Cystic fibrosis (CF) is a recessive disease that affects multiple organs. It is caused by mutations in CFTR. Animal modeling of this disease has been challenging, with species- and strain-specific differences in organ biology and CFTR function influencing the emergence of disease pathology. Here, we report the phenotype of a CFTR-knockout ferret model of CF. Neonatal CFTR-knockout ferrets demonstrated many of the characteristics of human CF disease, including defective airway chloride transport and submucosal gland fluid secretion; variably penetrant meconium ileus (MI); pancreatic, liver, and vas deferens disease; and a predisposition to lung infection in the early postnatal period. Severe malabsorption by the gastrointestinal (GI) tract was the primary cause of death in CFTR-knockout kits that escaped MI. Elevated liver function tests in CFTR-knockout kits were corrected by oral administration of ursodeoxycholic acid, and the addition of an oral proton-pump inhibitor improved weight gain and survival. To overcome the limitations imposed by the severe intestinal phenotype, we cloned 4 gut-corrected transgenic CFTR-knockout kits that expressed ferret CFTR specifically in the intestine. One clone passed feces normally and demonstrated no detectable ferret CFTR expression in the lung or liver. The animals described in this study are likely to be useful tools for dissecting CF disease pathogenesis and developing treatments.
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Introduction
Cystic fibrosis (CF) is the most common life-threatening autosomal recessive condition among people of mixed European descent, with approximately 1 in 3,500 newborns affected each year. CF is caused by mutations in an epithelial chloride channel encoded by the CFTR gene (1–3). Tissues affected in CF include the lung, pancreas, liver, intestine, gallbladder, sweat gland, and male reproductive tract (2, 3). Phenotypic variability in the severity of disease in these tissues can be influenced by modifier genes, the type of CFTR mutation, and the environment in ways that are only partially understood (3, 4). CF mice have been an invaluable system for dissecting the biology of CFTR function and for demonstrating that genetic background can significantly influence CF-related phenotypes in this species (5, 6). Recently, the description of the neonatal CF pig phenotype has expanded the potential for modeling CF disease (7). Interestingly, CF mice and pigs develop either less or more severe disease in certain organ systems than do humans with CF, a fact that highlights species-specific differences in organ physiology and CFTR function. Additional CF models may help understanding of how the pleiotropic functions of CFTR in multiple organs influence the progression of lung disease — the most life-threatening aspect of CF being chronic bacterial infections of the airways.

The domestic ferret (Mustela putorius furo) is a potentially attractive species for modeling CF for 2 major reasons: its lung anatomy and lung cell biology are similar to those in humans, and it reproduces rapidly (42-day gestation and 4–6 months to sexual maturity). With regard to lung anatomy and lung cell biology, it is important to note that ferrets and humans have submucosal glands throughout their cartilaginous airways, whereas mice possess these glands only in the proximal trachea (8, 9). Submucosal glands express abundant CFTR in the serous tubules, which facilitate fluid and mucous secretion into the airway (10, 11); given that these structures are thought to play an important role in protecting the airways from bacterial infection (11, 12), their distribution may be important for modeling CF disease. Additionally, the goblet cell is the predominant secretory cell type of the human and ferret proximal cartilaginous airways, whereas the Clara cell is the analogous secretory cell type in mice (9, 13). Although Clara cells are also present in humans and ferret airways, they are limited to the bronchioles rather than being distributed throughout the proximal and distal airways as in mice (13, 14). For these reasons, we recently developed ferrets heterozygous for a CFTR exon 10 deletion, using adeno-associated virus gene targeting in fibroblasts, coupled with somatic cell nuclear transfer (SCNT) (15). Here, we report the neonatal disease phenotype in CFTR-null ferrets.

CFTR-knockout neonatal ferrets developed many of the pathologies observed in humans with CF, including meconium ileus (MI), pancreatic disease, liver disease, severely impaired nutrition, and a predisposition to lung infections during the early postnatal period. Liver disease in CF ferrets, as evident by a rise in liver function tests (LFTs) during the early postnatal period, was treatable by bile acid replacement. Additionally, improved nutritional status in the ferret model of CF could be achieved by administering an oral proton-

Conflict of interest: Jill Ascher is employed by Marshall Farms Group Ltd. as a veterinarian. Marshall Farms Group Ltd. will be distributing the animal model described in this manuscript.

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Disease phenotype of a ferret CFTR-knockout model of cystic fibrosis

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The generation of a gut-corrected fatty acid–binding protein (FABP) in CFTR-knockout ferrets. Intestinal tissue from CFTR+/− kits expressed no CFTR protein as compared with that of CFTR−/− and CFTR+/− kits (Figure 1A). Within the first 24 hours of life, CFTR−/− kits were indistinguishable from CFTR+/− or CFTR−/− littermates, in terms of activity and weight (Figure 1B and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43052DS1). However, by 36 hours after birth, all CFTR−/− kits failed to thrive, and by 48 hours they were typically euthanized due to severe morbidity. Gross and histological pathologies indicated that the majority of kits (~75%) suffered from MI and failed to pass stool during the neonatal period (Figure 1C). Approximately half of the kits with MI died from intestinal perforation at the level of the ileum or colon (Figure 1C, arrows). Additionally, microcolon was observed in approximately 30% of the animals born with MI (Figure 1C). Interestingly, approximately 25% of newborn CFTR−/− kits passed meconium but nevertheless failed to thrive and died within 2–4 days after birth (Figure 1C). GI obstruction in CFTR−/− kits suffering from MI was associated with intestinal luminal mucus and mucous cell hyperplasia (Figure 1D). The occurrence of MI in ferret CFTR−/− kits (~75%) was greater than that for CF infants (~15%) (2, 16) and less than that seen in CFTR−/− pigs (100%) (7). Although CF mice do not present with classical MI at birth, they do suffer from gut obstruction at weaning, and this can significantly impair survival. Interestingly, the penetrance of gut obstruction in CF mice can vary significantly (0%–95% survival at weaning) depending on the background strain (5, 6), suggesting that genetic modifier genes significantly influence this phenotype in the mouse.

Analysis of the frequency of MI within the CF ferret colony suggested genetic influences for the development of severe intestinal complications at birth in CFTR−/− kits. Nine F2 generation male CFTR+/− hobs used for breeding gave rise to litters with MI frequencies ranging from 50%–100% (Table 1). Pearson’s χ2 test and Fisher’s exact test for association demonstrated a statistically significant dependence of the variable MI frequency in CF offspring on the CFTR+/− hobs used for breeding (P < 0.047 for both tests). Additionally, a logistic regression was run in which the response variable was the MI status of the offspring and the explanatory variable was the hob identification. This analysis also suggested that inheritance of the MI phenotype was influenced by the hob (P < 0.042). Inclusion of a pump inhibitor. Because the frequency of MI in both the new CF pig and ferret models significantly limits their use, we corrected the gut defect by generating a transgenic CFTR-knockout ferret that expresses a HA-tagged CFTR cDNA under control of the intestinal-specific fatty acid–binding protein (FABP) promoter. These studies led to the generation of a gut-corrected CFTR-knockout model that lacks MI at birth, in which expression of HA-tagged CFTR (Figure 1B) was expanded through a single generation of breeding, and their F1 CFTR+/− offspring gave rise to 313 kits containing 65 CFTR+/+, 171 CFTR−/−, and 77 CFTR+/− animals (Figure 1A). This ratio was not significantly different from the expected 1:2:1 inheritance of a recessive trait and indicates that prenatal lethality is not a consequence of deleting the CFTR gene in ferrets. Intestinal tissue from CFTR−/− kits expressed no CFTR protein as compared with that of CFTR+/− and CFTR+/− kits (Figure 1A). Within the first 24 hours of life, CFTR−/− kits were indistinguishable from CFTR+/− or CFTR+/− littermates, in terms of activity and weight (Figure 1B and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43052DS1). However, by 36 hours after birth, all CFTR−/− kits failed to thrive, and by 48 hours they were typically euthanized due to severe morbidity. Gross and histological pathologies indicated that the majority of kits (~75%) suffered from MI and failed to pass stool during the neonatal period (Figure 1C). Approximately half of the kits with MI died from intestinal perforation at the level of the ileum or colon (Figure 1C, arrows). Additionally, microcolon was observed in approximately 30% of the animals born with MI (Figure 1C). Interestingly, approximately 25% of newborn CFTR−/− kits passed meconium but nevertheless failed to thrive and died within 2–4 days after birth (Figure 1C). GI obstruction in CFTR−/− kits suffering from MI was associated with intestinal luminal mucus and mucous cell hyperplasia (Figure 1D). The occurrence of MI in ferret CFTR−/− kits (~75%) was greater than that for CF infants (~15%) (2, 16) and less than that seen in CFTR−/− pigs (100%) (7). Although CF mice do not present with classical MI at birth, they do suffer from gut obstruction at weaning, and this can significantly impair survival. Interestingly, the penetrance of gut obstruction in CF mice can vary significantly (0%–95% survival at weaning) depending on the background strain (5, 6), suggesting that genetic modifier genes significantly influence this phenotype in the mouse.

### Results

**CFTR-knockout kits exhibit MI of variable penetrance.** Eight heterozygous founder clones (CFTR+/−) were expanded through a single generation of breeding, and their F1 CFTR+/− offspring gave rise to 313 kits containing 65 CFTR+/+, 171 CFTR−/−, and 77 CFTR+/− animals (Figure 1A). This ratio was not significantly different from the expected 1:2:1 inheritance of a recessive trait and indicates that prenatal lethality is not a consequence of deleting the CFTR gene in ferrets. Intestinal tissue from CFTR−/− kits expressed no CFTR protein as compared with that of CFTR+/− and CFTR+/− kits (Figure 1A). Within the first 24 hours of life, CFTR−/− kits were indistinguishable from CFTR+/− or CFTR+/− littermates, in terms of activity and weight (Figure 1B and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43052DS1). However, by 36 hours after birth, all CFTR−/− kits failed to thrive, and by 48 hours they were typically euthanized due to severe morbidity. Gross and histological pathologies indicated that the majority of kits (~75%) suffered from MI and failed to pass stool during the neonatal period (Figure 1C). Approximately half of the kits with MI died from intestinal perforation at the level of the ileum or colon (Figure 1C, arrows). Additionally, microcolon was observed in approximately 30% of the animals born with MI (Figure 1C). Interestingly, approximately 25% of newborn CFTR−/− kits passed meconium but nevertheless failed to thrive and died within 2–4 days after birth (Figure 1C). GI obstruction in CFTR−/− kits suffering from MI was associated with intestinal luminal mucus and mucous cell hyperplasia (Figure 1D). The occurrence of MI in ferret CFTR−/−kits (~75%) was greater than that for CF infants (~15%) (2, 16) and less than that seen in CFTR−/− pigs (100%) (7). Although CF mice do not present with classical MI at birth, they do suffer from gut obstruction at weaning, and this can significantly impair survival. Interestingly, the penetrance of gut obstruction in CF mice can vary significantly (0%–95% survival at weaning) depending on the background strain (5, 6), suggesting that genetic modifier genes significantly influence this phenotype in the mouse.

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of the female parents (i.e., jills) as an explanatory variable did not reach significance in this logistic regression, since the number of CF kits born to each of the 40 jills used in this analysis was too small, and only 8 of the jills were bred twice. These findings suggest that hereditary factors influence the occurrence of MI in CFTR–/– kits.

CFTR-knockout kits exhibit pancreatic lesions similar to those seen in CF infants but no overt histopathology of the liver or gallbladder. The newborn CFTR–/– pancreas was indistinguishable from that of CFTR+/+ and CFTR+/– kits at the gross level, but histologic lesions were evident in all animals. Most, but not all, CFTR–/– acini and ductules were dilated with inspissated, eosinophilic zymogen secretions (Figure 2A). Microscopic changes in the pancreas are seen in approximately 75% of CF infants, with the majority of these cases (~72%) demonstrating histopathology similar to that seen in newborn CFTR–/– ferrets and a small minority of cases (~3%) demonstrating more severe lesions, with evidence of loss of exocrine tissue and the development of fibrosis (16, 17). Thus, the level of histopathology in the newborn CFTR–/– ferret pancreas appears to be quite similar to that seen in CF infants and significantly less severe than the extensive destruction observed in the exocrine pancreases of newborn CF pigs (7).

Table 1
Frequency of MI in CFTR–/– kits born to 9 CFTR+/– hobs

<table>
<thead>
<tr>
<th>Hob ID</th>
<th>910078S</th>
<th>910288S</th>
<th>910448S</th>
<th>910468S</th>
<th>910518S</th>
<th>910538S</th>
<th>910598S</th>
<th>910608S</th>
<th>910618S</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFTR–/– kits with MI</td>
<td>3</td>
<td>11</td>
<td>3</td>
<td>10</td>
<td>12</td>
<td>14</td>
<td>11</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>CFTR+/- kits without MI</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Total kits born to hob</td>
<td>6</td>
<td>12</td>
<td>6</td>
<td>10</td>
<td>14</td>
<td>15</td>
<td>15</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>MI (%)</td>
<td>50%</td>
<td>92%</td>
<td>50%</td>
<td>100%</td>
<td>86%</td>
<td>93%</td>
<td>73%</td>
<td>100%</td>
<td>71%</td>
</tr>
</tbody>
</table>

Data were derived from 56 litters born from 40 jills bred randomly with 9 hobs.

Figure 2
Primary organ pathologies observed in newborn CFTR–/– kits. (A) H&E-stained sections of the pancreas. Eosinophilic zymogen material filled exocrine acini (asterisks) of the CFTR–/– pancreas. (B) H&E-stained sections of the liver and gall bladder (GB). Arrowheads mark the bile-filled canaliculi infrequently seen in all genotypes. (C) H&E-stained section of the lung demonstrating the type of lung lesions observed in CFTR–/– newborn kits. The CFTR–/– examples are from the same animal that passed stool in Figure 1C and died at 48 hours. Boxed regions are enlarged in the bottom row and demonstrate evidence of fibrin deposition, necrosis, bacteria, and/or inflammation. (D) Blood chemistries for ALT, total bilirubin (Tbil), and cholesterol (CHOL) in animals of the indicated genotypes (WT, CFTR+/+ and CFTR+/–; CF, CFTR–/–). CF kits were divided into 2 groups with and without MI. BL, below limits of detection. Blood was drawn at the time of euthanasia. Values depict the mean ± SEM (n = 5–9 animals in each group) (see Supplemental Figure 3 for additional blood chemistry data). Scale bar: 200 μm (A, top row, B, top row, and C, bottom left and right panels); 25 μm (A, bottom row); 50 μm (B, bottom row); 500 μm (C, top row); 100 μm (C, bottom center panel).
The liver and gallbladder of newborn CFTR−/− kits were indistinguishable from those of CFTR+/+ or CFTR−/- littermates at the gross and histological levels (Figure 2B).

**CFTR-knockout kits have a degenerate or absent vas deferens at birth.** Nearly all male adults with CF suffer from infertility caused by bilateral absence of the vas deferens (18). Most males with CF have an intact vas deferens at birth; however, mucoid obstruction of the vas deferens has been noted in newborns with CF, and unilateral absence of the vas deferens has been detected as early as 2 years of age (17). These findings have led to the prominent hypothesis that obstruction of the vas deferens and/or altered secretions lead to progressive degeneration in patients with CF (19). Histopathologic examination of the vas deferens in newborn CFTR−/−, CFTR+/−, and CFTR−/- littermates demonstrated considerable pathology in the newborn CF kits. Of the 5 CFTR−/− kits evaluated, the vas deferens was completely absent in about 50% of the spermatic cords (Figure 3). In the remainder the vas deferens was segmentally absent, with remnant epithelium characterized as small and degenerate to tortuous and serpentine (Supplemental Figure 2). In 1 out of the 5 CFTR−/− kits, there was complete bilateral absence of the vas deferens, while a normal vas deferens was consistently detected in all of the 5 control CFTR+/+ and CFTR−/- kits.

**Newborn CFTR-knockout kits that escape MI fail to thrive and exhibit early lung infections and elevated LFTs.** Histopathologic analysis failed to ascertain the reason for early neonatal death of the 25% of CFTR−/− kits that passed meconium. Although the intestine appeared histologically normal in these animals, we noted evidence of bronchopulmonary pneumonia and aspiration (Figure 2C), which suggested that perhaps CFTR−/− kits were predisposed to aspiration-induced secondary infections. Blood chemistries in this subset of animals revealed that both plasma alanine aminotransferase (ALT) and bilirubin were consistently elevated while cholesterol levels were reduced (Figure 2D and Supplemental Figure 3); these findings suggested the potential for liver disease. Although clinically apparent liver disease in 1- to 5-year-old patients with CF is low (0.3%) (20), 53% of infants with CF demonstrate abnormally elevated LFTs, a feature that typically resolves itself by 2–3 years of age (21). Interestingly, these neonatal elevations in the levels of serum liver enzymes such as ALT were not associated with neonatal cholestasis when liver biopsies were evaluated (21). Such findings are similar to those seen in newborn CFTR−/− kits, which demonstrated no histopathologic lesions in the liver despite elevated LFTs (Figure 2B).

The reason for elevated LFTs in the majority of children with CF without histological signs of cholestasis remains unclear (21). However, children with CF frequently exhibit bile acid malabsorption by the intestine, which leads to altered enterohepatic circulation and hepatobiliary composition of bile acids (22–24). Excessive fecal bile acid loss has been proposed to influence fat and cholesterol absorption by the gut, bacterial flora in the gut, and the progression of liver disease through alterations in bile acid pool composition (24). Bile acids are synthesized from cholesterol by hepatocytes and transported into bile ducts in a conjugated form (25). Our findings of reduced serum cholesterol levels in 2- to 4-day-old CFTR−/− kits that escaped MI are consistent with impaired bile acid absorption by the gut and the depletion of cholesterol pools through protective fat absorption by the gut. Oral administration of the hydrophilic dihydroxylated bile acid ursodeoxycholic acid (UDCA) has been shown to normalize LFTs including serum ALT and bilirubin levels, and to improve the nutritional status in patients with CF (26, 27).

For these reasons, we tested whether oral UDCA therapy of CFTR−/− kits might normalize liver function and improve nutrition. Additionally, CFTR−/− kits were gavaged with Golytely within the first 12 hours to enhance rapid meconium clearance and treated with antibiotics to prevent secondary lung infection during the early neonatal period. Among 20 CFTR−/− kits treated in this manner, 16 died of MI and 4 survived for 5–9 days but failed to gain weight (Figure 4A). Gross necropsy demonstrated that although there was no intestinal obstruction in the CFTR−/− kits, fat stores throughout the animals were depleted, consistent with sustained malnutrition (Figure 4B and Supplemental Figure 4C). UDCA treatment normalized serum ALT and bilirubin levels, as is the case in CF infants (27), but failed to normalize serum cholesterol levels, which remained low in comparison with treated controls (Figure 4C).
Three out of the four CFTR–/– kits developed respiratory distress and showed signs of multifocal bronchopneumonia—with the lungs demonstrating intralobular neutrophils, macrophages, hemorrhage, fibrin, and bacterial colonies (Figure 4B and Supplemental Figure 4). Airways and alveoli were patent and showed no evidence of inflammation, hemorrhage, or bacterial colonization in control-treated CFTR+/+ and CFTR+/– kits. Although control lungs were free of pathology, it is currently unclear whether lung lesions in the CFTR–/– kit were due to the inability of the weakened animals (i.e., nutritionally compromised) to eradiate the bacteria associated with aspirated material. In a small subset of CFTR–/– kits (n = 3) that passed meconium, we also attempted pancreatic enzyme replacement, but this failed to improve either the nutritional status or the survival of these animals.

Oral administration of a proton-pump inhibitor improves nutrition in the CFTR-knockout model. Bile acids play an important role in intestinal lipid digestion and absorption, cholesterol homeostasis, and excretion of lipid-soluble xenobiotics (25). The solubilization and reabsorption of bile acids by the gut can also be influenced by fecal pH (28). Given that gut pH would likely be reduced in the context of CF, due to impaired pancreatic secretion of bicarbonate, we reasoned that raising the gut pH by oral administration of the proton-pump blocker omeprazole might allow for better bile acid–mediated absorption of fat in the gut and thereby improve the nutritional status of CF kits. Omeprazole has been used to enhance the function of pancreatic enzyme supplementation in CF patients with residual steatorrhea to improve fat absorption (29). Indeed, oral administration of UDCA and an omeprazole-containing liquid elemental diet led to significantly improved weight gain in a CFTR–/– kit during the first 16 days of treatment; weight gain was comparable to that of a CFTR+/+ littermate control that was treated identically. However, at 16 days the weight gain of the CFTR–/– kit began to slow in comparison to that of the control, and thus the animals were supplemented with oral pancreatic enzymes. After several weeks of increasing enzyme intake, the CFTR–/– kit recovered enough weight to nearly match...
that of the control kit (Figure 4D). Four additional CFTR−/− kits have been reared using this same approach and also had improved weight gain during the neonatal period; although in these 4 cases, weight gain was only approximately 25–50% of that of controls (Table 2). One of these CFTR−/− kits died acutely at 20 days, due to a massive aspiration while nursing. The second CFTR−/− kit died at 32 days, due to a rectal prolapse. The third and fourth CFTR−/− kits died at 7 and 13 days of age. The cause of death was undetermined in the 7-day-old kit, but it did have a small focus of pneumonia; the 13-day-old kit had intestinal obstruction noted during pathology examination. Histopathologic examination of these animals demonstrated increased pancreatic inflammation and loss of exocrine tissue consistent with progression of CF disease. In addition, localized atelectasis was not uncommon and complete obstruction of airways was at times detected (Supplemental Figures 5 and 6).

Bacteriology of bronchoalveolar lavage fluid and fecal samples. Despite improved weight gain in treated CFTR−/− kits that lack MI, approximately half still died within the first week of life. To evaluate the types of bacteria found in the lungs of these and other CFTR−/− kits that died within the first month of life, we performed bacteriologies on bronchoalveolar lavage (BAL) fluid. To control for the type of bacteria deposited in the lung due to gut obstruction, we also evaluated CFTR−/− kits that died from MI (Table 3). As anticipated, those animals that succumbed to MI had a much higher abundance of enteric bacterial flora in their lungs. By contrast, 2 additional species (Staphylococcus and Streptococcus) were found in the BAL of 2-day-old CFTR−/− kits without MI at higher abundance in comparison with that of wild-type and heterozygous controls (Table 3 and Supplemental Figure 7). In CFTR−/− kits that died after the first week of life, the density of bacteria in the lung was similar to or only slightly greater than that of controls animals. In the oldest CFTR−/− animal that died at 32 days, Streptococcus spp, alpha haemolytic was the only species found in the BAL, and this differed from those found in the control. We also evaluated the types of bacteria found in feces from the oldest surviving CFTR−/− and littermate control ferrets; however, no obvious differences were found (Table 3).

<table>
<thead>
<tr>
<th>Genotype (experiment)</th>
<th>Birth weight in g</th>
<th>Peak weight in g (age d)</th>
<th>Weight at death/euthanasia in g (age d)</th>
<th>Apparent cause of death</th>
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<tr>
<td>+/− (1)</td>
<td>9.7</td>
<td>901 (180)</td>
<td>901 (180)*</td>
<td>Alive</td>
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<tr>
<td>−/− (1)</td>
<td>11.3</td>
<td>1,194 (165)</td>
<td>1,033 (180)*</td>
<td>Alive</td>
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<tr>
<td>+/+ (2)</td>
<td>8.6</td>
<td>88.7 (19)</td>
<td>89.2 (20)</td>
<td>Acute aspiration</td>
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<tr>
<td>−/− (2)</td>
<td>8.9</td>
<td>31.9 (19)</td>
<td>29.8 (20)</td>
<td>(rare neutrophils)</td>
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<tr>
<td>+/− (3)</td>
<td>8.6</td>
<td>168.2 (29)</td>
<td>157.1 (32)</td>
<td>Rectal prolapse</td>
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<tr>
<td>−/− (3)</td>
<td>8.4</td>
<td>76.9 (27)</td>
<td>71.7 (32)</td>
<td>(focus of pneumonia noted)</td>
</tr>
<tr>
<td>+/+ (4)</td>
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<td>41.7 (10)</td>
<td>62.1 (13)</td>
<td>Unknown</td>
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<tr>
<td>−/− (4)</td>
<td>9.0</td>
<td>14.9 (10)</td>
<td>14.2 (13)</td>
<td>(intestinal obstruction noted)</td>
</tr>
<tr>
<td>+/− (5)</td>
<td>10.4</td>
<td>28.9 (7)</td>
<td>28.9 (7)</td>
<td></td>
</tr>
<tr>
<td>−/− (5)</td>
<td>10.1</td>
<td>11.8 (6)</td>
<td>10.2 (7)</td>
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</tbody>
</table>

*Animals were healthy at the time this most recent weight was taken.

Tracheas from CFTR-knockout ferrets demonstrate defects in cAMP-induced chloride permeability and submucosal gland secretions. Lung disease is the most life-threatening aspect of CF (2, 3). Defective CFTR-mediated anion transport and enhanced fluid absorption by the surface airway epithelium is thought to hinder bacterial clearance (3). Additionally, submucosal glands of the cartilaginous airways express abundant CFTR and have been proposed to play an important role in the pathogenesis of CF lung disease, as a consequence of defective secretion of antibacterial factor containing fluid into the airway lumen (3, 11, 30). Although the natural progression of lung disease in adult CFTR−/− ferrets remains to be delineated, functional studies on newborn CFTR−/− ferret tracheas demonstrate that this model features abnormalities characteristic of the proximal airways of CF patients, including defective cAMP-induced chloride permeability and submucosal gland fluid secretion (Figure 5). Using transepithelial potential difference (TEPD) measurements in an ex vivo tracheal xenograft model, we found that CFTR−/− tracheas lack cAMP-dependent changes in CI− permeability (Figure 5, A and B), a feature that is characteristic of TEPD defects seen in the nasal epithelium of human patients with CF (3). No significant difference was observed in amiloride-sensitive TEPD among the various genotypes; although this finding differs from those of studies of the human CF nasal epithelium that demonstrate elevated amiloride-sensitive changes in TEPD in comparison with non-CF (3), it is consistent with findings from human CF and non-CF tracheal xenografts (31), and thus may be a consequence of functional differences in the region (i.e., nose vs. trachea) of airway epithelium evaluated or a specific feature of the xenograft model. Fluid secretion from submucosal glands of CFTR−/− tracheas was also significantly reduced — secretion in response to 3 μM forskolin was reduced by 7.4 fold and secretion in response to 1 μM carbachol was reduced by half (Figure 5, C and D, and Supplemental Figure 8). Glandular secretory responses in human CF proximal airways show this same pattern — a large reduction in response to cAMP agonists and a smaller reduction in response to Ca2+ agonists (11, 30). There was no significant difference between CF and non-CF ferret tracheal xenografts, in terms of the glandular area relative to the unit length of the surface airway epithelium (P = 0.679), demonstrating that the observed differences in secretion were not due to altered gland size between genotypes.

A gut-corrected transgenic CFTR-knockout ferret model corrects MI at birth. Similar to the situation for the CF pig and mouse models, the severity of intestinal complications in the CFTR−/− ferret model significantly hinders its application as a research model. In CF mice, correction of intestinal complications by expressing the human CFTR cDNA under the direction of the FABP1 promoter has proven invaluable (32). Taking advantage of this approach, we generated a transgene cassette that expresses the CFTR cDNA under the direction of the FABP1 promoter (Figure 6A). This CFTR cDNA contains a HA-tag inserted into the fourth extracellular...
loop and demonstrated normal chloride channel function in vitro (data not shown). The linear fragment of the FABPi-HA-CFTR-PGK-Zeo cassette was transfected into primary fibroblasts derived from a female CFTR−/− 28-day-old embryo, and selected pools of the transgenic cells were used for SCNT. Four transgenic FABPi-HA-CFTR/CFTR−/− clones were born harboring the transgene cassette in their genomic DNA (Figure 6C), but only 1 survived the early postnatal period and passed stool normally (clone-1). The 3 additional clones born had to be euthanized within 36 hours after birth due to MI (Figure 6B, clone-2, -3, and -4). The surviving clone-1 was also euthanized to determine the expression pattern of recombinant CFTR, and this clone had a grossly normal intestine, lacking any signs of MI (Figure 6B, clone-1). Analysis of intestinal CFTR expression in the 4 clones demonstrated that clone-1 expressed the highest levels of CFTR protein (Figure 6D), correlating with the lack of a neonatal MI phenotype in this clone. Additionally, primary fibroblasts, liver, and lung were harvested from clone-1 to confirm tissue-specific expression of CFTR prior to nuclear transfer recloning and expansion of the line. Results from this analysis demonstrated that FABPi-HA-CFTR/CFTR−/− clone-1 expressed CFTR in the intestine but not in the lung and liver (Figure 6E). Endogenous CFTR protein was expressed in all of these organs from CFTR+/− kits but not CFTR−/− kits (Figure 6E).

Currently, SCNT is being performed to expand the clone-1 line.

Discussion

Although the gene responsible for CF was discovered more than 2 decades ago, it still remains unclear how CFTR defects lead to the airway disease that is responsible for most CF deaths. Such slow progress can be attributed to the lack of an adequate animal model of CF airway disease. Although CF mice have illuminated so many aspects of CF pathophysiology, they do not display a human-like CF airways disease. The need for different animal models of CF prompted the creation of CF pig and ferret models. We believe this is the first report of early phenotypic features in a genetically engineered ferret model of CF, demonstrating that it shares many of the abnormalities seen in newborn humans with CF. To our knowledge, CF is the first human disease for which directed engineering has generated 2 non-rodent knockout models, and comparative studies on organ-specific CF disease phenotypes in 4 species (human, mouse, ferret, and pig) will greatly expand our understanding of CF pathogenesis.

The greatest cause of CF neonatal mortality in all species is intestinal obstruction, but with important differences in penetrance, age at onset, and phenotype. Human with CF and CF pigs and ferrets display a true MI at birth, whereas CF mice develop intestinal complications at a different stage in development (i.e., weaning to solid chow). The penetrance of MI varies greatly among humans with CF (~15%), CF ferrets (~75%), and CF pigs (100%), and a wide

### Table 3

**Bacteriology of BAL and fecal samples**

<table>
<thead>
<tr>
<th>Animal (experiment)</th>
<th>CFTR genotype</th>
<th>Age (d)</th>
<th>Sample</th>
<th>Density of growth</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1)</td>
<td>+/-</td>
<td>2</td>
<td>BAL</td>
<td>+</td>
<td>Streptococcus spp, alpha haemolytic</td>
</tr>
<tr>
<td>2 (1)</td>
<td>+/-</td>
<td>2</td>
<td>BAL</td>
<td>-</td>
<td>No growth</td>
</tr>
<tr>
<td>3 (1)</td>
<td>+/-</td>
<td>2</td>
<td>BAL</td>
<td>+</td>
<td>Actinomyces spp</td>
</tr>
<tr>
<td>4 (1)</td>
<td>+/-</td>
<td>2</td>
<td>BAL</td>
<td>+++</td>
<td>Staphyloccocus spp; Enterococcus spp</td>
</tr>
<tr>
<td>5 (1)</td>
<td>+/- (MI)</td>
<td>2</td>
<td>BAL</td>
<td>+</td>
<td>Enterococcus spp</td>
</tr>
<tr>
<td>6 (1)</td>
<td>+/- (MI)</td>
<td>2</td>
<td>BAL</td>
<td>+++</td>
<td>Bacillus spp</td>
</tr>
<tr>
<td>7 (2)</td>
<td>+/-</td>
<td>2</td>
<td>BAL</td>
<td>+</td>
<td>Corynebacterium spp; Enterococcus spp; Actinomyces spp</td>
</tr>
<tr>
<td>8 (2)</td>
<td>+/-</td>
<td>2</td>
<td>BAL</td>
<td>+</td>
<td>Streptococcus spp, alpha haemolytic; Enterococcus faecalis; Proteus mirabilis</td>
</tr>
<tr>
<td>9 (2)</td>
<td>+/- (MI)</td>
<td>2</td>
<td>BAL</td>
<td>+++</td>
<td>Enterococcus faecalis; Coagulase negative Staph group; Corynebacterium spp; Chryseobacterium indologenes; Clostridium perfringens</td>
</tr>
<tr>
<td>10 (2)</td>
<td>+/-</td>
<td>2</td>
<td>BAL</td>
<td>+++</td>
<td>Streptococcus spp, alpha haemolytic; Enterococcus species; Enterococcus faecalis; Staphylococcus epidermidis; Staph pseudointermies; Proteus mirabilis</td>
</tr>
<tr>
<td>11 (3)</td>
<td>+/-</td>
<td>7</td>
<td>BAL</td>
<td>+</td>
<td>Staph pseudointermies; Streptococcus spp, alpha haemolytic</td>
</tr>
<tr>
<td>12 (3)</td>
<td>+/-</td>
<td>7</td>
<td>BAL</td>
<td>++</td>
<td>Streptococcus spp, alpha haemolytic; Coag negative Staph group; Clostridium perfringens; Proteus mirabilis</td>
</tr>
<tr>
<td>13 (4)</td>
<td>+/-</td>
<td>20</td>
<td>BAL</td>
<td>+</td>
<td>Enterococcus faecalis; Staph pseudointermies</td>
</tr>
<tr>
<td>14 (4)</td>
<td>+/-</td>
<td>20</td>
<td>BAL</td>
<td>++</td>
<td>Enterococcus faecalis; Staph pseudointermies</td>
</tr>
<tr>
<td>15 (5)</td>
<td>+/-</td>
<td>32</td>
<td>BAL⁵</td>
<td>+</td>
<td>Coagulase negative Staph group; Non-fermenter species; Haemophilus spp</td>
</tr>
<tr>
<td>16 (5)</td>
<td>+/-</td>
<td>32</td>
<td>BAL⁵</td>
<td>+</td>
<td>Streptococcus spp, alpha haemolytic</td>
</tr>
<tr>
<td>17 (6–8)</td>
<td>+/-</td>
<td>174⁶; 176⁶; 178⁶ Feces</td>
<td>+++⁺⁺⁺</td>
<td>Proteus vulgaris⁶; Proteus mirabilis⁶; Streptococcus spp, alpha haemolytic⁶; E. coli⁶; Enterococcus faecalis⁶; Enterococcus faecium⁶; Clostridium perfringens⁶; Corynebacterium spp⁶</td>
<td></td>
</tr>
<tr>
<td>18 (6–8)</td>
<td>+/-</td>
<td>174⁶; 176⁶; 178⁶ Feces</td>
<td>+++⁺⁺⁺</td>
<td>Proteus mirabilis⁶; Streptococcus spp, alpha haemolytic⁶; E. coli⁶; Enterococcus faecalis⁶; Enterococcus spp⁶; Enterococcus faecium⁶; Clostridium perfringens⁶</td>
<td></td>
</tr>
</tbody>
</table>

*Evaluation of growth was based on bacterial colonies appearing in quadrants 1 (+), 2 (++), 3 (+++), 4 (++++) of the nonselective bacterial cultures plates.

*Animals that died from MI are denoted by "(MI)." All other CFTR−/− animals passed stool. Single lobe lavages were used to retain integrity of histopathology. Letters D, E, and F indicate a correlation between the day in age of collection and the presence of the bacteria found on that day. Feces were collected every other day for a total of 3 collections.
The variance in intestinal obstruction is also observed in CF mice (0%–100%) depending on the strain background (5, 6). Modifier genes appear to influence the occurrence of MI in infants with CF based on monozygous and dizygous CF twin/triplet studies (33), and our studies also suggest that the hob exerts a genetic influence for development of MI in CFTR−/− ferrets. Complete penetrance of MI in the CFTR−/− piglets studied to date may be a consequence of their intestinal anatomy and/or their inbred status.

The severity of CF pancreatic pathology at birth differs widely across the species, with pigs being most severe (7), ferrets and humans being similar (16, 17), and mice being least severe (6). The sparing of the pancreas in newborn mice has been attributed to alternative Ca2+-activated chloride channels in this organ (34), and it will be interesting to determine whether this single factor can account for the variation across all 4 species. The vas deferens is another tissue that demonstrates variable CF disease pathology between the species. Congenital bilateral absence of the vas deferens is diagnosed in nearly all (~99%) of adult males with CF (18). Even though “congenital absence” implies that this structure is not present at birth, most infant males with CF have an intact vas deferens at birth (17), and increased detection rate in younger patients suggests that disease in this tissue is a degenerative process that may begin prior to birth in some individuals and culminate with destruction by adulthood (19). Interestingly, with the exception of the CF ferret that demonstrates an absent or degenerate vas deferens at birth, other CF animal models have not replicated this phenomenon — CF mice have minor histologic changes to the vas deferens but are fertile (35), and CF pigs appear to have intact vas deferens at birth (7). Whether CF pigs will develop disease of the vas deferens in adulthood remains to be determined.

Of all species, CF newborn ferrets have the most severely impaired nutritional status at birth. The ferret intestinal tract is unique in comparison with those of humans, pigs, and mice. As an obligate carnivore, ferrets lack a cecum, which is found in humans, pigs, and mice; this structure is known to assist in the digestion of plant material. The ferret also has a shorter intestinal transit time in comparison to other species (36, 37). These features likely impose special nutrient requirements, which to date remain to be defined.
at the molecular level. The complete lack of weight gain in new- 
born CF ferrets suggests that intestinal CFTR plays a critical role in 
nutritional absorption in this species. Additionally, the pancreatic 
pathology and bile acid–dependent liver abnormalities observed 
at birth in CF ferrets may also play a role in their poor nutrition.

Oral administration of a proton-pump inhibitor significantly 
improved nutrition in CF ferrets, suggesting that CFTR-depen- 
dent perturbations in GI pH may have a significant impact on 
the absorption of fat and/or other key nutrients in this species. Proton-
pump inhibitors have been extensively used in CF patients suffering 
from sustained steatorrhea while on pancreatic enzymes, and these 
drugs appear to improve nutritional status according to some 
reports (29, 38). Furthermore, reduced GI pH in a Cfr−/− mouse model has been suggested to impair lipolysis and fat absorp- 
tion by the intestine (wild type, 94% ± 0.3% absorption vs. knock- 
out, 89.7% ± 1.2% absorption), despite normal lipase and bicarbo- 
nate secretion by the pancreas (39), and this defect was corrected 
by oral administration of a proton-pump inhibitor. Interest- 
ingly, elevated bile salt secretion into the intestine was also observed in Cfr−/− mice, despite normal levels of bile salt secretion by the 
biliary system (39). The authors of this study concluded that Cfr−/− mice suffer from fat mal- 
absorption due to impairment of the duodenal bicarbonate production that is required for efficient lipolysis and uptake of fatty acids (39). It is interesting, 
however, that AF508-CFTR mice do not suffer from the same 
lipolysis and fatty acid uptake defects seen in Cfr−/− mice (39). 
Although further studies on intestinal biology in the ferret 
are needed to understand how CFTR in this organ might directly 
fluence lipid absorption in this species, it seems plausible 
that CFTR−/− ferrets suffer from a relatively pronounced intestinal 
ph imbalance that influences fat absorption in a similar manner 
as in Cfr−/− mice. These and other issues will likely be clari- 

cation of the FAPBi promoter as shown. (D) Detection of CFTR protein levels in intestinal lysates from the 4 FABP- 
HA-CFTR/Cftr−/− clones and a CFTR−/− kit by CFTR immunoprecipitation, followed by in vitro phosphorylation in the presence of [γ-32P]ATP and protein kinase A. (E) Comparison of CFTR protein levels using in vitro phosphorylation of 
immunoprecipitated CFTR from the intestine, lung, and liver of FABP-HA-CFTR/Cftr−/− clone-1. Lanes 
show results for CFTR−/−, CFTR+/−, and FABP-HA-CFTR/Cftr−/− clone-1 kits. The fully glycosylated band 
C and partially processed band-B forms of CFTR are shown (note that migration of transgenic CFTR is 
slightly shorter than that of endogenous CFTR, due to the presence of the HA-tag).

Figure 6

Generation of a gut-corrected transgenic CFTR−/− ferret by SCNT. (A) Schematic diagram of the FAPBi- 
HA-CFTR-PGK-Zeo cassette used to generate transgenic ferrets expressing HA-tagged CFTR under 
the control of the FAPBi promoter (FABP-Pr) and bovine growth hormone (BGH) poly-A. pFABP, plasmid FABP. (B) Primary fibroblasts were transfected with the linear transgenic fragment shown in A, and 
selected pools were used for SCNT. Four cloned kits were born, and the gross morphology of the intestine 
is shown. Clone-1 passed stool normally within 24 hours of birth, while clone-2, -3, and -4 suffered from 
MI and failed to pass stool. St, stomach. (C) DNA PCR genotyping of the 4 transgenic FABP-HA-CFTR/Cftr−/− 
cloned kits served as a negative control, while plasmid DNA (pCFTR) 
harborizing the transgene cassette was used as a positive control. The PCR reactions were designed to 
specifically detect a segment of the HA-tag and CFTR cDNA or the rat FABPi promoter as shown. (D) 
Expression of CFTR protein levels in intestinal lysates from the 4 FABP- 
HA-CFTR/Cftr−/− clones and a CFTR−/− kit by CFTR immunoprecipitation, followed by in vitro phosphorylation in the presence of 
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show results for CFTR−/−, CFTR+/−, and FABP-HA-CFTR/Cftr−/− clone-1 kits. The fully glycosylated band 
C and partially processed band-B forms of CFTR are shown (note that migration of transgenic CFTR is 
slightly shorter than that of endogenous CFTR, due to the presence of the HA-tag).
first week, lung infections did not appear to be the primary cause of death in CFTR−/− kits. It is interesting to speculate that neonatal aspiration events may serve as inoculation mechanisms, and defective bacterial eradication compromises the ability of the CF lung to clear normal early pathogens like control animals. Although the number of animals analyzed remains low, our observations of early fatal lung infections and rectal prolapse in CFTR−/− kits is also similar to clinical observations in CF infants during the early 1950s (40). In this case study of 68 CF infants, over 60% died before the age of 1 year, while only 15% survived 2–13 years. More than half of the number of animals analyzed remains low, our observations of death in CFTR−/− offspring and failure to thrive during the neonatal period despite a ravenous appetite was common. These observations are quite similar to our findings with CFTR−/− kits. As with humans, the improved use of broad-spectrum antibiotics during the neonatal period may help to improve survival in this ferret model of CF. These initial findings in the lung and other organs of CFTR−/− ferrets, suggest that the ferret may be a useful model for dissecting CF pathophysiology and developing therapies. This report also describes the production of what we believe to be the first transgenic ferret using SCNT, and such methods will expand opportunities for genetically dissecting CF pathogenesis in the ferret model.

Methods

Animals and genotyping. All animal experimentation was performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Iowa. Eight CFTR−/− (F0) male ferret clones of sable coat color background were used to expand the colony by breeding. PCR products (100 bp and 180 bp) were resolved on a 1.5% agarose gel. PCR amplification of genomic ferret using SCNT, and such methods will expand opportunities for genetically dissecting CF pathogenesis in the ferret model.

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trends for stimulatory condition b were intermediate to conditions a and c. Condition b was tested to evaluate the synergy of cAMP and Ca²⁺-dependent pathways for glandular secretion that have previously been described (43). Digital images of the spherical mucous bubbles under mineral oil were obtained at 5-minute intervals, based on which the secretory rates for individual glands were calculated using ImageJ software (http://rsweb.nih.gov/ij/). Typically, approximately 10–15 glands were imaged per xenograft tissue sample. The average number of gland bubbles observed per mm² was 1.8 ± 0.7 for non-CF samples and 1.9 ± 0.3 for CF samples. To confirm that differences in glandular secretory rates between CF and non-CF xenografts were not due to differences in gland size, we performed morphometric analysis on the samples analyzed. After functional assays were completed, the xenograft tissue was fixed in formalin and paraffin embedded, and 5–6 sections at approximately 500-μm intervals through the depth of the trachea were obtained. The unit length of the surface airway basal lamina and the glandular area were measured for each section using Metamorph software. The total length of the surface airway epithelium (basal lamina) was then divided by the glandular area for each sample to produce a glandular size index for each sample. On average, approximately 75 photomicrographs were evaluated for each xenograft sample (n = 5 for non-CF and n = 6 for CF). Statistical comparison of the glandular size indexes using the Student’s t-test demonstrated that the glandular size did not differ significantly between the 2 genotypes.

Neonatal clinical care of CFTR+/− kits. Two treatment protocols were developed to enhance postnatal survival and reduce the risk of intestinal obstruction caused by MI. Both protocols included administering metronidazole (5 mg/kg in 100 μl dextrose saline, s.c.) 4 times daily beginning at the time of birth, starving the animals for the first 3 hours after birth, and then administering a 150- to 200-μl Golytely gavage (Braintree Laboratories Inc.) using a fire-polished microloader pipette tip (Eppendorf Inc.) (see Supplemental Figure 9 for example of gavage tube components). The gavage was repeated every 6 hours, up to 3 times, until meconium was passed. After the CF kits passed meconium, they were fed 150–200 μl Elecare (Abbott Laboratories) supplemented with UDCA (5 mg/kg) and/or omeprazole (5 mg/kg) 4 times daily by gavage. When the diet was supplemented with pancreatic enzymes, Viokase-V was used and dosed according to lipase units as specified in the legend for Figure 4. Once genotypes were known, typically at 12 to 18 hours after birth, the litter size was reduced, such that only the CFTR+/− and/or CFTR−/− kits and an equal number of controls (CFTR+/+ and/or CFTR+++) were kept. When kits were weaned at 5 weeks of age, they were fed a slurry of Elecare hydrated solid chow (Marshall Farms) twice daily, mixed with 1 lipase unit of Viokase-V per 10 grams of body weight (the amount of food eaten with each feeding was recorded for these calculations).

Blood chemistries. Blood chemistries were performed using a VetScan VS2 (Abaxis), using blood drawn at the time of clinical death and necropsy. Mammalian Liver Profile rotors from Abaxis were used to assess plasma levels of alkaline phosphatase, ALT, bile acids, total bilirubin, total cholesterol, gamma glutamyl transferase, and blood urea nitrogen. Total and direct bilirubin levels in the plasma were measured using 2 kits from Diazyme Inc. for total bilirubin (DZ150A-K) and direct bilirubin (DZ151A-K); to find the level of indirect bilirubin in the plasma, we used the following formula: indirect bilirubin = total bilirubin − direct bilirubin.

Histopathology. Standard histopathology analysis was performed on paraffin sections from formalin-fixed tissues. Tissues were collected at the time of euthanasia (clinical death) and immediately placed in 10% neutral buffered formalin for at least 72 hours. Tissues were then paraffin embedded, sectioned (4–5 μm), and stained with H&E or periodic acid-Schiff. A modified Gram-Twort procedure was used to identify Gram-positive and Gram-negative bacteria in tissue sections (44). Briefly, this technique uses a Gram stain protocol, followed by application of Twort solution (containing nuclear fast red). This procedure results in Gram-positive bacteria staining blue/black and Gram-negative bacteria staining red/pink. Histopathological examination was performed by a veterinary pathologist, and age-matched CFTR+/− and CFTR−/− or CFTR+++ controls were used.

Bacteriology of BAL fluid and fecal samples. BAL was collected when animals were euthanized and performed in a laminar flow hood under sterile surgical conditions. A small incision was made to expose the trachea, and a 23-gauge angiocatheter was inserted into the proximal end of the trachea and stabilized using surgical sutures. Sterile saline (1–2 ml) was used to lavage the lung. This was repeated 3 times, and BAL fluid was pooled. Fecal samples were collected from the oldest CFTR+/− and control animal, and 0.2 grams were resuspended in 3 ml of sterile saline by vortexing with glass beads. Three percent of each BAL fluid or fecal sample was plated onto various types of bacterial growth media for bacteriologies. Fecal and BAL samples were streaked directly and were plated from enrichment broth onto selective and nonselective agar media (blood agar, anaerobic blood agar, MacConkey agar, colistin and nalidixic acid agar, anaerobic colistin and nalidixic acid reducible agar, chocolate agar [for BAL only], Hec-toen enteric agar [for fecal only], brilliant green agar [for fecal only], XLT4 agar [for fecal only], tetrathionate broth, chopped meat glucose broth, brain heart infusion broth). Cultures were incubated aerobiologically and anaerobiologically for 24–48 hours at 35°C. All plates were examined for bacterial growth and visually assessed for bacterial types and quantity. Growth was measured semiquantitatively based on colony distribution on the plates within the 4-quadrant streak pattern. Growth on nonselective media was ranked for growth based on the occurrence of colonies in quadrants 1–4 (+++), 1–3 (++), 1–2 (+), or 1 only (+). Representative colonies from mixed cultures were subcultured to purity onto appropriate media. Bacterial identification was based on colony morphology, gram stain, and biochemical reactions (45). Extended bacterial identification was performed if needed using an API 20E test kit.

SCNT cloning of a CFTR−/− transgenic ferret with intestine-specific expression of wild-type ferret CFTR. A transgene cassette containing the rat FABP fibroblast promoter (32) (gift of Jeffrey Whitsett, University of Cincinnati, Cincinnati, Ohio, USA) driving the expression of a HA-tagged CFTR cDNA with a bovine growth hormone poly-A was generated in plasmid pCDNA3.1. This cassette also contained a floxed PGK promoter-driven Zeocin/SV40–poly-A resistance gene cassette for selection of transfected cells. The wild-type CFTR DNA was engineered to contain a 3xHA tag in extracellular loop 4 by PCR-mediated cloning, and its sequence was based on that previously described for generating 3xHA-tagged human CFTR (46). The cDNA sequence of the 3xHA-tag was 5′-aggtcactcacTCTGAAGTCCAAGGCACCTTCGAT-TACGCTTACCTTACGAGTTCCTGATTACGCTGCTAGCTACCCTTACGACGTTCCTGAT-3′ (sequences in lowercase letters show homology to the junctional CFTR sequence, and those in uppercase letters represent the 3xHA-tag sequence). The amino acid sequence of this tag is as follows: S7QSLEPYPIEPDVDPYDAASYTPIDPVDPYDAASVYSS (italicized amino acids are junctional CFTR amino acids, and non-italicized amino acids are the HA tag sequence; the underlined letters are the 3 HA amino acid epitopes). The linear fragment encompassing the FABP-HA-CFTR/PKG-Zeocon transgenic cassette was isolated by Pmel digestion of the pFABP-HA-CFTR-Zeo plasmid, followed by agarose-gel purification. This gel-puriﬁed fragment was then transfected into primary ﬁbroblasts generated from a female 28-day-old CFTR−/− kit. The transfected CFTR−/− fibroblasts were selected in 50 μg/ml Zeocin (Invitrogen) for 3 weeks, and the zeocin-resistant cells from this pool were used as donors for SCNT according to previously described protocols (15, 47). Cloned kits were evaluated for the presence of the transgene in their genomic DNA using PCR and phenotyped for MI and tissue expression of the HA-CFTR transgene.
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Statistics. Statistical significance for all comparisons (with the exception of inheritance studies) was assessed using an unpaired, 2-tailed Student’s t test. Inheritance of the M1 phenotype was performed using the Pearson’s χ2 test, Fisher’s exact test, and logistic regression. For logistic regression, the response variable was the M1 status of the offspring, and the explanatory variable was the hob and/or jill identification. In all statistical analyses, P values of less than 0.05 were considered significant.