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Porcine models of cystic fibrosis reveal male reproductive tract phenotype at birth

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Title: Porcine Models of Cystic Fibrosis Reveal Male Reproductive Tract Phenotype at Birth

Short title: CBAVD phenotype in porcine cystic fibrosis models

Summary sentence: Vas deferens and epididymis abnormalities are present in *CFTR*$^{-/-}$ and *CFTR*$^{\Delta F508/\Delta F508}$ pigs at birth.

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Abstract

Nearly all male cystic fibrosis (CF) patients exhibit tissue abnormalities in the reproductive tract, a condition that renders them azoospermic and infertile. Two porcine CF models have been reported recently that include respiratory and digestive manifestations that are comparable to human CF. The goal of this study was to determine the phenotypic changes that may be present in the vas deferens of these porcine CF models. Tracts from *CFTR*$^{-/-}$ and *CFTR*$^{\Delta F508/\Delta F508}$ neonates revealed partial or total vas deferens and/or epididymis atresia at birth, while wild-type (WT) littermates were normal. Histopathological analysis revealed a range of tissue abnormalities and disruptions in tubular organization. Vas deferens epithelial cells were isolated and electrophysiological results support that *CFTR*$^{-/-}$ monolayers can exhibit Na^+ reabsorption but reveal no anion secretion following exposure to cAMP-generating compounds, suggesting that CFTR-dependent Cl^- and/or HCO_3^- transport is completely impaired. SLC26A3 and SLC26A6 immunoreactivities were detected in all experimental groups, indicating that these two chloride-bicarbonate exchangers were present, but were either unable to function or their activity is electroneutral. In addition, no signs of increased mucus synthesis and/or secretion were present in the male excurrent ducts of these CF models. Results demonstrate a causal link between *CFTR* mutations and duct abnormalities that are manifested at birth.

Introduction

Cystic fibrosis (CF), a life-threatening human disease with multi-organ symptomology, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [1, 2]. CFTR is a chloride and bicarbonate channel that is most commonly expressed and functional at the apical membrane of epithelia throughout the body [2, 3]. CF pathology includes debilitating respiratory and gastro-intestinal symptoms [2], and a male reproductive tract abnormality of high penetrance. Greater than 95% of men carrying two alleles harboring *CFTR* mutations exhibit a reproductive condition termed congenital bilateral absence of the vas deferens (CBAVD), which is characterized by vas deferens and epididymal tissue atresia that renders CF patients with permanent post-testicular azoospermia and infertility [4-7].

Mouse models of CF have been made, but this species does not reproduce major symptomatology observed in humans [8]. Neither *Cftr* knockout ($Cftr^{-/-}$) mice nor mice carrying homozygous ΔF508 mutations (the most frequent human CF mutation) in the *Cftr* gene ($Cftr^{\Delta F508/\Delta F508}$) were reported to exhibit CBAVD [9]. Alternatively, a single report states that a vas deferens could not be identified in long-lived $Cftr^{-/-}$ mice [10]. Nonetheless, much more successful outcomes have rewarded recent efforts in the development of CF animal models. A $CFTR^{-/-}$ pig revealed a nearly complete spectrum of organ pathology: airway, pancreas, liver and intestinal diseases were manifested at birth [11-13]. A porcine CF model carrying homozygous ΔF508 mutations ($CFTR^{\Delta F508/\Delta F508}$) [14], as well as a ferret CF model [15] have also been generated. Initial reports indicated that the male reproductive tract in $CFTR^{-/-}$ pigs was apparently normal at birth [13].

The role of CFTR and other bicarbonate transporters in vas deferens epithelia is of primary interest and focus in this laboratory, and models of adult porcine and human vas

deferens epithelia were generated and characterized [16-18]. In addition, luminal pH in the adult porcine vas deferens can increase rapidly in vivo, via a bicarbonate secretion mechanism that is thought to include CFTR, bicarbonate exchangers and a bicarbonate co-transporter [19, 20]. Thus, characterization of the porcine *CFTR*$^{-/-}$ and *CFTR*$^{\Delta F508/\Delta F508}$ vasa deferentia was hypothesized to be a valuable system that could be used to elucidate the role of CFTR - and of other bicarbonate transporters when CFTR function is muted - in the male duct. Close examinations of porcine CFTR$^{-/-}$ reproductive tracts conducted by this laboratory revealed that tissue abnormalities are present in the vas deferens and epididymis at birth. Therefore, more extensive and systematic evaluations of the CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$ male reproductive tracts were conducted. Here we report that both porcine CF models reveal vas deferens and epididymal atresia, a phenotype that recapitulates human CBAVD. Histopathological, histochemical, electrophysiological and protein immunoreactivity results are also reported to provide an initial description of the abnormalities that arise in the male reproductive tract in the absence of normal CFTR function.

Material and Methods

Animal models and tissue procurement. The CF porcine models generated at the University of Iowa [11-14] were used in this study, and tissues were obtained in accordance with an animal protocol approved by that institution. Neonatal males were genotyped, sacrificed and their reproductive tracts excised. Tracts were immersed in buffered physiological medium, packaged in a cooled container and shipped to Kansas State University, where they were finely dissected, inspected, photographed and employed in the studies reported here. Tissues were in transit for less than 24 hs.

Histological preparations and stains. Vas deferens and epididymal tissues were fixed by immersion in either 4% paraformaldehyde or Carnoy's solution (9 ethanol : 3 chloroform : 1 glacial acetic acid) for 24-48 hs. Tissues were paraffin embedded, sectioned at 4 μm and collected on slides for subsequent staining. Sections were subjected to either automated hematoxylin and eosin (H&E) stain procedure or were PAS stained as per the manufacturer's instructions (Sigma, St. Louis, MO), with the exception that no hematoxylin counterstain was conducted on PAS stained tissues.

Neonatal vas deferens epithelial cell isolations and culture. Vas deferens epithelial cells were isolated using methods described previously with minor modifications [16]. It should be noted that $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ vasa, which were employed for cell isolations in this study, were often not full length due to apparent duct atresia. Briefly, following excision from the male tract, vasa were cannulated (35 gauge) and Hank's balanced salt solution (HBSS) was used to flush the lumen. The duct was filled with an enzyme preparation (collagenase/trypsin) and both distal and proximal ends were clamped with hemostats. Tissues were incubated at 37°C for 30 minutes, while immersed in HBSS. Cannulation and flushing were used again to harvest

epithelial cells that had detached. Cells were centrifuged at 1000 rpm for 5 minutes, suspended in culture medium [16] and allowed to attach and grow on solid supports for up to 5 days. Cells were lifted and seeded on 0.33 cm^2 permeable supports (Corning Inc., Lowell, MA) where they typically attached, grew and organized to become confluent monolayers. Cultured cells, which are denominated here as primary neonatal porcine vas deferens (n1^{o}PVD) epithelial monolayers, were employed in experiments 10-14 days after seeding on permeable supports. In a subset of experiments, n1^{o}PVD were kept either untreated or were exposed to dexamethasone (100 nM; Sigma, Saint Louis, MO) for 72-96 hours prior to assay.

Electrophysiology and data analysis. n1^{o}PVD cell monolayers were mounted in modified Ussing flux chambers, bathed symmetrically in Ringer solution (composition in mM: 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.83 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$), maintained at 39^{o}C and bubbled with 5% CO_2-95% O_2. Monolayers were clamped to 0 mV and short-circuit current (I_{SC}) was measured continuously with multi-channel voltage-clamp apparatuses (model 558C, University of Iowa [Iowa City, IA] or model VCCMC8 [Physiologic Instruments, San Diego, CA]). Data were acquired digitally at either 1 or 5 Hz on Intel-based computers with AcqKnowledge (version 3.7.3, BIOPAC Systems) or Acquire and Analyze (version 2.3.159; Physiologic Instruments), respectively. Monolayers were exposed to different compounds: amiloride, norepinephrin, adenosine, PGE2 and bumetanide (Sigma); forskolin (Calbiochem, La Jolla, CA) and DASU-02 (synthesized de novo [21]). Recorded data were compiled and subjected to statistical analysis that included paired or unpaired two-tailed *t*-tests (Microsoft Office Excel 2003, Microsoft Corporation, Redmond, WA). All graphs were made with SigmaPlot (version 10.0; Systat Software Inc., Point Richmond, CA).

Western blotting and sera. Protein lysates were generated from n1^{o}PVD and Calu-03 cell monolayers via direct lysis in a 2X-Laemmli sample buffer containing 4% SDS and 125 mM Tris-HCl, at pH 6.8. Following homogenization, samples were aspirated repeatedly through 25 or 30 gauge needles and centrifuged at 14000 rpm, for 15 min and at 14°C. Protein concentration was determined by the BCA method at 562 nm (ND-8000 NanoDrop Products, Wilmington, DE) using concentration standards of bovine serum albumin (Pierce, Rockford IL). Prior to electrophoresis, DTT was added to lysates (50 mM) and samples incubated for 1 hour at 40°C. Equal protein masses from each sample were resolved on 4-20% Tris-HEPES-SDS gradient gels (Pierce). Blotting onto PVDF membranes (Millipore, Billerica, MA) was conducted at 100V, for 4 hours, at 4°C. Membranes were blocked in 5% molecular grade dry milk overnight and subsequently incubated with primary antibodies. Anti-CFTR (clone M3A7, Millipore), anti-SLC26A3 (clone N12, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-SLC26A6 (clone C17, Santa Cruz Biotechnology) were employed to probe membranes at concentrations of 0.5, 1 and 5 μg/ml, respectively. Suitable peroxidase conjugated secondary antibodies (Pierce) were used at 50 ng/ml. Chemiluminescent signals were obtained with a suitable substrate (Pierce) and acquired digitally (Imagestation 4000R, Eastman Kodak Co., Rochester, NY). Typically, membranes were probed with one of these primary antibodies, then stripped (Pierce) and reprobed for another of the target proteins, and ultimately reprobed with anti-β-actin (Sigma) to assess the degree of protein mass homogeneity loaded in each lane.

Results

CFTR$^{-/-}$ *and CFTR*$^{\Delta F508/\Delta F508}$ *piglets reveal atresia of the male excurrent duct at birth*

Initial examinations of the male reproductive tracts from porcine *CFTR*$^{-/-}$ neonates conducted by this laboratory suggested that anatomical abnormalities of the vas deferens were present in this CF model at birth, although the degree of impact appeared variable. Thus, more extensive examinations were conducted in CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$ male tracts, as well as heterozygous and wild type *CFTR* homozygous samples, which are referred to as WT.

Macroscopic inspections of CFTR$^{-/-}$ tracts revealed that 32 of 34 samples exhibited vas deferens and/or epididymis atresia, while all 50 WT littermates examined had a normal phenotype (Fig. 1A-1F). Vas deferens atresia was often localized to the distal region of the duct (prostatic end), while complete absence of a vas deferens in both spermatic cords was detected in some individuals. In 1 of the 32 abnormal samples, absence of vas deferens was unilateral. In 4 samples, there were no apparent macroscopic abnormalities of the vas deferens, but these samples presented some degree of atresia in the epididymes. Epididymal atresia was observed most often in the corpus, but in some cases, the cauda epididymis was also affected. A rudimentary caput epididymis was present in all CFTR$^{-/-}$ tissues in which an epididymal phenotype was present (Fig. 1E-F).

CFTR$^{\Delta F508/\Delta F508}$ piglets also exhibited vas deferens and/or epididymis atresia in 17 of 19 samples, while all 26 WT littermates presented normal anatomy. CFTR$^{\Delta F508/\Delta F508}$ vas deferens and epididymal phenotypes were similar to that of CFTR$^{-/-}$, and complete absence of the vas deferens was documented in tissues derived from some *CFTR*$^{\Delta F508/\Delta F508}$ pigs (Fig. 1G-H).

These observations demonstrate that, much like the gross pathology described in CF male patients, these porcine CF models exhibit a male reproductive tract phenotype of high penetrance.

Histopathological analysis reveals that vas deferens and epididymis tissue organization and structure are altered in porcine CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$ neonates

To determine the histological features of the tissues and possibly to provide insights into the pathophysiology of the anatomical abnormalities detected, segments of vas deferens and the epididymis from all experimental groups were paraffin-embedded, sectioned and stained with H&E. WT tissues revealed normal vas deferens histology (Figs 2A-D). Although principal cells occupied most of the epithelium height ranging from the basal lamina to the apical membrane lining the lumen, basal cells were also present and their nuclei were localized next to a defined basal lamina, which separated the epithelium from a robust muscular layer. Peripherally, connective tissue, blood vessels and nerves were present as expected. However, CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$ tissues revealed several abnormalities. Those most severely altered segments of the spermatic cord, seen macroscopically with no signs of duct structure, revealed a homogeneous unorganized mass of fibrous tissue (Fig. 2E). Variable levels of vas deferens duct structure and organization were detected in tissues from other animals (Figs. 2F-G and 2I-L). Additional histopathological abnormalities that were identified in a minority of the *CFTR$^{-/-}$* and *CFTR$^{\Delta F508/\Delta F508}$* piglets analyzed included round areas of apparently empty space in or next to the epithelium (e.g. Fig. 2H) and epithelial cells with degenerative and apoptotic features (data not shown).

Epididymal tissues were analyzed histologically and results confirmed the macroscopic observations. The rudimentary caput epididymides exhibited by *CFTR$^{-/-}$* and *CFTR$^{\Delta F508/\Delta F508}$*

neonates had tubular profiles composed by a regular basal lamina, epithelial cells with apparently normal morphology and a lumen free of debris or detached epithelia (data not shown). The number of tubular profiles in this region appeared to be less than that of WT, as more connective tissue appeared to interspace tubular profiles in $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$, although no systematic morphometric analyses were conducted. All $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ samples that revealed atresia in the cauda region macroscopically, revealed fibrous tissue histologically (data not shown), much like that seen in the spermatic cord.

cAMP-induced anion secretion is altered in $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ vas deferens epithelial monolayers

Histological results described above revealed that lumens lined with epithelial cells were present in the neonatal WT vas deferens. Apparently similar cells were present in $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ vas deferens segments that had maintained a duct structure as seen macroscopically. Thus, it was hypothesized that, if isolated and placed in culture on permeable supports, these remaining epithelial cells may serve as an in vitro model to test for anion secretion mechanisms that have been demonstrated previously in adult porcine vas deferens epithelial cells [16, 20]. Tissues from 34 $CFTR^{-/-}$ and 19 $CFTR^{\Delta F508/\Delta F508}$ neonates were used in attempts to derive neonatal primary porcine vas deferens epithelial cell monolayers ($n1^{o}$PVD). However, confluent monolayers exhibiting a minimum basal transepithelial resistance (R_{TE}) of 100 Ω cm^{-2} after at least 10 days of culture on permeable supports were derived only from 4 $CFTR^{-/-}$ and 2 $CFTR^{\Delta F508/\Delta F508}$ piglets. Therefore electrophysiological data from these $n1^{o}$PVDs, along with WT $n1^{o}$PVDs derived from littermates were acquired with the Ussing flux chamber technique and are reported here.

Initial experiments aimed to determine whether n1^{o}PVDs respond to agonists known to induce rapid I_{SC} increases in adult porcine vas deferens epithelial cells. In these initial assays, n1^{o}PVD monolayers were first exposed to apical amiloride, to which no responses were detected. Following amiloride, WT n1^{o}PVD produced responses to forskolin or receptor agonists (Fig. 3A-C) that were consistent with observations derived from adult porcine vas deferens epithelial cells. WT n1^{o}PVD exhibited rapid increases in I_{SC} that formed a peak, followed by a sustained plateau, which was partially sensitive to bumetanide (Fig. 3A). However, CFTR$^{-/-}$ n1^{o}PVD monolayers were not responsive to forskolin or any of the receptor agonists tested (Figs. 3 D and E).

Given the total absence of cAMP-induced increases in I_{SC} presented by CFTR$^{-/-}$ n1^{o}PVD monolayers, subsequent experiments were designed to test whether amiloride-sensitive I_{SC} could be detected after exposure to corticosteroids prior to assay. The primary rationale for testing this possibility was that adult 1^{o}PVD monolayers are responsive to corticosteroids [22] and CF epithelial are aften reported to exhibit elevated Na^{+} absorption. Thus, we speculated that CFTR$^{-/-}$ n1^{o}PVD would exhibit amiloride-sensitive I_{SC} if they had preserved a degree of epithelial phenotype. Dexamethasone-treated WT n1^{o}PVDs presented mean basal I_{SC} greater than untreated WT n1^{o}PVDs, although this difference did not reach statistical significance ($P < 0.09$, Fig. 4A). Dexamethasone-treated CFTR$^{-/-}$ n1^{o}PVDs exhibited mean basal I_{SC} that was significantly greater than their respective untreated pairs ($P \leq 0.05$). Apical amiloride caused a rapid significant decrease in I_{SC} of all monolayers, both WT and CFTR$^{-/-}$ n1^{o}PVDs that had been cultured in the presence of dexamethasone ($P \leq 0.05$, Figure 4 A and B). Again, no responses to forskolin by CFTR$^{-/-}$ n1^{o}PVDs were observed. Nine CFTR$^{\Delta F508/\Delta F508}$ n1^{o}PVD monolayers have been derived successfully from the vasa deferentia of two *CFTR*$^{\Delta F508/\Delta F508}$ piglets. Fourteen WT monolayers were derived concurrently from three littermates. This set of CFTR$^{\Delta F508/\Delta F508}$-

monolayers and their respective WT pairs were subject to the same experimental design involving dexamethasone exposure, as well as basal and pharmacological tests in Ussing chambers. Figure 4C depicts a summary of the results derived from these monolayers. Consistent with observations from $CFTR^{-/-}$, amiloride induced I_{SC} changes $CFTR^{\Delta F508/\Delta F508}$ monolayers that were exposure to corticosteroids. Interestingly, $CFTR^{\Delta F508/\Delta F508}$ monolayers also responded to forskolin, although responses were suggestively smaller than those of WT (Figure 4B-C). All nine I_{SC} tracings derived from $CFTR^{\Delta F508/\Delta F508}$ monolayers exhibited responses to forskolin (data not shown).

Together these results suggest that neonatal porcine vas deferens epithelia exhibit epithelial cellular differentiation that includes mechanisms to accomplish electrolyte absorption and secretion in a manner similar to that of the adult tissue [16-18], and that cAMP-induced electrogenic anion secretion is muted if WT CFTR is not expressed. Furthermore, these results suggest that $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ neonatal vas deferens exhibits the capacity to absorb sodium, and that $CFTR^{\Delta F508/\Delta F508}$ monolayers can carry out a degree of anion secretion that is not sufficient to prevent the mechanisms leading to anatomical abnormalities in the male reproductive tract.

Bicarbonate exchangers SLC26A3 and SLC26A6 are expressed in cultured neonatal porcine vas deferens in the presence or absence of CFTR expression.

Adult WT porcine vas deferens epithelia express CFTR, SLC26A3 and SLC26A6 both in vivo and in culture [16, 23] and these transporters are thought to serve as apical pathways for bicarbonate secretion across vas deferens epithelium and into the luminal environment. To determine whether SLC26A3 and SLC26A6 are expressed in $n1^{o}$PVD derived from these CF

models, western blots were conducted with cellular lysates from WT, $CFTR^{-/-}$, $CFTR^{\Delta F508/\Delta F508}$, and specific cell lines employed as controls. CFTR-immunoreactivity was not detected in $CFTR^{-/-}$ or $CFTR^{\Delta F508/\Delta F508}$, whereas both SLC26A3 and SLC26A6 immunoreactivities were present in all of the $n1^{o}$PVD samples tested (Fig. 5). These results provide evidence to support the conclusion that cellular differentiation in neonatal $CFTR^{-/-}$, $CFTR^{\Delta F508/\Delta F508}$ and WT vas deferens epithelia reaches a stage in which expression of other Cl^- and HCO_3^- epithelial transporters are present.

$CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ vasa deferentia and epididymides do not exhibit histological signs of increased mucus synthesis, secretion, or accumulation

Luminal mucus accumulation that causes obstruction is reported in the respiratory and gastrointestinal tracts of $CFTR^{-/-}$ pigs [11-13]. In addition, mucins are expressed in the human male epididymis and vas deferens [24]. Thus, it was hypothesized that mucus synthesis, secretion, or accumulation may be increased in the vas deferens of the neonatal *$CFTR^{-/-}$* and *$CFTR^{\Delta F508/\Delta F508}$* pigs. To assess this possibility, histological sections of neonatal WT, $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ vas deferens and epididymal tissues, as well as adult porcine lung tissues, were subject to histochemistry by the PAS method. Results revealed no differences in the pattern or quantity of PAS positive staining among neonatal WT, $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ reproductive ducts (Fig. 6). These results suggest that it is unlikely that enhanced mucus accumulation contributes to the physiopathological mechanism(s) that culminate in the male reproductive tract abnormalities reported here for the *$CFTR^{-/-}$* and *$CFTR^{\Delta F508/\Delta F508}$* neonatal pigs.

Discussion

Results reported here describe the effects that *CFTR* deletion or Δ*F508*-homozygozity has on the male reproductive duct of two porcine CF models that recapitulate human symptomology [11, 13, 25]. Both of these models present male duct atresia that is most often localized to the distal vas deferens and corpus epididymis. Complete vas deferens atresia was sometimes present and in these cases, not only corpus but also cauda epididymis atresia was detected. Caput epididymides with rudimentary morphology were present in all observations, while testis exhibited an unremarkable macroscopic anatomy. These results demonstrate that these CF porcine models recapitulate male reproductive pathology that has been described in humans [4, 6, 7]. Abnormalities of the porcine male reproductive tract were detected already at birth and are temporally consistent with vas deferens abnormalities reported recently in another non-rodent CF animal model, the *CFTR*-null ferret [26]. The simplest interpretation of these data is that the lack of normal CFTR expression in the male excurrent duct either impairs normal duct development and/or induces pathological mechanisms during gestation. Thus, data reported here enhance our understanding of CF male pathology and infertility, as they suggest that there may be mechanisms, and timing to their occurrence that have not been identified by previous studies aimed at elucidating CBAVD pathophysiology.

CBAVD has penetrance greater than 95% among male CF patients and is typically diagnosed at reproductive age [27]. When considering a time-line for the progression of human CBAVD, evidence from human fetuses and pre-pubertal patients suggest that male duct anatomical changes occur postnatally. For instance, two human fetuses that were Δ*F508*-homozygous exhibited no signs of vas deferens abnormalities at 16-18 weeks of gestation [28]. A second study with a larger number of observations and based on sonographic examination of

male children with CF revealed less frequent male reproductive tract abnormalities than in CF adult males [29]. However, no single patient in the cohort studied was homozygous for the Δ*F508* mutation. Therefore, whether there is a marked difference for the time of vas deferens abnormalities development between CF patients and these porcine models or whether this is dictated by the types of mutations, these remain as open questions.

Moreover, respiratory and digestive tract epithelia of CF patients and CF animal models reveal deficient clearance with retained, inspissated and accumulated mucus, which is thought to be among the primary factors causing luminal obstructions and the consequent organ pathologies that follow [11, 13]. Tissues exhibiting a basic ductal structure (i.e. epithelia lining a lumen) were also detected in the affected male reproductive tracts, and these portions did not exhibit any signs of luminal obstructions. Moreover, the *CFTR*-null ferret, which also models CF organ disease to a high degree, manifests vas deferens atresia at birth [26]. Together, data reported here and those from the *CFTR*-null ferret [26] suggest that the pathophysiology of human CBAVD may involve important prenatal events.

Mucus-filled pancreatic ducts and gallbladder, as well as meconium and extremely thick mucoid depositions on the apical aspect of intestinal epithelia are common observations in the $CFTR^{-/-}$ newborn piglet [13]. Increased mucus in obstructed airways is also detected in $CFTR^{-/-}$ pigs at 3-4 months of age [11]. Similar digestive and respiratory tract phenotypes are being described for the $CFTR^{\Delta F508/\Delta F508}$ porcine model [25]. Expression of mucins has been documented in human vas deferens and epididymal epithelia [24], however the segments of vas deferens and epididymis that were present in $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ newborn piglets had lumens free of any obstructing materials and no obvious difference in PAS positive stain was detected when compared visually to WT littermates. Collectively, these data suggest that porcine

male duct pathology evolves downstream from the lack of normal CFTR function in a manner that is independent of inspissated mucus retention and/or luminal duct clogging. One argument against this conclusion could be formulated by proposing that obstructed segments of the duct were phagocytosed and remodeled by inflammatory cells. However, this seems unlikely because none of the histological sections derived from portions of the $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ vas deferens and epididymis that exhibited ductal architecture revealed signs of duct obstruction and/or a collection of inflammatory cells populating the luminal space. Inflammatory cells were only seen in low single digit counts, isolated and in very few of the histopathological sections we derived from these tissues. Furthermore, this near total absence of inflammatory cells in $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ vas deferens and epididymis contrasts with observations derived from the $CFTR^{-/-}$ liver, which exhibited significant inflammatory cell populations at birth [13]. Ultimately, the pathophysiological mechanisms accounting for porcine male duct abnormalities remain unknown. Additional investigation to elucidate these mechanisms will require analyses of fetal male duct tissues to determine the earliest stage in ductal development at which structural disruptions become apparent. Supported by the data reported here, we would expect to observe such initial disruptions in ductal structure to occur while the lumen of the developing male duct remains free of any obstructive material.

The most prevalent localization of duct atresia in the vas deferens of animals examined in this study was distal, near the prostate. Distal vas deferens epithelia express high levels of cyclooxygenase-2 (COX-2 or PTGS2) under testosterone regulation [30]. In addition, during male fetal development, the testosterone peak induces COX-2 expression in human distal vas deferens epithelial cells [31]. COX expression and prostaglandin synthesis is upregulated by testosterone in epididymal and vas deferens epithelial cells, where this pathway induces anion

secretion that is largely CFTR-dependent [32, 33]. Thus, it is likely that in the absence of CFTR, a reduction in anion secretion and duct luminal flow occurs, generating a state of reduced local clearance, and consequent increase in the levels of prostaglandins, cellular stress and apoptosis. Reduced local clearance could also increase signaling by growth factors such as TGFβ, which is synthesized and secreted by epithelial cells lining the epididymis [34]. TGFβ signaling can induce pathological epithelial-to-mesenchymal transformation and tissue remodeling [35, 36]. Furthermore, a recent report indicates that $CFTR^{-/-}$ piglets have significantly reduced serum levels of insulin-like growth factor 1 (IGF-1) at birth [37]. In mice, IGF-1-knockout is associated with anatomical abnormalities of the male excurrent duct and seminal vesicles [38], suggesting that IGF-1 could have a role in the pathophysiology of CBAVD.

Results from cell isolation and culture experiments reported here demonstrate that cells derived from both $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ vasa deferentia can reorganize and establish epithelial monolayers in culture (although the success rate in achieving resistive monolayers with these tissues types was substantially less than with cells derived from WT littermates). Results from electrophysiological assays demonstrated that these monolayers exhibited an epithelial phenotype at the time of assay, as assessed by their R_{TE} and I_{SC} properties. $CFTR^{-/-}$ monolayers revealed no signs of I_{SC}-increases, unlike WT monolayers derived from littermates that responded to cAMP-generating compounds such as forskolin, norepinephrine, adenosine and PGE2. $CFTR^{-/-}$, $CFTR^{\Delta F508/\Delta F508}$ and WT n1^{o}PVDs responded to dexamethasone and amiloride with changes in I_{SC} that were consistent with ENaC expression and blockade. Note that amiloride-sensitive I_{SC} was present in these monolayers only after a period (72 hs or longer) of corticosteroid exposure, which is consistent with previous observations derived from adult porcine vas deferens epithelial cells [22]. These results demonstrate that neonatal porcine vas

355 deferens epithelia are responsive to corticosteroids and that sodium might be absorbed in the prenatal $CFTR^{-/-}$ male duct while anion secretion is severely hindered. These factors could account for very little water flux into the ductal lumen, leading to reduced luminal volume and nearly absent flow along the excurrent duct. Obviously this scenario is not conducive to the maintenance of a ductal lumen and could contribute to increased concentrations of
360 prostaglandins and growth factors, as discussed previously. Interestingly, nine $CFTR^{\Delta F508/\Delta F508}$ $n1^{o}$PVDs isolated from 2 piglets revealed a degree of forskolin-induced anion secretion that appears greater than that in $CFTR^{-/-}$ $n1^{o}$PVDs (which is nil) and less than one third of that in WT $n1^{o}$PVDs. These results suggest that the native male reproductive duct epithelia of $CFTR^{\Delta F508/\Delta F508}$ piglets are capable of limited anion secretion that is not sufficient to prevent the
365 mechanisms that lead to the anatomical abnormalities detected at birth in these animals. It should be noted that no cells were available from the tissues that were most affected (tissues lacking a duct) and that many attempts to culture resistive cell monolayers from $CFTR^{-/-}$ ducts were unsuccessful. Thus, when one examines the spectrum of tissue atresia, the cultured cells that were assessed in the Ussing chamber may represent the 'high functioning' cells. Recent
370 expression and functional studies conducted with intestinal and airway epithelia from newborn $CFTR^{\Delta F508/\Delta F508}$ piglets reveal that reduced anion secretion in those epithelia are linked to reduced ΔF508-mRNA and -protein expression levels, as well as less ΔF508-protein expressed apically when compared to WT controls [39]. Overall, the results derived from the $CFTR^{\Delta F508/\Delta F508}$ vas deferens epithelia and reported here are consistent with the current
375 knowledge that unlike human ΔF508-CFTR, the porcine version of this protein can be processed, apically expressed and exhibits some anion conductance.

In porcine vas deferens epithelia, CFTR is thought to function as an apical conductance for HCO_3^- and/or Cl^-. HCO_3^- secretion onto the lumen is also supported by the basolateral Na^+-HCO_3^- co-transporter SLC4A4 and the apical Cl^--HCO_3^- exchangers SLC26A3 and SLC26A6 [20, 23]. Data reported here reveal that $CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F508}$ monolayers exhibit SLC26A3 and SLC26A6 immunoreactivities, and thus an apparent apical route for HCO_3^- secretion. These results do not, however, ultimately determine that these exchangers are expressed and functionally active at the apical membranes of n1°PVDs or native neonatal porcine vas deferens epithelia, as it has been demonstrated for cultured adult porcine vas deferens epithelia [40]. However, assuming that they are present and active would lead to the proposition that SLC26A3 and SLC26A6 are not electrogenic, which is consistent with a recent report that investigated SLC26A3 activity in native and recombinant systems [41]. Results from western blot analyses also indicated that the anti-CFTR employed in this study does not recognize porcine ΔF508-CFTR from protein lysates derived from n1°PVDs. This is so despite the fact that the epitope employed to generate this antibody maps to amino acids 1365-1395 of the human CFTR sequence and ΔF508-CFTR exhibits a single phenylalanine deletion at position 508.

In conclusion, we report that both *CFTR*$^{-/-}$ and *CFTR*$^{\Delta F508/\Delta F508}$ neonatal piglets exhibit reproductive phenotypes that model human CBAVD. Results suggest that major events of CF male tract pathology have taken place prenatally and do not involve inflammatory cell-mediated tissue remodeling or mucus obstructions.

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Figure Legends

Figure 1. Male excurrent duct atresia is observed in *CFTR*$^{-/-}$ and *CFTR*$^{\Delta F508/\Delta F508}$ neonatal pigs. **A-C**. Vas deferens (arrow heads) are present in full length, from the cauda epididymis (CD) to the prostate (P) in all wild type (WT) piglets examined. Likewise, the corpus (CO) and caput (arrows) epididymes were present and anatomically robust. **D, G**. Atresia in the vas deferens was often localized in CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$ tissues, at the distal segment of the duct, near the the prostate (P). Only fine fibers of macroscopically visible connective tissue and/or blood vessels were present. The spermatic cords (SC) of these pigs were typically composed by a cord of nearly translucent tissue with no duct structure present. A dashed line across a SC in D indicates a typical plan of histological section, and the histological features of this tissue are shown in Fig. 2E. **E-F**, **H-I**. Images reveal atresia of proximal vas deferens, corpus and cauda epididymis. The spermatic cords (SC) exhibit a lengthwise patch of more opaque tissue with no clear duct structure. Rudimentary caput epididymides (arrows) are consistently present. Images of tissues from a single animal are shown for each genotype. Most profound examples of apparent reproductive duct atresia are shown. Size bars are 5 mm.

Figure 2. Histopathology reveals abnormalities in CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$ neonatal vasa deferentia that range from complete absence of duct structure to presence of an epithelial-like layer and lumen. **A-D**. Cross-sections of WT vasa deferentia revealed ample lumens, lined by columnar pseudo-stratified epithelia (EPI), outwardly surrounded by elongated smooth muscle cells (SM). Images shown are derived from 3 different piglets and representative of 15 WT piglets analyzed. A number of sections derived from WT, CFTR$^{-/-}$, and CFTR$^{\Delta F508/\Delta F508}$

revealed luminal content (*) that was either amorphous and eosinophilic (**C**) or appeared to be composed of detached epithelium (**D**). **E**. Cross section of CFTR$^{-/-}$ spermatic cord presents no duct structure (inset) and magnified view reveals fibrous tissue (FT) with small caliber blood vessels (arrows). Section shown is representative of observations acquired from 3 of 8 *CFTR*$^{-/-}$ piglets analyzed. Similar profiles were also seen in 2 out of 9 *CFTR*$^{\Delta F508/\Delta F508}$ piglets analyzed (data not shown). **F-G**, **K-L**. CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$ neonatal vasa deferentia presented profiles in which the muscular layer (SM) enclosed a basal lamina (arrow heads), epithelial-like cells, and the lumen were either absent or small. Smooth muscle cells are not elongated and organized concentrically, a feature present in all homozygous mutants analyzed. **H**. Abnormalities in CFTR$^{-/-}$ included round empty spaces (arrows). **I-J**. CFTR$^{\Delta F508/\Delta F508}$ spermatic cords exhibited profiles in which smooth muscle (SM) was seen without epithelia or a basal lamina in 3 piglets. Typically, further sectioning changed the profile to include epithelium and a lumen. Size bar in inset is 200 μm, in A it is 100 μm and applies to I also, and in E (applies to all other images) it is 25 μm.

Figure 3. Epithelial cell monolayers derived from primary cultures of neonatal porcine vas deferens (n1^{o}PVD) exhibit typical anion secretion if WT CFTR is expressed. **A-C**. WT n1^{o}PVDs respond with short circuit current (I_{SC}) increases when stimulated with norepinephrine (10 μM), PGE2 (1 μM), adenosine (1 μM) and forskolin (2 μM, not shown). Bumetanide (20 μM) reduces cAMP-induced I_{SC} partially and is thought to inhibit a basolateral chloride-loading component. **D-E**. Typical I_{SC}-tracings from CFTR$^{-/-}$ n1^{o}PVDs, which failed to respond to these compounds.

Figure 4. CFTR$^{-/-}$ n1^{o}PVDs exhibit amiloride-sensitive I_{SC} after corticosteroid exposure, but no forskolin-induced responses. **A**. Dexamethasone- (dex) treated CFTR$^{-/-}$ and WT n1^{o}PVDs respond to amiloride (10 μM) significantly, but CFTR$^{-/-}$ monolayers presented no changes in I_{SC} after stimulation with forskolin. *, **, ***, # and ## indicate statistical significance ($P < 0.05$). n = 5 WT piglets (7 monolayers in untreated and 7 monolayers in dex-treated) and 4 *CFTR*$^{-/-}$ piglets (7 monolayers in untreated and 10 monolayers in dex-treated). **B**. Typical outcomes from CFTR$^{-/-}$, CFTR$^{\Delta F508/\Delta F508}$ and WT n1^{o}PVDs assayed in Ussing flux chambers. CFTR$^{\Delta F508/\Delta F508}$ not only respond to amiloride (if dex-treated, arrowheads 1) but also present consistent I_{SC}-increases that are indicative of anion secretion in response to forskolin (arrowheads 2). Forskolin-induced increases in I_{SC} were partially sensitive to the CFTR inhibitor DASU-02 (100 μM, arrowheads 3). **C**. Summary of observations derived from CFTR$^{\Delta F508/\Delta F508}$ monolayers (from 2 piglets) and WT littermates (3 piglets). Monolayers were assigned to experimental groups as follows: WT (6 monolayers), WT exposed to dexamethasone (8 monolayers), CFTR$^{\Delta F508/\Delta F508}$ (3 monolayers) and CFTR$^{\Delta F508/\Delta F508}$ exposed to dexamethasone (6 monolayers).

Figure 5. Immunoreactivities from bicarbonate transporters SLC26A3 and SLC26A6 are present in n1^{o}PVD regardless the presence or absence of WT CFTR expression. **A**. Lanes loaded with protein lysates derived from 3 WT and 3 *CFTR*$^{-/-}$ piglets reveal presence and absence of CFTR immunoreactivity, respectively, and as expected. CFTR immunoreactivity of same mobility is detected on lane loaded with Calu-3 cellular lysate. All images were sampled from the same western blot membrane. SLC26A3 and SLC26A6 immunoreactivities are present in both WT and CFTR$^{-/-}$. **B**. Immunoreactivity of CFTR is not detectable in CFTR$^{\Delta F508/\Delta F508}$, while SLC26A3 and SLC26A6 immunoreactivities are present in all n1^{o}PVD tested. Beta-actin

immunoreactivities of expected mobility and similar densities were detected on all lanes of each western blot. Results are representative of 10 WT, 6 *CFTR*$^{-/-}$ and 2 *CFTR*$^{\Delta F508/\Delta F508}$ neonates analyzed.

Figure 6. Periodic acid-Schiff (PAS) stain pattern and quantity are similar between WT, CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$, and suggest that no relevant changes in mucus abundance had occurred in the male excurrent duct of *CFTR*$^{-/-}$ and *CFTR*$^{\Delta F508/\Delta F508}$ piglets by birth. **A-C**, **G-I**. Most common PAS stain pattern detected in WT, CFTR$^{-/-}$ and CFTR$^{\Delta F508/\Delta F508}$ neonatal vasa deferentia and epididymides. No distinct PAS positive tissue or subcellular components are present. **D-F**, **J-L**. In few of the male tracts analyzed (7 out of 17 WT, 1 out of 9 CFTR$^{-/-}$ and 1 out of 9 CFTR$^{\Delta F508/\Delta F508}$) more distinct PAS positive subcellular components of the epithelia or the luminal content were present and these images illustrate their pattern. No CFTR$^{-/-}$ or CFTR$^{\Delta F508/\Delta F508}$ profiles from the vasa deferentia or epididymides exhibited signs of PAS positive material clogging the lumens. **M-O**. Adult porcine lung tissues (unrelated to neonatal male reproductive tissues) revealed intense PAS positive stain along the apical surface of bronchial epithelia, which is typical and consistent with secreted mucus (arrow heads) and in cells of the submucosal glands (arrows). Size bar (A) is 25 μm and applies to all images.