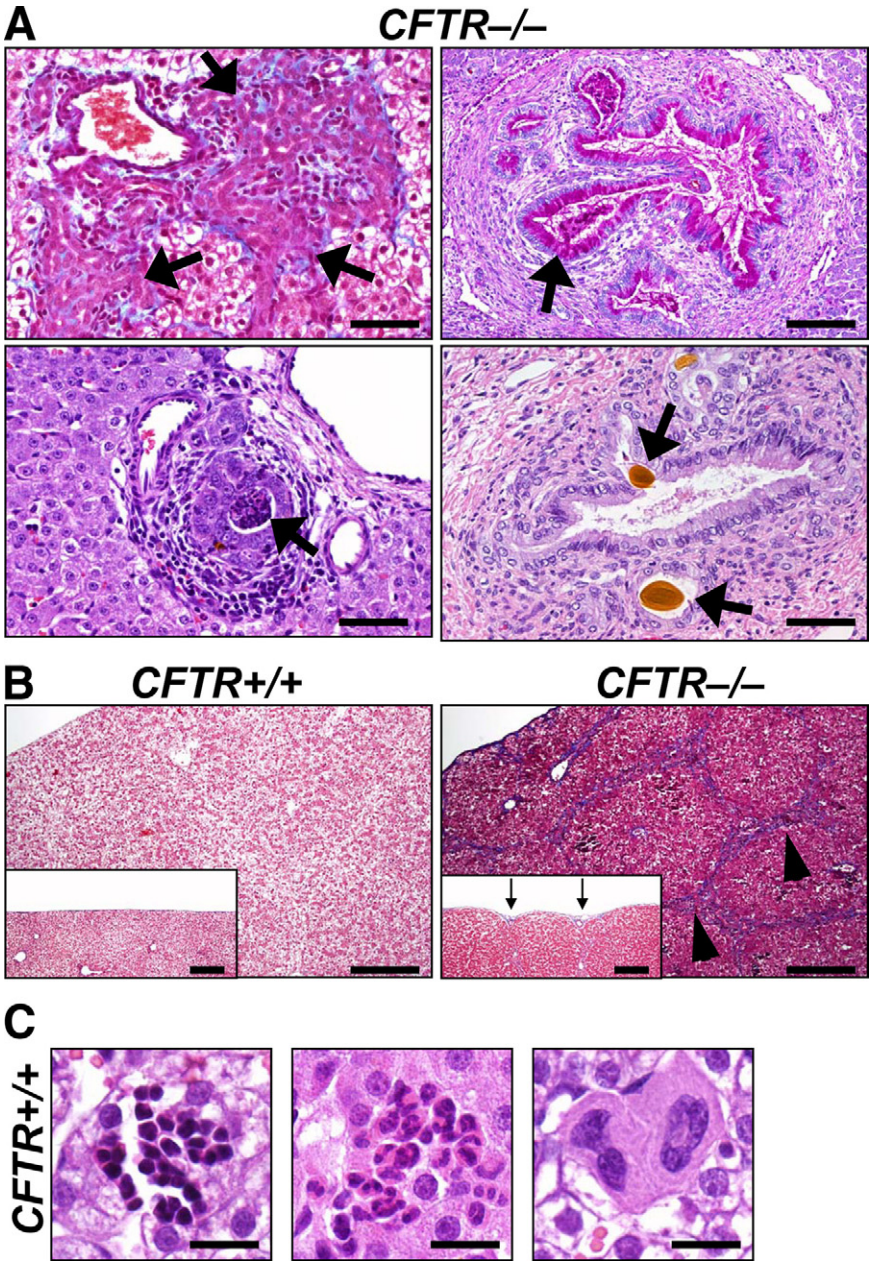

Figure 6. Rare lesions in *CFTR*$^{-/-}$ liver and incidental findings in all genotypes. **A:** Infrequent to rare findings in *CFTR*$^{-/-}$ liver. Biliary tracts had expansive and florid proliferation (**arrow**, **top left panel**, MT stain) with adjacent fibrosis (blue) and inflammation. Intrahepatic ducts had mucinous change (**arrow**, **top right panel**, PAS stain), mucocellular plugs (**arrow**, **left bottom panel**, HE stain), or choleliths (**arrows**, **right bottom panel**, HE stain). Scale bars = 55 μm. **B:** Widespread bridging (**arrowheads**) of triads by biliary hyperplasia, inflammation, and fibrosis (blue staining) was rarely detected in *CFTR*$^{-/-}$ pigs. Scale bars = 220 μm. In these cases, the serosal surface was often retracted causing a slightly irregular surface (**arrows**, **inset**, Scale bars = 197 μm). MT stain. **C:** Foci of extramedullary hematopoiesis were composed of erythroid, granulocytic, and megakaryocytic lineage aggregates (respective panels) in liver of all genotypes. HE stain. Scale bars = 17.5 μm.

obstruction and stenosis.[5,52,53] Bodian[5] noted that in fourteen cases of CF gallbladder disease, the cystic duct was consistently obstructed, yet the extrahepatic ducts were patent. Similarly, *CFTR*$^{-/-}$ pig cystic ducts were consistently obstructed by mucocellular material (Figure 9B), bile, and at times proliferative mucosa folds that formed cystic-like structures containing luminal material. Inflammatory changes were similar to that seen in the gallbladder. We

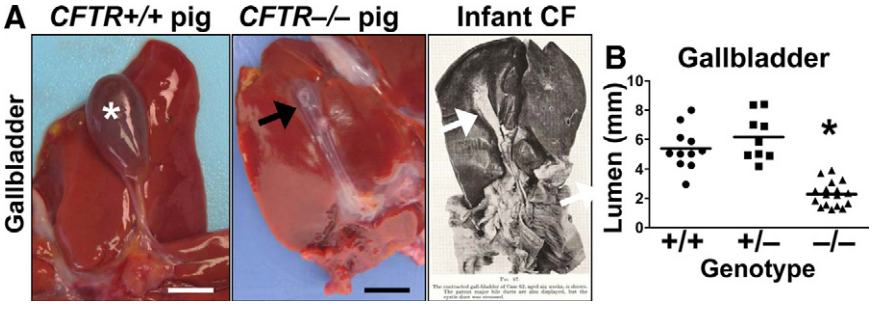

Figure 7. Microgallbladder in *CFTR*$^{-/-}$ pigs. **A:** *CFTR*$^{+/+}$ pig gallbladders (**asterisk**, Scale bar = 10.5 mm) were typically distended with bile, whereas microgallbladder (**arrows**, Scale bar = 7.4 mm) was seen in the *CFTR*$^{-/-}$ pig. **Right panel** shows microgallbladder from infant with CF as reported by Bodian.[5] Reprinted from Fibrocystic Disease of the Pancreas: a Congenital Disorder of Mucus Production-Mucosis, Martin Bodian, Page 112, Copyright 1953. **B:** Histological measurements showed that porcine *CFTR*$^{-/-}$ gallbladder was smaller than *CFTR*$^{+/+}$ or *CFTR*$^{+/-}$ ($P < 0.001$ respectively, one-way analysis of variance with Bonferroni post test, Scale bar = mean).

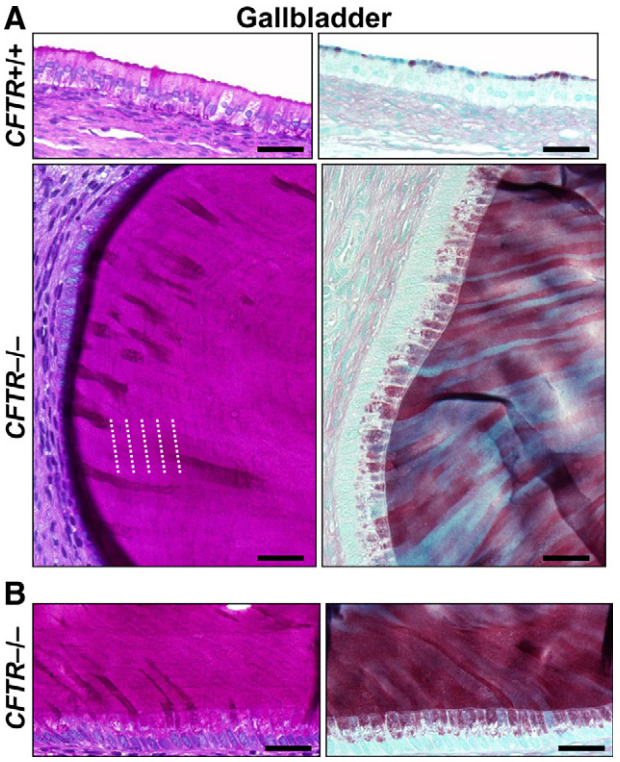

Figure 8. Mucus cell proliferation and mucus accumulation in *CFTR*$^{-/-}$ gallbladder. **A:** Nominal apical mucus staining in *CFTR*$^{+/+}$ gallbladder epithelium (**top panels**, Scale bars = 38 μm). *CFTR*$^{-/-}$ gallbladder typically had diffuse mucinous change (**bottom panels**, Scale bars = 26 μm) with contrasting morphological characteristics of mucus including lamellar striations (see **white dotted lines**) parallel to the epithelium (**left panel**, PAS stain) and ribbons of resilient mucus perpendicular to the epithelium (**right panel**, ABPY stain). **B:** Serial sections of *CFTR*$^{-/-}$ gallbladder contained similar morphological changes. Scale bars = 32 μm.

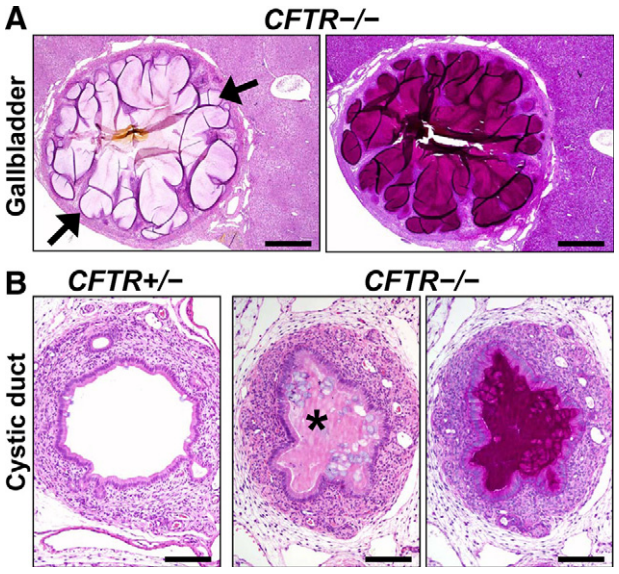

Figure 9. Gallbladder and cystic duct lesions. **A:** The *CFTR*$^{-/-}$ gallbladder mucosa sometimes formed folds of proliferative epithelium to form cyst-like structures (**arrows**) along the mucosa. HE (**left**) and PAS (**right**) stain. Scale bars = 550 μm. **B:** *CFTR*$^{-/-}$ cystic ducts were variably obstructed (**asterisk**) and stenotic compared with controls. HE (**left** and **middle**) and PAS (**right**) stains. Scale bars = 116 μm.

examined the common bile duct at necropsy in a few cases, and no obstruction was detected.

Discussion

Disease Severity in Humans with CF and CFTR$^{-/-}$ *Pigs*

The most prominent difference between disease in newborn babies with CF and newborn *CFTR*$^{-/-}$ pigs was that the disease was more severe in the pigs. For example, meconium ileus occurred in 100% of the pigs, but ≈15% of humans[19–22]; gallbladder abnormalities were present in 100% of *CFTR*$^{-/-}$ pigs, but ≈20% to 30% of humans[2,51–53]; up to 42% of newborn *CFTR*$^{-/-}$ pigs had lesion parameters consistent with FBC, whereas ≈14% to 43% of CF infants show similar morphological changes[2,5,48,52]; and all *CFTR*$^{-/-}$ pig pancreata (100%) were readily distinguished from controls by gross inspection at necropsy, whereas in CF infants, morphological detection of pancreatic disease is up to 93% successful only when using extensive histological examination and quantitative morphometric analysis.[55]

What accounts for the increased disease severity in the *CFTR*$^{-/-}$ pigs? There are several considerations. First, the pigs have a null mutation, whereas the majority of humans have at least one ΔF508 or one missense mutation, either of which might generate a very small amount of CFTR function. Perhaps only a small amount of CFTR function is sufficient to slow the progression of disease in humans relative to *CFTR* null pigs. Consistent with this possibility, many aspects of the disease, including meconium ileus, have a significant genotype/phenotype correlation.[56] Second, genetic modifiers might be distinct in humans and these pigs. Genetic modifiers are known to influence the course of CF in humans.[56–59] Although not inbred to the extent defined for mice, the *CFTR*$^{-/-}$ pigs are currently much more inbred than humans, and it is possible that by out-breeding the pigs we will be able to modify their phenotype. Consistent with this speculation, the severity of pathological changes varied between individual animals. Furthermore, study of human *CFTR* genotype to phenotype correlation in twins suggests modifier genes play an important role for both causative and preventive effects.[56] Third, it is possible that humans express another anion channel that compensates, at least in small measure, for the loss of CFTR and thereby partly mitigates the pathological developments. In this regard, it has been hypothesized that an alternative Cl^- channel partly replaces CFTR function in *CFTR*$^{-/-}$ mice.[60] Fourth, it has been hypothesized that a misfolded CFTR protein, especially CFTR-ΔF508, might elicit an unfolded protein response that contributes to CF disease in humans.[61] Because CF disease tends to be milder in humans than in pigs, our data suggest that if an unfolded protein response contributes to differences in severity between the species, then it may attenuate disease severity. Fifth, it seems unlikely that anatomical differences between the species (eg, the spiral colon in pigs versus the ascending colon in humans) are entirely responsible because differences in severity between the

species occurred in multiple organs. In fact, the greater severity of disease in pigs versus humans suggest that whatever the cause, it likely involves a global difference in phenotype of CFTR-deficient epithelia that is manifest in many organs before birth. The same is likely to be true in mice. Although *CFTR*$^{-/-}$ mice do not develop classical meconium ileus like humans with CF, at an early age, most *CFTR*$^{-/-}$ mice develop intestinal mucus obstruction. Histopathological changes in the pancreas, liver, and gallbladder are nominal and may show some disease progression in advanced age.[60,62] We propose that comparing the pathophysiological features of these three species may yield novel insight into the varying lesion severity (pig > human ≫ mice) and hence, the pathogenesis of CF disease.

Tissue Remodeling and Mucus Accumulation

In general, affected organs in newborn pigs shared four features: a) prominent epithelial mucus producing cells, b) mucus accumulation, c) tissue remodeling, and d) inflammation.

Epithelial Mucus Cells

Epithelia in the affected porcine organs often showed a prominent increase in mucus cells. Mucinous epithelial changes also occur in human tissues, which led Bodian[5] to use the term "mucosis" to describe the changes in various CF organs.

Mucus Accumulation

Accumulation of mucus in pancreatic ducts, gallbladder/cystic duct, and intestine was a consistent finding in *CFTR*$^{-/-}$ pigs. Likewise, early observations in CF infants reported mucus accumulation as a common pathological feature.[3,4] The prominent mucus accumulation even caused some early investigators to suggest the cause of CF was a "mucinase deficiency."[4] In *CFTR*$^{-/-}$ pigs, the morphological character of the mucus was striking. The concentrically lamellar striations of mucus (gallbladder, pancreas ducts, and intestine) suggest that luminal accumulation formed by serial secretions. Consistent with this speculation, the cytoplasm of mucus-producing cells was usually distended with mucus suggesting that the secretion process occurred at a modest rate that did not deplete mucus from the cells.[63] The mucus secretions were also congealed and resilient, retaining a relatively fixed macroscopic structure even when it migrated to a new location. Whether the structure of the mucus was a result of stasis attributable to a long-lasting obstruction or a consequence of altered transepithelial ion transport attributable to the lack of CFTR is unknown.

Tissue Remodeling

Tissue remodeling (eg, hyperplastic, metaplastic, connective tissue changes) occurs as a response to chronic disease, and many of these parameters were evident in the neonatal *CFTR*$^{-/-}$ organs. This implies that affected tissues undergo significant remodeling even in the absence of environmental stimuli and that remodeling was a dynamic component of fetal life.

Inflammation

Inflammation was detected in affected organs and was characterized by mild to moderate patchy cellular infiltrates adjacent to tissue lesions. The heterogeneous character and localization of cellular infiltrates suggests that multiple mechanisms/roles for inflammation may be at work.

The observations of increased epithelial mucus cells, luminal mucus accumulation, tissue remodeling, and inflammation in multiple organs raise questions about the pathogenic sequence of events. One idea might be that inflammation triggered the conversion of a normal epithelium to one with increased numbers of mucus cells, and that they then secreted mucus that obstructed the lumen. In that case, the stimulus for inflammation would presumably be an inherent inflammatory state caused by lack of CFTR; such an event has been postulated to exist in CF.[64] Although this study cannot fully address the role of inflammation, our finding that profound intestinal mucinous change occurred in the absence of overt cellular inflammation suggests that the presence of leukocytes may not be a requirement for mucus production in all CF tissues. Another possibility is that CF luminal mucus had an intrinsic abnormal structure thereby causing tissue injury and inflammation, which then further drove mucus cell production. However, mucus cells are not routinely present in epithelia at some anatomical sites (eg, small ducts of the pancreas), and thus mucus production would be absent or limited in the initial stages of the disease pathogenesis. Nevertheless, the quantity of mucus required for obstruction might be very small. A third, related scenario is that defective electrolyte transport attributable to loss of CFTR might change the consistency of luminal secretions, thereby causing luminal mucoid plugs. Obstruction by altered secretions could cause direct physical irritation of lining cells leading to a cycle of mucinous metaplasia and further mucus production. Consistent with this scenario, Tucker et al[40] suggested that altered zymogen secretions, rather than mucus, caused the initial detectable obstruction of pancreatic ducts; then, mucus metaplasia occurred and contributed to the obstruction and exocrine atrophy. In addition, mucinous metaplasia of the gallbladder epithelium is chronologically correlated with gallstone formation, and the physical presence of luminal gallstones may synergistically perpetuate metaplastic change in the gallbladder epithelium.[65] Studies of fetal *CFTR*$^{-/-}$ pigs may shed further light on some of these issues.

We also found that smooth muscle layers (eg, intestinal tunica muscularis and gallbladder wall) were hypertrophic in *CFTR*$^{-/-}$ pigs. Recently, CFTR has been detected in smooth muscle, and functional studies suggested that CFTR may play a role in their relaxation.[66–68] Although hypertrophy could be a secondary response of tissue remodeling, it could also represent an intrinsic conse-

quence due to loss of CFTR (ie, a persistent contracted state). Thus, the absence of CFTR might cause altered smooth muscle physiology that could contribute to CF disease pathogenesis; however, the mechanism and extent of this effect remain elusive.

Relationship to Lung Disease

Even though CFTR is absent, the lungs of newborn *CFTR*$^{-/-}$ pigs appeared similar to those of their *CFTR*$^{+/+}$ littermates.[16] The discrepancy between the marked pathology in the gastrointestinal organs described here and the lack of pulmonary disease in newborn pigs may provide some hints about CF pathogenesis and allows for some speculation.

There are several feature(s) of the fetal lung that might potentially prevent it from developing CF disease before birth. The fetal lung is filled with liquid that originates from respiratory epithelia and amniotic fluid.[69] The composition of fetal lung liquid may be less prone to congealing and causing obstruction than that in the ducts of gastrointestinal organs. Furthermore, impaired mucociliary clearance is speculated to initiate CF airway disease,[70] but in the relatively quiescent fetal lung, there is no apparent requirement for mucociliary clearance. This situation contrasts with the functional activity of fetal gastrointestinal organs, as hepatobiliary and pancreatic secretions are constituents of meconium that is moved through the intestines via peristalsis. It is also possible that compared with affected gastrointestinal organs, the composition or concentration of material in fetal lung liquid is insufficient to incite disease. Perhaps only after breathing air, when the volume of airway surface liquid is very small, is abnormal transepithelial electrolyte transport able to modify its composition in a way that initiates CF airway disease.

Comparing gastrointestinal organ pathology with the lack of abnormalities in lungs also has implications for two other aspects of CF that are well known to contribute to the disease, submucosal glands and inflammation. Submucosal glands have been hypothesized to generate mucus with abnormal properties that contribute to lung disease.[71] Mucus metaplasia or hyperplasia with dramatic mucus accumulation was detected in affected gastrointestinal organs. In some cases (eg, pancreatic ducts and intestinal epithelia), the luminal mucus derived from metaplastic surface epithelia, and glands were not apparently required for disease. In contrast, the CFTR-rich Brünner glands of the duodenal submucosa showed only minimal abnormalities compared with other portions of the intestine. These observations lead us to wonder about the relative contribution of mucus derived from submucosal glands versus surface epithelia in the development of CF lung disease.

Inflammation was a feature in some affected gastrointestinal organs of newborn pigs, but not in the airways, despite the fact that epithelia in all these organs express CFTR. While it is certainly possible that lack of CFTR may initiate an overly exuberant inflammatory response,[72] our results lead us to speculate that inflammation may not be an inherent property of a *CFTR*$^{-/-}$ cell or tissue in the absence of a stimulus, but with a stimulus it plays an important role in progression of the disease.[73]

This work sets the stage for studies that look forward as animals mature and look backward to the fetus to assess early pathogenic changes. Similarities and differences between pathological changes in the pig, humans, and mice should provide an opportunity to better understand the pathogenesis of this devastating disease.

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