

# Early Embryonic Renal Tubules of Wild-Type and Polycystic Kidney Disease Kidneys Respond to cAMP Stimulation with Cystic Fibrosis Transmembrane Conductance Regulator/ $\text{Na}^+$ , $\text{K}^+$ , $2\text{Cl}^-$ Co-Transporter–Dependent Cystic Dilation

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Metanephric organ culture has been used to determine whether embryonic kidney tubules can be stimulated by cAMP to form cysts. Under basal culture conditions, wild-type kidneys from embryonic day 13.5 to 15.5 mice grow in size and continue ureteric bud branching and tubule formation over a 4- to 5-d period. Treatment of these kidneys with 8-Br-cAMP or the cAMP agonist forskolin induced the formation of dilated tubules within 1 h, which enlarged over several days and resulted in dramatically expanded cyst-like structures of proximal tubule and collecting duct origin. Tubule dilation was reversible upon withdrawal of 8-Br-cAMP and was inhibited by the cAMP-dependent protein kinase inhibitor H89 and the cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor CFTR<sub>inh</sub>-172. For further testing of the role of CFTR, metanephric cultures were prepared from mice with a targeted mutation of the *Cftr* gene. In contrast to kidneys from wild-type mice, those from *Cftr*  $-/-$  mice showed no evidence of tubular dilation in response to 8-Br-cAMP, indicating that CFTR  $\text{Cl}^-$  channels are functional in embryonic kidneys and are required for cAMP-driven tubule expansion. A requirement for transepithelial  $\text{Cl}^-$  transport was demonstrated by inhibiting the basolateral  $\text{Na}^+$ , $\text{K}^+$ , $2\text{Cl}^-$  co-transporter with bumetanide, which effectively blocked all cAMP-stimulated tubular dilation. For determination of whether cystic dilation occurs to a greater extent in PKD kidneys in response to cAMP, *Pkd1*<sup>mlBe1</sup>  $-/-$  embryonic kidneys were treated with 8-Br-cAMP and were found to form rapidly CFTR- and  $\text{Na}^+$ , $\text{K}^+$ , $2\text{Cl}^-$  co-transporter–dependent cysts that were three- to six-fold larger than those of wild-type kidneys. These results suggest that cAMP can stimulate fluid secretion early in renal tubule development during the time when renal cysts first appear in PKD kidneys and that PKD-deficient renal tubules are predisposed to abnormally increased cyst expansion in response to elevated levels of cAMP.

*J Am Soc Nephrol* 17: 3424–3437, 2006. doi: 10.1681/ASN.2006030295

**A**utosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder that is characterized by the growth of numerous renal tubule-derived, fluid-filled cysts, which enlarge over a period of years and eventually lead to a decline in kidney function and often to renal failure (1–5). Cyst formation involves increased cAMP-dependent cell proliferation (6–11), but as cysts expand, they become isolated structures and can increase in size only when there is concomitant transepithelial fluid secretion (12,13). It is not known when embryonic renal tubules acquire the capacity for fluid secretion or when fluid secretion first becomes important for cyst formation and enlargement. The capacity to secrete

fluid has been demonstrated in normal adult renal tubules (14–16); however, its significance is unknown.

Cultured epithelial cells from cysts of ADPKD kidneys secrete fluid in response to cAMP-driven transepithelial  $\text{Cl}^-$  transport that is mediated by cystic fibrosis transmembrane conductance regulator (CFTR) (12,13,17–21). However, the extent to which cyst formation depends on CFTR and whether CFTR is functional in embryonic kidneys at a time when cyst growth begins in PKD has not been known. The expression of CFTR isoforms has been demonstrated by reverse transcriptase–PCR and immunohistochemistry in developing epithelial structures of embryonic kidneys, including the early proximal tubule and ureteric bud (22–25). Later in development and in the mature kidney, CFTR has been found in all nephron segments (17,21,22,25,26). The functional role of CFTR in the kidney is unclear, because patients with cystic fibrosis (CF) do not seem to have significantly impaired renal function (21,25,26). However, CFTR-dependent fluid secretion may be a factor in PKD, because patients who have both CF and PKD seem to have somewhat milder cystic disease (27,28).

Received March 30, 2006. Accepted September 26, 2006.

Published online ahead of print. Publication date available at [www.jasn.org](http://www.jasn.org).

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While investigating early kidney development in mouse metanephric organ culture, we discovered that cyst-like tubular dilations appeared sporadically in wild-type kidneys under certain culture conditions. We reasoned that these dilations were due to fluid accumulation in the lumens of the developing tubules. However, cultured metanephric kidneys largely are avascular and therefore do not support glomerular filtration (29–31). This suggests that any luminal fluid accumulation would result from fluid transport across the tubular epithelium. Therefore, to determine whether embryonic kidney tubules are capable of secreting fluid and by which mechanism, we examined metanephric organ cultures after treatment with cAMP agonists. We found that early metanephric kidneys formed cyst-like tubular dilations after cAMP treatment, using a mechanism that depended on CFTR. We also demonstrated that transepithelial  $\text{Cl}^-$  transport was required for cyst formation by inhibiting the  $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$  co-transporter (NKCC1) with bumetanide, which effectively blocked all cAMP-stimulated tubule dilation. In *Pkd1*  $-/-$  embryonic kidneys, 8-Br-cAMP caused the rapid formation of very large cysts from both proximal tubules and collecting ducts, in a CFTR- and NKCC1-dependent process. These results demonstrate that cAMP-stimulated fluid secretion occurs early in embryonic renal tubule development in wild-type and PKD kidneys at the time when renal cysts first appear in ADPKD, suggesting that a cAMP-driven mechanism may be involved in the initial stages of cyst formation in ADPKD.

## Materials and Methods

### Mice

Wild-type CD1 mice or mice that were heterozygous for a *Cftr* knockout allele on a C57BL/6 background were used for timed breedings. *Cftr*<sup>m1UNC</sup> (S489X) mice produce no stable CFTR mRNA or protein and therefore are considered to have a null phenotype (32,33). *Pkd1*<sup>m1Bet</sup> mice were obtained from the Mutant Mouse Regional Resource Center (University of North Carolina, Chapel Hill, NC) and were stabilized onto a C57BL/6 background (>10 backcrosses). This mouse has a point mutation (T to G at 9248 bp) that causes an M to R substitution that affects the first transmembrane domain of polycystin-1 (34). The mice were genotyped using PCR primers and TaqMan Chemistry (Applied Biosystems, Foster City, CA) real-time PCR. Double-heterozygous mice (*Pkd1*  $+/-$ :*Cftr*  $+/-$ ) were produced in our laboratory and crossed to produce *Pkd1*  $-/-$ :*Cftr*  $-/-$  embryos. Embryos were procured at embryonic day 13.5 (E13.5), E14.5, or E15.5. All methods followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Organ Culture

Metanephroi were dissected from embryonic mice and placed on transparent Falcon 0.4-mm cell culture inserts (29–31,35,36). DMEM/F12-defined culture medium (supplemented with 2 mM L-glutamine, 10 mM HEPES, 5  $\mu\text{g}/\text{ml}$  insulin, 5  $\mu\text{g}/\text{ml}$  transferrin, 2.8 nM selenium, 25 ng/ml prostaglandin E, 32 pg/ml T3, 250 U/ml penicillin, and 250  $\mu\text{g}/\text{ml}$  streptomycin) was added under the culture inserts, and organ cultures were maintained in a 37°C humidified  $\text{CO}_2$  incubator for up to 6 d. Upon culturing and approximately 24 h later (1 d) and each day after (2 to 5 d), kidneys were photographed using a 2 $\times$  or 4 $\times$  objective, and the images were acquired and analyzed using the analySIS imaging program (Soft Imaging System, Münster, Germany).

### Treatment Regimens

Reagents (100  $\mu\text{M}$  8-Br-cAMP, 10  $\mu\text{M}$  forskolin, 10  $\mu\text{M}$  H89, 100  $\mu\text{M}$  CFTR<sub>inh</sub>172, 10  $\mu\text{M}$  fluorescein, 100  $\mu\text{M}$  bumetanide, and 100  $\mu\text{M}$  benzamil) were added to the culture medium in the lower chamber. In previous studies, 100  $\mu\text{M}$  bumetanide was found in cell culture to inhibit NKCC1-mediated  $\text{Cl}^-$  and fluid transport (14,15,37–39). For fluorescein labeling (40), the dye was added to the culture medium for 1 h, the medium was replaced, and photographs were taken 2 h later.

### Histology

Organ cultures were fixed with Karnovsky fixative and embedded in plastic. Sections that were 4  $\mu\text{m}$  thick were cut and stained with 1% Toluidine blue. Images were collected using a Leica DC200 digital camera (Wetzler, Germany).

### Quantification of Dilated Tubule Area

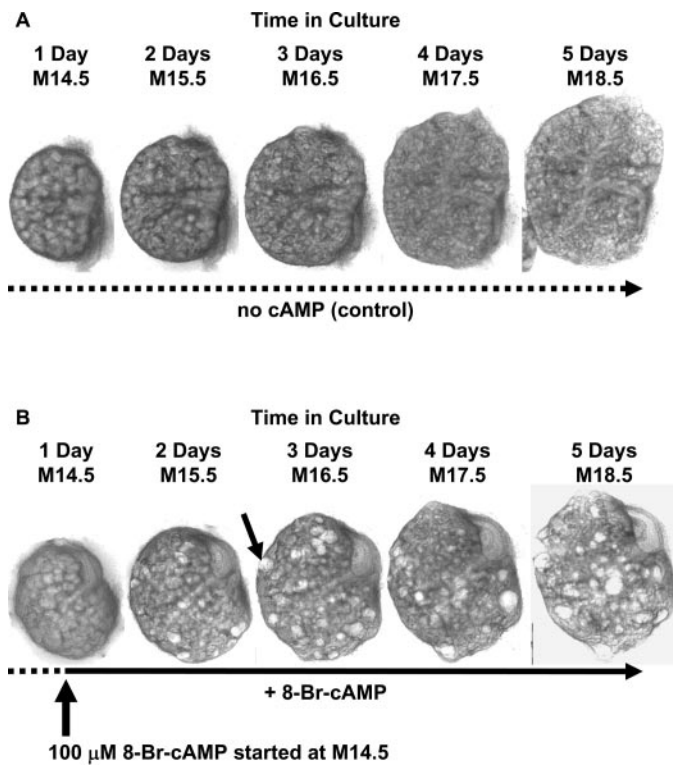
Quantification was performed on captured images using analySIS software. The wand tool was used to select a pixel within the image of a dilated tubule, which highlighted all of the pixels of similar density within the dilated tubule. This process was repeated within the dilation until the highlighted area comprised the entire dilation. The highlighted area then was selected and saved by the software. Each dilation was analyzed in the same manner. When all of the dilated tubules had been selected and saved, a measurement sheet was generated for statistical analysis. For total kidney area, the freehand polygon tool was used to trace around the kidney (excluding the ureter), and the area within the tracing was determined. Fractional cyst area was calculated as total tubule dilation area divided by total kidney area.

### Lectin and NKCC1 Labeling

For whole-mount lectin labeling, kidneys were fixed in 100% methanol, incubated with Dolichos biflorus agglutinin (DBA)-FITC (1:50; Vector Laboratories, Burlingame, CA), washed, and photographed. In other staining protocols, cultured kidneys were fixed in 4% paraformaldehyde, and frozen sections were cut. For staining of NKCC1, sections were subjected to heat retrieval and incubated with NKCC1 antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with anti-goat Texas Red (1:100; Jackson Immunoresearch, West Grove, PA) and DBA- or Lotus tetragonobolus agglutinin (LTA)-FITC. Incubation of the NKCC1 antibody overnight with the blocking peptide completely eliminated staining (data not shown).

## Results

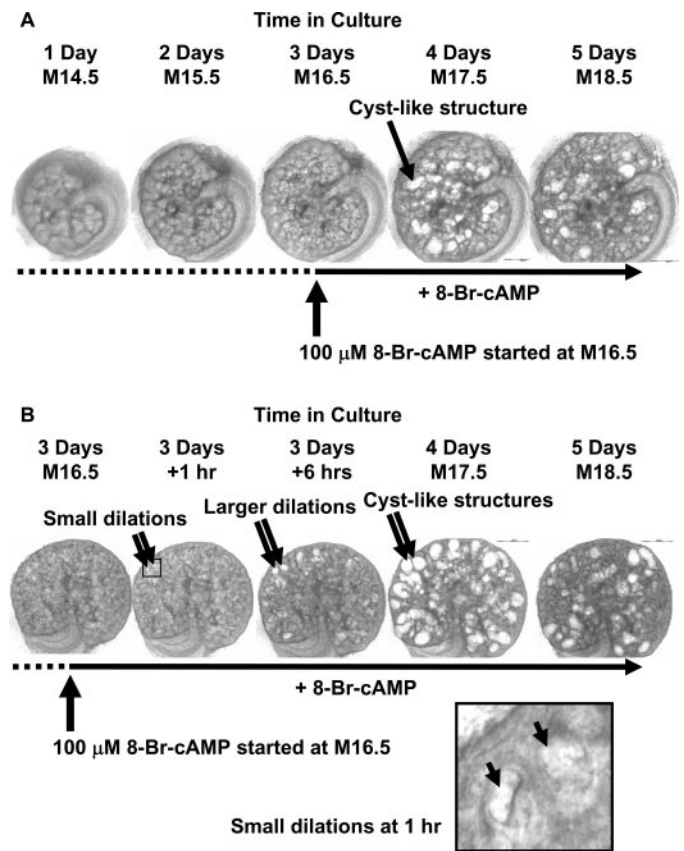
Embryonic kidneys from E13.5 mice were cultured for 5 d under a variety of treatment regimens and were monitored periodically by light microscopy for the presence of tubular lumen dilations. Under basal culture conditions, the E13.5 renal explants increased in size and continued ureteric bud branching and tubule formation over 5 d in culture (Figure 1A) (29–31,35,36). Treatment with 8-Br-cAMP (Figure 1B) or the cAMP agonist forskolin (data not shown) produced numerous dilated tubules, which enlarged over several days in culture, resulting in dramatically expanded cyst-like structures throughout the kidneys. Addition of 8-Br-cAMP after 3 d of culture also caused cyst formation (Figure 2A). Tubule dilation occurred rapidly, with visible expansions of the tubules within 1 h after cAMP treatment (Figure 2B, inset). These dilations continued to enlarge over the next 6 to 24 h. Tubule expansion was reversible rapidly upon withdrawal of 8-Br-cAMP (data not shown). In



**Figure 1.** cAMP treatment of embryonic kidneys. Kidneys from CD1 mice were removed and placed in culture at embryonic day 13.5 (E13.5). Twenty-four hours later at metanephric day 14.5 (M14.5) and at each subsequent day thereafter (M15.5 through M18.5), the kidneys were photographed. Cultures were either left untreated (no cAMP control; A) or were treated with 100  $\mu\text{M}$  8-Br-cAMP after 1 d in culture (B). Both untreated and cAMP-treated kidneys increased in size during the 5 d in culture. Each series of photographs represents the same kidney on successive days in culture. The M14.5 (B) photograph was taken immediately before 8-Br-cAMP was added to the culture on that day. All kidneys are at the same scale. Arrow, cyst-like structure; M, metanephric day.

some cultures, there was a spontaneous reduction in the size of some of the dilations after 4 to 5 d in culture (Figure 2, 4 *versus* 5 d), although in most kidneys, this was not the case. A comparison of kidneys that were placed in culture at E13.5, E14.5, and E15.5 (Figure 3) showed that cAMP induced cystic dilation at all of these developmental stages; however, in general, the response to cAMP declined as the kidneys became developmentally more advanced (Figure 3A), with there being a small but significant reduction in fractional cyst area at E15.5 (Figure 3B).

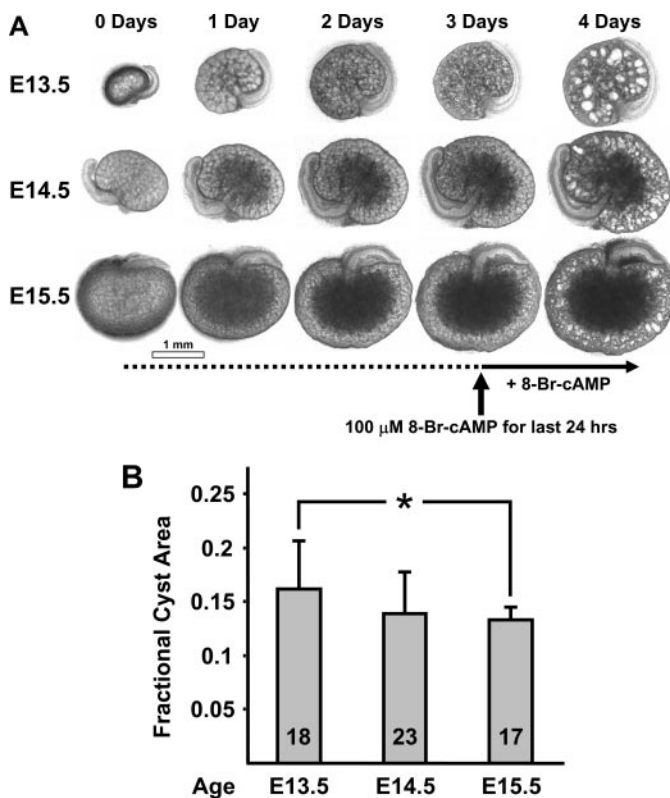
Representative histologic sections of control and cAMP-treated metanephric kidneys confirmed the presence of cyst-like tubular dilations after cAMP treatment (Figure 4). In some kidneys, the dilated tubules seemed to have a brush border, suggesting that they were derived from proximal tubules (data not shown). For determination of the origin of the cyst-like structures, metanephric kidneys were loaded with the organic anion fluorescein, which is transported specifically by proximal tubule cells using the organic anion transport (OAT) system



**Figure 2.** Time course for cAMP-mediated tubular dilation. Kidneys from CD1 mice were placed in culture at E13.5 and treated with 100  $\mu\text{M}$  8-Br-cAMP after 3 d in culture at M16.5. Kidneys were photographed immediately before cAMP treatment and at M17.5 and M18.5 (A) or at 1, 6, 24 (M17.5), and 48 h (M18.5) after cAMP addition (B). (B) Small dilations began to develop after 1 h in cAMP (see enlarged area in inset taken from the 1-h kidney) and continued to enlarge for at least 24 h (series of arrows showing small dilations, larger dilations, and cyst-like structures). After 5 d in culture, some dilations seemed to shrink in size.

(40–43). As shown in Figure 5, there was low-level fluorescence in kidneys that were not treated with 8-Br-cAMP. This was due to cellular uptake of the fluorescein in proximal tubular cells. Treatment of kidneys for 2 d with 8-Br-cAMP resulted in the formation of large, fluid-filled dilations, which became labeled intensely by the dye when fluorescein was present in the culture medium. The cystic dilations retained their fluorescence for at least 2 h after washout of the dye (Figure 5), suggesting that fluorescein had accumulated in the cyst lumens by being trapped in these dilated structures. It also can be seen that whereas some cystic dilations became intensely fluorescence (large arrow), others were only weakly fluorescence (small arrow) or did not seem to take up fluorescein.

To determine whether activation of protein kinase A (PKA) by cAMP is required for tubule dilation, we treated cultured kidneys with the PKA inhibitor H89. As shown in Figure 6A (middle), the size and the number of dilated tubules were reduced significantly by H89. Morphometric analysis of the



**Figure 3.** Comparison of E13.5, E14.5, and E15.5 kidneys. Kidneys from CD1 mice were placed in culture at E13.5, E14.5, or E15.5. (A) Kidneys were photographed after being placed in culture (0 d) and each subsequent day thereafter. The cultures were treated with 100  $\mu$ M 8-Br-cAMP for the last 24 h. Open bar = 1 mm. (B) Quantification of fractional cyst area in control and treated kidneys at 24 h. The bars show the average fractional cyst areas  $\pm$  SEM (\* $P$  < 0.05 by one-way ANOVA). The numbers within the bars are the numbers of kidneys analyzed.

cyst area as a fraction of total kidney area indicated that H89 reduced the fractional cyst area by 36% (Figure 6B). *In vitro* evidence suggests that fluid secretion during cyst growth in ADPKD kidneys is driven by cAMP- and PKA-dependent activation of CFTR  $\text{Cl}^-$  channels that are located on the apical surface of cyst epithelial cells. To test whether CFTR is involved in cAMP-mediated metanephric cyst formation, we treated kidneys with the thiazolidinone CFTR inhibitor CFTR<sub>inh</sub>172 (44). As shown in Figure 6, A (right) and B, this CFTR inhibitor reduced the size and the number of the tubular dilations by >50%, suggesting that CFTR  $\text{Cl}^-$  channels have a role in the cyst-forming process. When the inhibitor was washed out of the cultures, cystic dilations rapidly re-formed (data not shown), indicating that CFTR<sub>inh</sub>172 acts in a reversible manner.

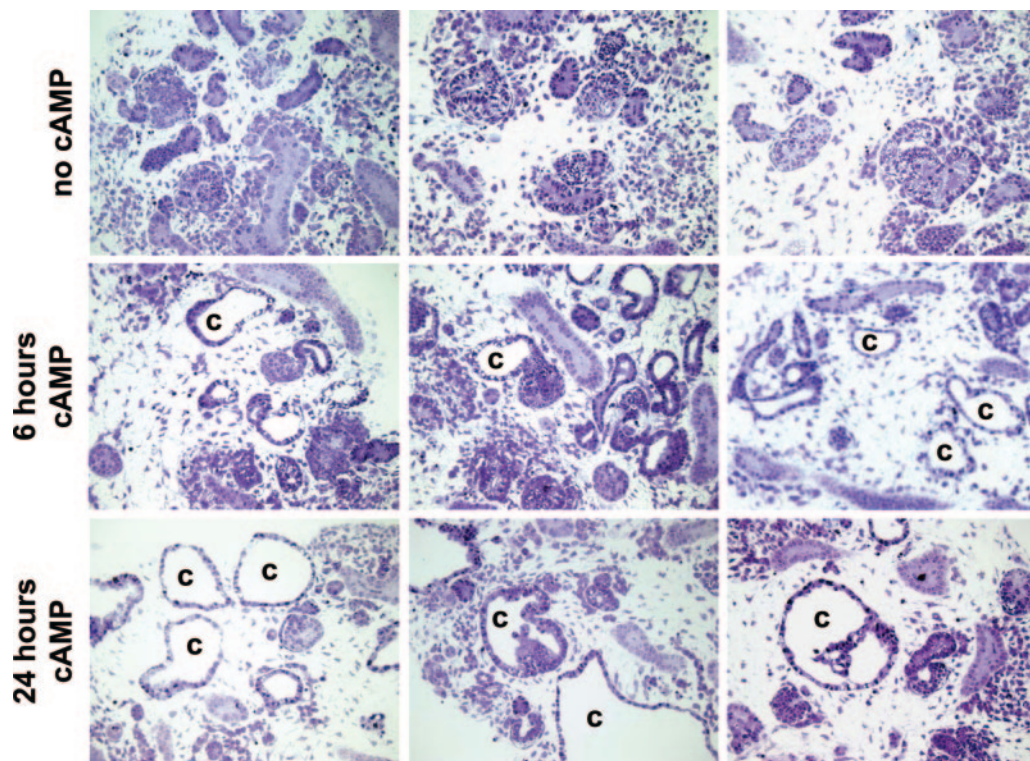
To test the role of CFTR further, we prepared metanephric cultures from mice with a targeted mutation of the *Cftr* gene. In contrast to *Cftr*  $+/+$  kidneys (Figure 7) and *Cftr*  $+/-$  kidneys (Figure 7B), which showed dramatic formation of cyst-like dilations in response to 8-Br-cAMP, the *Cftr*  $-/-$  kidneys showed no evidence of tubular dilation (Figure 7). These results indicate that CFTR is present and functional in embryonic

kidneys and that cAMP-mediated tubule expansion depends on the expression of CFTR. Also of interest is the observation that *Cftr*  $+/-$  kidneys had a small but significant reduction in the fractional cyst area as compared with *Cftr*  $+/+$  kidneys, suggesting that the capacity for cAMP-driven tubular dilation depends on *Cftr* gene dosage.

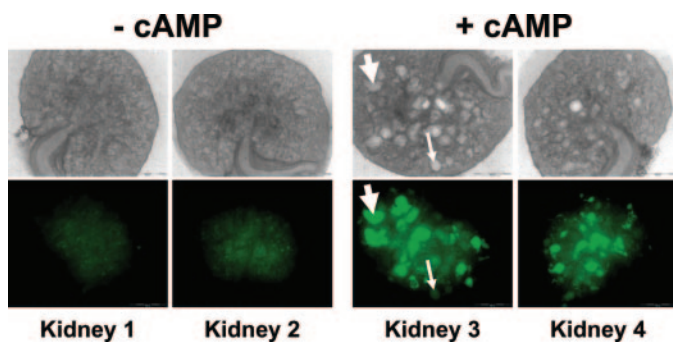
CFTR is both a conductance regulator and an anion channel. To determine whether  $\text{Cl}^-$  transport is an important component of the cyst-forming process, we treated embryonic kidneys with bumetanide, an inhibitor of NKCC1. NKCC1 has been shown by *in situ* hybridization to be expressed in early embryonic kidneys (45) and could provide basolateral transport of  $\text{Cl}^-$  into the cell above its electrochemical gradient for passive efflux through apical CFTR  $\text{Cl}^-$  channels. As shown in Figure 8A, cultured embryonic kidneys acquire bumetanide sensitivity during the 3-d period from E13.5 to E15.5. At E13.5 approximately 20% of the fractional cyst area was inhibited by bumetanide, but by E15.5, virtually all cyst formation was inhibited. Many of the bumetanide-resistant cysts at E13.5 were of proximal tubule origin, as demonstrated by their ability to transport fluorescein (Figure 8B). Immunostaining (Figure 9) showed the presence of NKCC1 in the metanephric mesenchyme, tubules, and cysts of cAMP-treated CD1 E13.5 kidneys. Thus, NKCC1 is widely present in early embryonic kidneys and is likely to play an important role in the cyst-forming process even at E13.5. Experiments also were carried out with the epithelial  $\text{Na}^+$  channel inhibitor benzamil, which was found to increase cyst size further in the cAMP-treated kidneys (data not shown), suggesting the possibility that there is a functional fluid absorption mechanism in these early embryonic kidneys.

Cyst formation in ADPKD begins during fetal development, and cysts are evident in mouse *Pkd1*  $-/-$  kidneys at E15.5. To determine whether cAMP stimulates PKD cyst enlargement, we cultured embryonic kidneys from C57BL/6 *Pkd1*<sup>m1bei</sup> mice. These mice have a single amino acid substitution in the first transmembrane domain of polycystin-1, which results in a null phenotype in *Pkd1*  $-/-$  animals (34). When first placed in culture at E15.5, the *Pkd1*  $-/-$  kidneys were seen to have numerous, very small tubular dilations but no large cysts (Figure 10A). Treatment with 8-Br-cAMP resulted in rapid cyst expansion in the *Pkd1*  $-/-$  kidneys by 24 h (day 1), which continued through day 4 of metanephric culture (Figure 10A). Comparison of wild-type and *Pkd1*  $-/-$  kidneys after 4 d in culture showed that the *Pkd1*  $-/-$  kidneys developed extremely large cysts that had three to six times the fractional cyst area as compared with *Pkd1*  $+/+$  kidneys (Figure 10, B and C). Thus, *Pkd1*  $-/-$  kidneys are predisposed to abnormally pronounced cAMP-driven cyst growth, forming significantly larger cystic dilations than their wild-type counterparts.

To test whether *Pkd1*  $-/-$  cysts depend on CFTR-mediated  $\text{Cl}^-$  secretion, we crossed *Pkd1*  $+/-$ :*Cftr*  $+/-$  mice to produce embryos that were deficient in *Pkd1*, *Cftr*, or both. As shown in Figure 10, A and B, the *Cftr*  $-/-$  genotype completely blocked cyst formation in *Pkd1*  $-/-$  kidneys. Inhibition of NKCC1 by bumetanide also was effective in blocking cyst enlargement (Figure 10C), suggesting that basolateral  $\text{Cl}^-$  transport is essen-



**Figure 4.** Histologic examination of cyst-like expansions of tubule lumens. Kidneys from CD1 mice were placed in culture at E13.5 and were either left untreated (no cAMP; top) or treated with 100  $\mu$ M 8-Br-cAMP (cAMP) after 3 d in culture (middle and bottom). Kidneys were harvested for semithin sectioning at 6 (middle) or 24 h (bottom) after cAMP treatment. Tubule lumens in untreated kidneys (top) were very small and difficult to see in most cases. In the cAMP-treated kidneys, there were numerous cyst-like (C) dilations. Each panel shows a different region of either the same or a different kidney (two different kidneys are represented in each row). Magnification,  $\times 400$ .

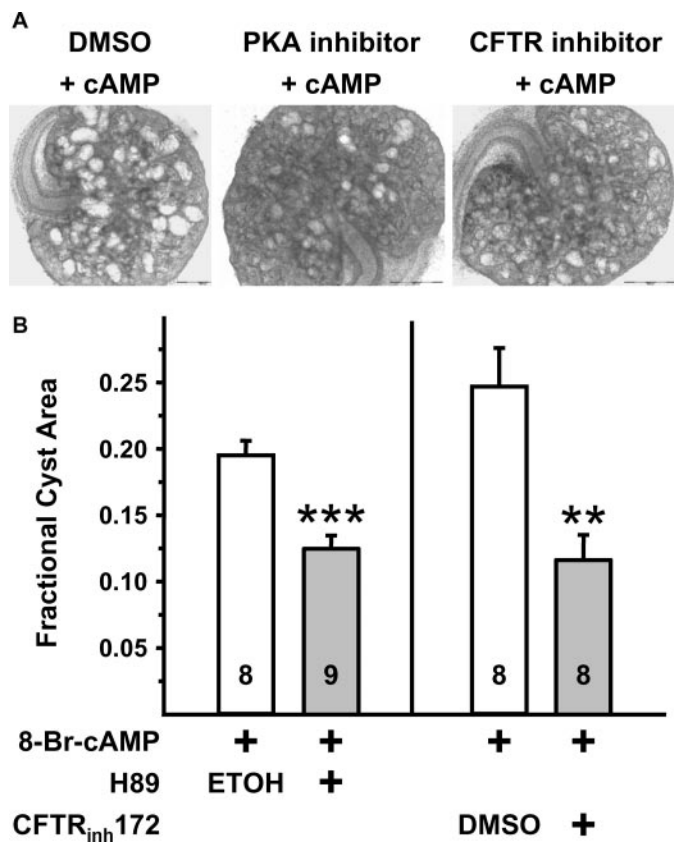


**Figure 5.** Fluorescein labeling of metanephric organ cultures. Embryonic kidneys from CD1 mice were placed in culture at E13.5, and after 3 d (M16.5), the kidneys were either left untreated ( $-$  cAMP) or treated with 100  $\mu$ M 8-Br-cAMP ( $+$  cAMP). Two days later (M18.5), the kidneys were loaded with the organic anion fluorescein for 1 h. Fresh medium (without fluorescein) was added with or without 100  $\mu$ M 8-Br-cAMP. Photographs were taken 2 h later. Shown are bright-field and fluorescence images. The low-level fluorescence in the control kidneys ( $-$  cAMP) represents cellular uptake of fluorescein. The intense fluorescence (arrows) in the cAMP-treated kidneys represents fluorescein that has accumulated in the cyst fluid of the dilated proximal tubules. Some (e.g., wide arrow) but not all (e.g., narrow arrow) of the dilated structures were labeled with fluorescein, identifying those of proximal tubular origin.

tial for the cAMP-stimulated cyst-forming process in these *Pkd1* $^{-/-}$  kidneys.

Bumetanide inhibition of cyst formation suggests that NKCC1 is expressed in the cyst-forming tubules of the C57BL/6 mice. As shown in Figure 11, A through C, NKCC1 is localized to the early collecting ducts (CD; see merge) of *Pkd1* $+/+$  metanephric kidneys, as well as the metanephric mesenchyme, and is present in the ureteric bud at somewhat lower levels. NKCC1 also is present in the expanded tubules of *Pkd1* $^{-/-}$  kidneys that were treated with cAMP (Figure 11D, Cyst).

CFTR is known to be localized to the ureteric bud tips in embryonic kidneys. Therefore, to determine whether a lack of CFTR expression would affect ureteric bud development in metanephric organ culture, we stained the ureteric buds and collecting ducts of cultured E15.5 kidneys with DBA. At whole-mount resolution (Figure 12), the DBA-stained structures in *Cftr* $^{-/-}$  kidneys were similar in appearance to the DBA-stained structures in *Cftr* $+/+$  kidneys, with or without cAMP treatment. Likewise, treatment with bumetanide (Figure 13A, bottom) did not result in any observable abnormality in the pattern of collecting duct DBA staining. The cyst indicated by the arrow (Figure 13A, top right) was examined at higher magnification (Figure 13, B through D); it appeared DBA positive, suggesting a collecting duct origin. Cysts also were of proximal tubule origin in both *Pkd1* $+/+$  and *Pkd1* $^{-/-}$  kid-

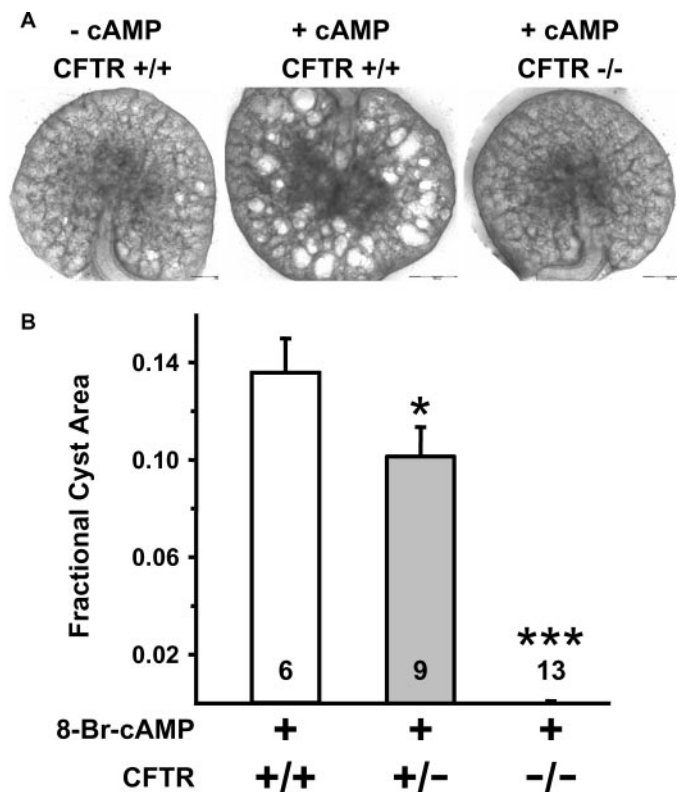


**Figure 6.** Effect of inhibition of protein kinase A (PKA) or cystic fibrosis transmembrane conductance regulator (CFTR) on cAMP-mediated tubular dilation. Embryonic kidneys from CD1 mice were placed in culture at E13.5 and after 3 d were pretreated for 1 h with vehicle (ethanol for H89; DMSO for CFTR inhibitor), with 10  $\mu$ M H89 (PKA inhibitor), or with 100  $\mu$ M CFTR<sub>inh</sub>172 (CFTR inhibitor). After pretreatment, 100  $\mu$ M 8-Br-cAMP (+ cAMP) was added to the medium, and the kidneys were examined at 1, 6, 24, and 48 h after cAMP addition. (A) Representative photographs from the 24-h time point. (B) Quantification of fractional cyst area in control and treated kidneys at 24 h. The bars show the average fractional cyst areas  $\pm$  SEM (\*\* $P$  < 0.005, \*\*\* $P$  < 0.001 by unpaired  $t$  test analysis *versus* control). The numbers within the bars are the numbers of kidneys analyzed. Visual inspection and data analysis indicated that the numbers and the sizes of dilated tubules were significantly reduced by inhibition of either PKA or CFTR at all time points.

neys (Figure 13, E and F) as shown by positive LTA staining (+Cyst) of some of the cysts.

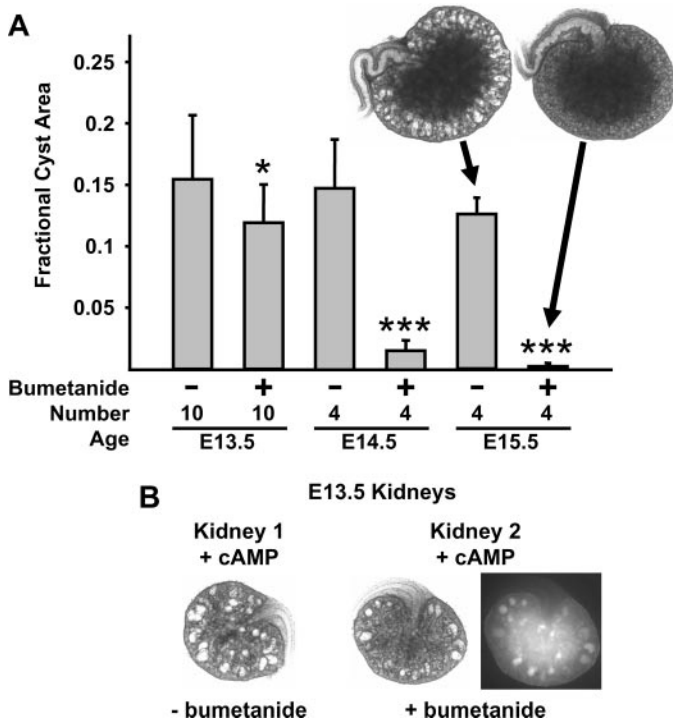
### Discussion

Normal adult renal tubules have a number of well-characterized transport mechanisms for salt and fluid absorption, including a variety of Na<sup>+</sup>-dependent transporters, the absorptive NKCC1, the Na<sup>+</sup> and Cl<sup>-</sup> co-transporter, and the epithelial Na<sup>+</sup> channel (46–48). Renal tubules also have mechanisms for secretion (16). Beyenbach and associates (49–51) found evidence for cAMP-stimulated Cl<sup>-</sup> and fluid secretion in proximal tubules of both glomerular and aglomerular fish. cAMP-depen-



**Figure 7.** Effect of cAMP on tubule dilation in *Cfr*  $-/-$  kidneys. Embryonic kidneys from C57BL/6 wild-type (CFTR  $+/+$ ) or *Cfr* knockout (CFTR  $+/-$  and CFTR  $-/-$ ) mice were placed in culture at E13.5. After 3 d, the kidneys were either left untreated ( $-$  cAMP) or treated with 100  $\mu$ M 8-Br-cAMP (+ cAMP) and were photographed after 48 h. (A) Representative photographs of  $+/+$  and  $-/-$  kidneys. (B) Quantification of fractional cyst area in  $+/+$ ,  $+/-$ , and  $-/-$  kidneys at 48 h. The bars show the average fractional cyst areas  $\pm$  SEM (\* $P$  < 0.05, \*\*\* $P$  < 0.001 by one-way ANOVA with Student-Newman-Keuls multiple comparison test *versus*  $+/+$ ). The numbers within the bars are the numbers of kidneys analyzed. Loss of functional CFTR prevented cAMP-mediated tubule dilation, as there was no evidence of tubule dilation in the *Cfr*  $-/-$  (CFTR  $-/-$ ) kidneys.

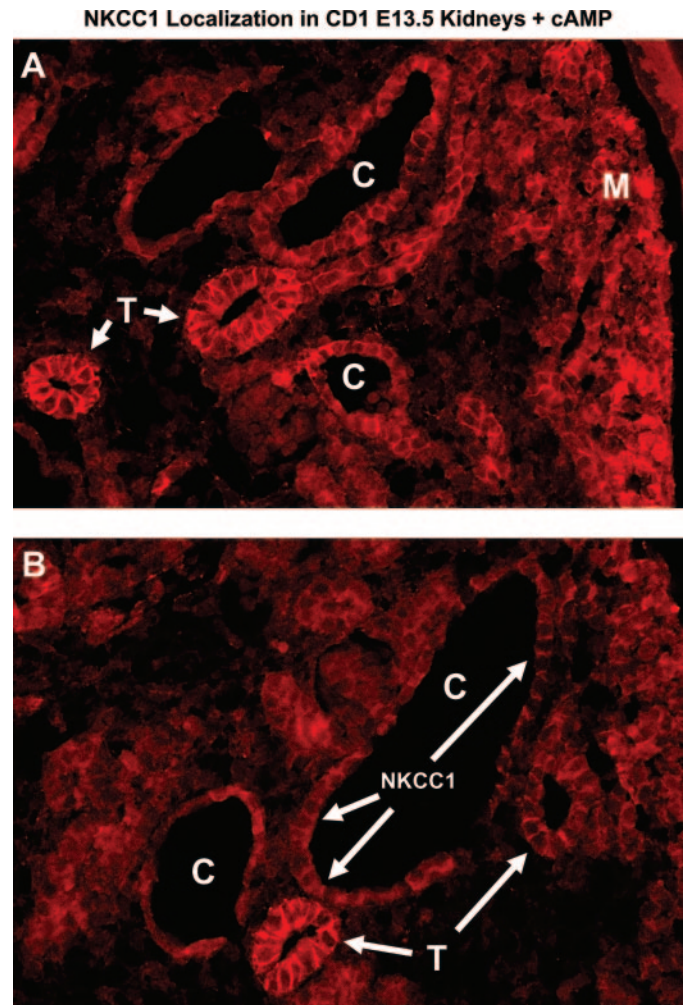
dent Cl<sup>-</sup> and fluid secretion also have been demonstrated in isolated rat collecting ducts (14,52,53) and in primary cultures of inner medullary collecting duct cells from rat (54) and human kidneys (15). In contrast to the adult kidney, less is known about the development of tubular transport mechanisms in the embryonic kidney. The expression of CFTR has been demonstrated in the developing tubules of embryonic kidneys, including the early proximal tubules and ureteric buds, and in all nephron segments later in renal development (17,21–26). Recently, NKCC1 transcripts were shown by *in situ* hybridization to be expressed in early embryonic kidneys (45). However, it was not known whether CFTR or NKCC1 is functional in embryonic kidneys. Our studies using metanephric organ culture now demonstrate that the early mammalian kidney tubule has an intrinsic capacity to secrete fluid in response to cAMP, using a CFTR- and NKCC1-dependent mechanism, showing



**Figure 8.** Effect of bumetanide inhibition on cyst formation. Embryonic kidneys from CD1 mice were placed in culture at E13.5, E14.5, or E15.5 and treated with 100  $\mu\text{M}$  8-Br-cAMP + DMSO or 100  $\mu\text{M}$  8-Br-cAMP + 100  $\mu\text{M}$  bumetanide for 4 d. (A) Kidneys were photographed for quantification of fractional cyst area  $\pm$  SEM (\* $P < 0.05$ , \*\*\* $P < 0.001$  by unpaired  $t$  test). Shown are representative photographs of E15.5 kidneys with cAMP, with or without bumetanide. (B) E13.5 kidneys were placed in culture and after 2 d were pretreated overnight with DMSO (–bumetanide) or bumetanide, then treated with 8-Br-cAMP for 24 h. The kidneys were loaded with the organic anion fluorescein for 1 h. Fresh medium (without fluorescein) was added with 8-Br-cAMP, with or without bumetanide. Photographs were taken 2 h later. Kidney 1 shows a bright-field image without bumetanide treatment, and Kidney 2 shows bright-field and fluorescence images with 8-Br-cAMP with bumetanide treatment. The fluorescence in the cAMP + bumetanide–treated kidneys represents accumulated fluorescein in the fluid of dilated proximal tubules, which seem to be the only cystic structures remaining after treatment.

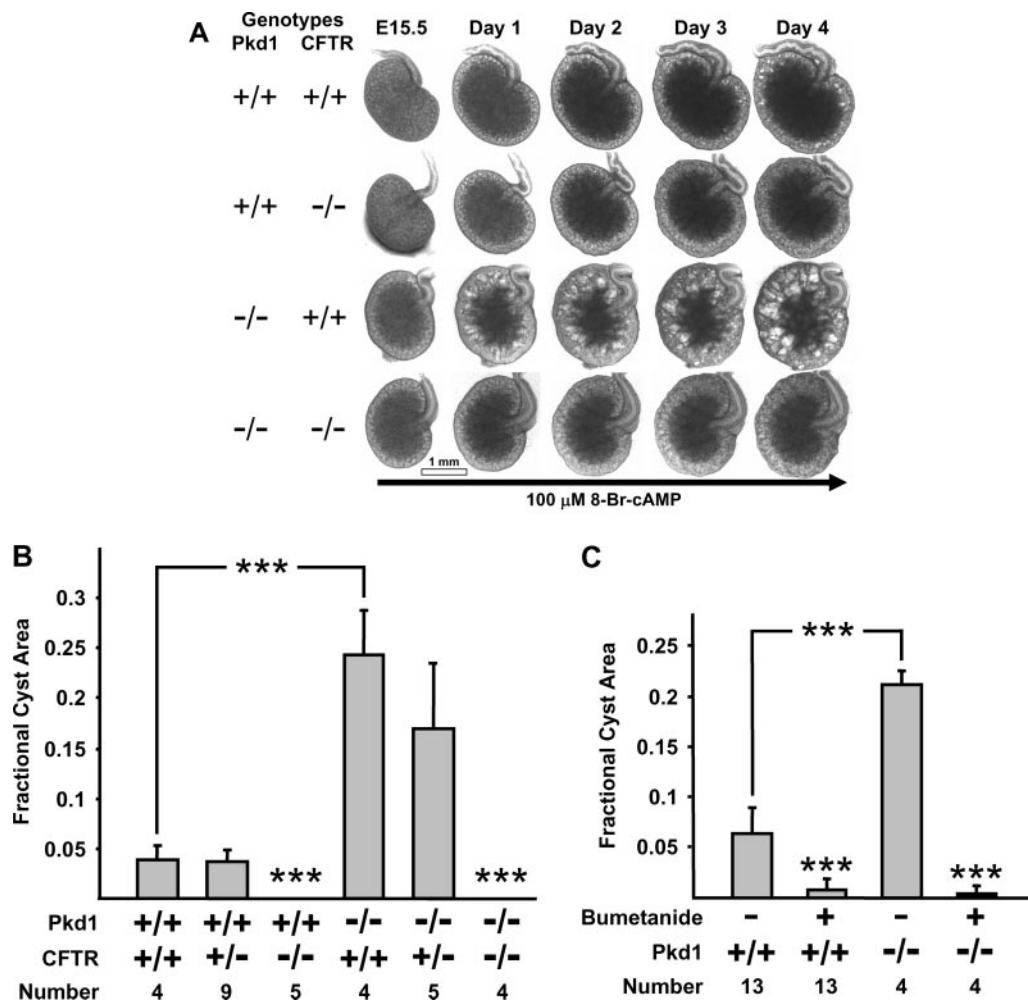
that these transporters are functional in early metanephric development.

In ADPKD, cysts are thought to form through a process that involves an increase in cAMP-driven cell proliferation (6,9,10), together with an increase in cAMP-driven net fluid secretion (12,13). In ADPKD, cysts initiate by focal dilation of the nephron, but as these cysts increase in cell number and grow in size, they often pinch off from the parent tubule and continue to grow as isolated, fluid-filled sacs (7,8). Increased fluid secretion is thought to maintain the turgidity of the expanding tubule as it grows and enlarges. Thus, while increased cyst growth in ADPKD continues to require cell proliferation, it also requires fluid secretion to fill the enlarging cyst cavity.



**Figure 9.**  $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$  co-transporter (NKCC1) localization in cultured metanephric kidneys. Embryonic kidneys from CD1 mice were placed in culture at E13.5, and after 3 d (M16.5), the kidneys were treated with 100  $\mu\text{M}$  8-Br-cAMP for 24 h. The kidneys then were harvested for sectioning and were labeled with an NKCC1 antibody. (A) The undifferentiated mesenchyme (M) shows strong labeling, along with the maturing ureteric buds (UB) and tubules (T). Cells lining the cystic structures (C) also clearly label with the NKCC1 antibody. (B) Labeling of another section showing tubules (T) and cysts (C) with pronounced lateral NKCC1 staining in the cyst-lining cells.

A driving force for fluid secretion during cyst growth is thought to be cAMP-dependent transepithelial  $\text{Cl}^-$  transport that involves activation of CFTR  $\text{Cl}^-$  channels that are located on the apical surface of cyst-lining epithelial cells (12,13,17–21). In consideration of this, a potential therapeutic intervention for ADPKD is the use of cAMP antagonists (55), which would be expected to slow fluid secretion, as well as cell proliferation, and thereby slow cyst expansion. Indeed, recent studies in animal models of PKD have demonstrated that AVP receptor antagonists, which reduce renal cAMP levels, markedly slow or reverse cystic disease (56–58). CFTR inhibitors also have been considered for therapeutic intervention in ADPKD (13,17–19), in part on the basis of evidence that cyst growth *in vitro* can be



**Figure 10.** Cyst formation in metanephric *Pkd1*  $-/-$  kidneys. Embryonic kidneys from matings of *Pkd1*  $+/-$  mice or double-heterozygous *Pkd1*  $+/-$ :*Cftr*  $+/-$  mice were placed in culture at E15.5 and treated with 100 μM 8-Br-cAMP. (A) Representative pictures of kidneys from the various genotypes are shown. Open bar = 1 mm. (B) Quantification of cyst area at 4 d in wild-type or *Pkd1*  $-/-$  kidneys, with zero, one, or two wild-type *Cftr* alleles. The bars show the average fractional cyst areas  $\pm$  SEM ( $***P < 0.001$  by one-way ANOVA versus the respective *Pkd1*  $+/+$  and/or *Cftr*  $+/+$  control). The numbers under the bars are the numbers of kidneys analyzed. Loss of functional CFTR prevented cAMP-mediated tubule dilation, as there was no evidence of tubule dilation in the *Cftr*  $-/-$  kidneys. (C) *Pkd1*  $+/+$  or *Pkd1*  $-/-$  kidneys were placed in culture at E15.5 and treated for 4 d with 100 μM 8-Br-cAMP, with or without 100 μM bumetanide (DMSO was used in the  $-$  bumetanide cultures). The bars show the average fractional cyst areas  $\pm$  SEM ( $***P < 0.001$  by one-way ANOVA versus the respective *Pkd1*  $+/+$  and/or  $-$  bumetanide control).

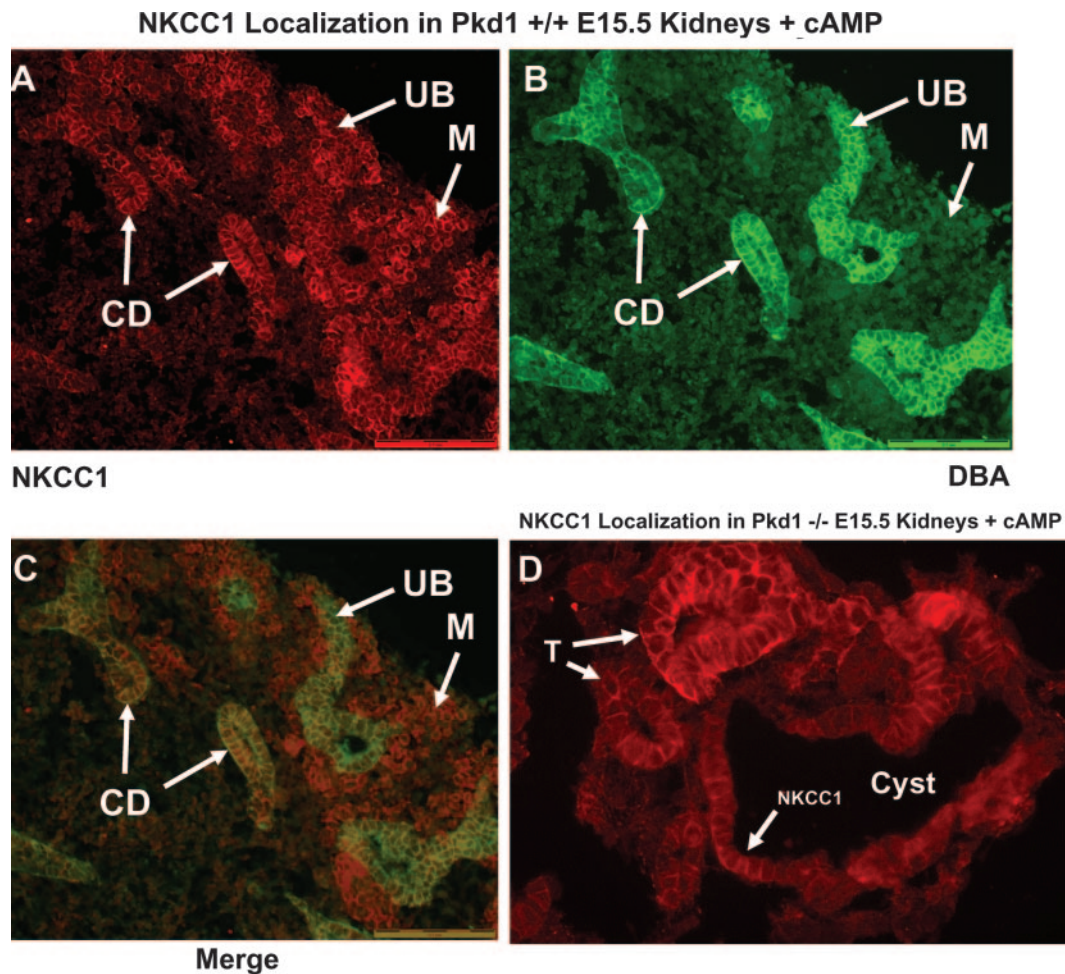
blocked by CFTR inhibition, which seems to target specifically the fluid secretion component of cyst enlargement (59). Although the success of these treatments suggests that it is possible to ameliorate PKD by treating fluid secretion, the question that remains is how early in kidney development fluid secretion occurs, because intervention to prevent cyst growth at the earliest possible time should be the goal.

Cysts are thought to initiate *in utero*. Indeed, in homozygous *Pkd1* or *Pkd2* null mouse models, cysts begin to develop at approximately E15.5 (60–64). However, the extent to which cyst filling can be attributed to glomerular filtration cannot be judged without knowing whether embryonic tubules have the capacity to secrete fluid. To determine whether embryonic kidneys are capable of CFTR-dependent fluid secretion, we treated

wild-type mouse metanephric kidneys with 8-Br-cAMP and found that many of the developing renal tubules formed cystic dilations. E13.5 to E15.5 mouse metanephric kidneys continue to support mesenchymal induction, tubule formation, and branching morphogenesis, in the absence of functioning glomeruli, over a period of days in culture (29–31,35,36,65). As such, the earliest stages of tubule formation and development can be seen to occur *in vitro* in a culture system that is amenable to treatment with small molecule agonists or antagonists.

Our studies are the first to demonstrate that embryonic kidney tubules have an intrinsic capacity for cAMP-driven fluid secretion in the absence of glomerular filtration. Treatment with either 8-Br-cAMP or the cAMP agonist forskolin resulted in the rapid expansion of cyst-like tubular dilations. cAMP-depen-



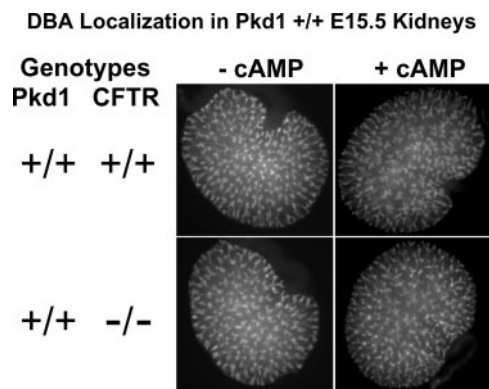


**Figure 11.** NKCC1 localization in *Pkd1* <sup>+/+</sup> and *Pkd1* <sup>-/-</sup> metanephric kidneys. Embryonic kidneys from matings of *Pkd1* <sup>+/-</sup> mice were placed in culture at E15.5 and treated with 100  $\mu$ M 8-Br-cAMP (cAMP) for 4 d (M19.5). Sections from *Pkd1* <sup>+/+</sup> and *Pkd1* <sup>-/-</sup> kidneys were co-labeled with NKCC1 antibody and DBA-FITC. (A) In a *Pkd1* <sup>+/+</sup> kidney, NKCC1 localizes to the maturing collecting ducts (CD), undifferentiated mesenchyme (M), and, to a lesser extent, the UB. (B) DBA labels the maturing CD and UB. (C) Merge of NKCC1 (red) and DBA (green) shows co-localization in the maturing CD and UB. (D) In a *Pkd1* <sup>-/-</sup> kidney, NKCC1 localizes in tubules (T) and in cells surrounding cysts (Cyst).

dent tubule dilation was reduced by the PKA inhibitor H89 and by the CFTR inhibitor CFTR<sub>inh</sub>172. Although in both cases, tubule dilation was not blocked completely by these inhibitors, there was a significant degree of inhibition, which suggested that cAMP-dependent activation of PKA and CFTR was important to the process and that the inhibitors were only partially effective. To examine further the role of CFTR, we prepared metanephric cultures from mice with a targeted mutation of the *Cftr* gene. In contrast to mice with at least one wild-type *Cftr* allele, which showed formation of cyst-like tubular dilations in response to 8-Br-cAMP, the *Cftr* <sup>-/-</sup> mice showed no evidence of tubular expansion, indicating that CFTR is functional in embryonic kidneys and that it is required for cAMP-mediated tubule dilation. Although these results do not rule out the presence of other cellular pathways that could drive fluid secretion in embryonic tubules, they do suggest that all cAMP-driven fluid secretion depends on CFTR. However, that *Cftr* <sup>-/-</sup> metanephric kidneys had no visible (microscopic or mac-

roscopic) tubule dilations suggests that if other fluid secretion pathways exist in embryonic tubules, then they are not visibly operative under our culture conditions. It is interesting that quantification of fractional cyst area indicated that there was a small but significant decrease in dilated tubule area in *Cftr* <sup>+/-</sup> kidneys as compared with *Cftr* <sup>+/+</sup> kidneys (Figure 7B; see also Figure 10B). Almost equally effective in blocking cyst formation was inhibition of NKCC1 by bumetanide, in particular at E15.5, suggesting a mechanism for cAMP-stimulated fluid secretion in embryonic kidneys that depends on NKCC1-mediated basolateral Cl<sup>-</sup> entry.

We observed that there is a decline in cyst-forming potential with increasing developmental age from E13.5 to E15.5 (Figures 3B and 8A) and that E13.5 kidneys produce larger cysts than do E15.5 kidneys (Figure 3A). There also were strain differences between CD1 and C57BL/6 kidneys (compare fractional cyst areas for the control CD1 kidneys in Figures 3B, 6B, 7B, and 8A with those for control C57BL/6 kidneys in Figure 10A). The



**Figure 12.** DBA localization in *Pkd1*  $+/+$  metanephric kidneys. Embryonic kidneys from matings of double-heterozygous *Pkd1*  $+/-$ :*Cftr*  $+/-$  mice were placed in culture at E15.5 and treated with (or without) 100  $\mu$ M 8-Br-cAMP (+ cAMP) for 4 d (M19.5). The kidneys were harvested, DBA-FITC labeled, and photographed to visualize the pattern of UB branching, which did not seem to be affected by the 8-Br-cAMP or the lack of CFTR (or both).

greatest cyst-forming potential, however, was observed in *Pkd1*  $-/-$  kidneys, which responded to cAMP treatment by rapidly filling with large cystic dilations that were three- to six-fold larger than those of wild-type kidneys (Figure 10). This observation suggests that, despite their relatively benign appearance at E15.5, the *Pkd1*  $-/-$  tubules are poised to respond to elevations in cAMP with unusually pronounced cyst expansion. As a possible contributing factor, it was reported recently that abnormal polycystin-1 function may increase plasma membrane expression of CFTR, thereby potentiating cAMP-stimulated  $Cl^-$  and fluid secretion in PKD renal tubules (66). As with CD1 wild-type cysts, the *Pkd1*  $-/-$  cysts were completely dependent on CFTR and almost totally inhibited by the NKCC1 blocker bumetanide (Figure 10, B and C).

In agreement with a recent report that showed NKCC1 RNA expression in the developing mouse kidney (45), we now have shown that NKCC1 protein is widely expressed in all tubule segments and in the metanephric mesenchyme, as early as E13.5. Both the bumetanide result and visual inspection of NKCC1-stained *Pkd1*  $-/-$  kidneys suggest that all cyst-forming structures are NKCC1 positive. It was reported in one study (67) that one third of the ADPKD cysts examined were NKCC1 positive and that all of these were CFTR positive. It may be possible that both NKCC1 and CFTR are expressed initially in all ADPKD cysts early in the disease process but that over time NKCC1 expression decreases as the disease progresses and the cysts undergo dedifferentiation and suffer further secondary change. If so, then bumetanide may be more effective in reducing cyst growth early in the disease process, rather than later.

CFTR-dependent fluid secretion may be a factor in PKD, because patients who have both CF and ADPKD have less severe renal enlargement (27,28). Our studies suggest that CF carriers ( $+/-$ ) also may have a somewhat milder clinical PKD course, although the effect could be difficult to detect and quantify, but it also should be noted that patients who have

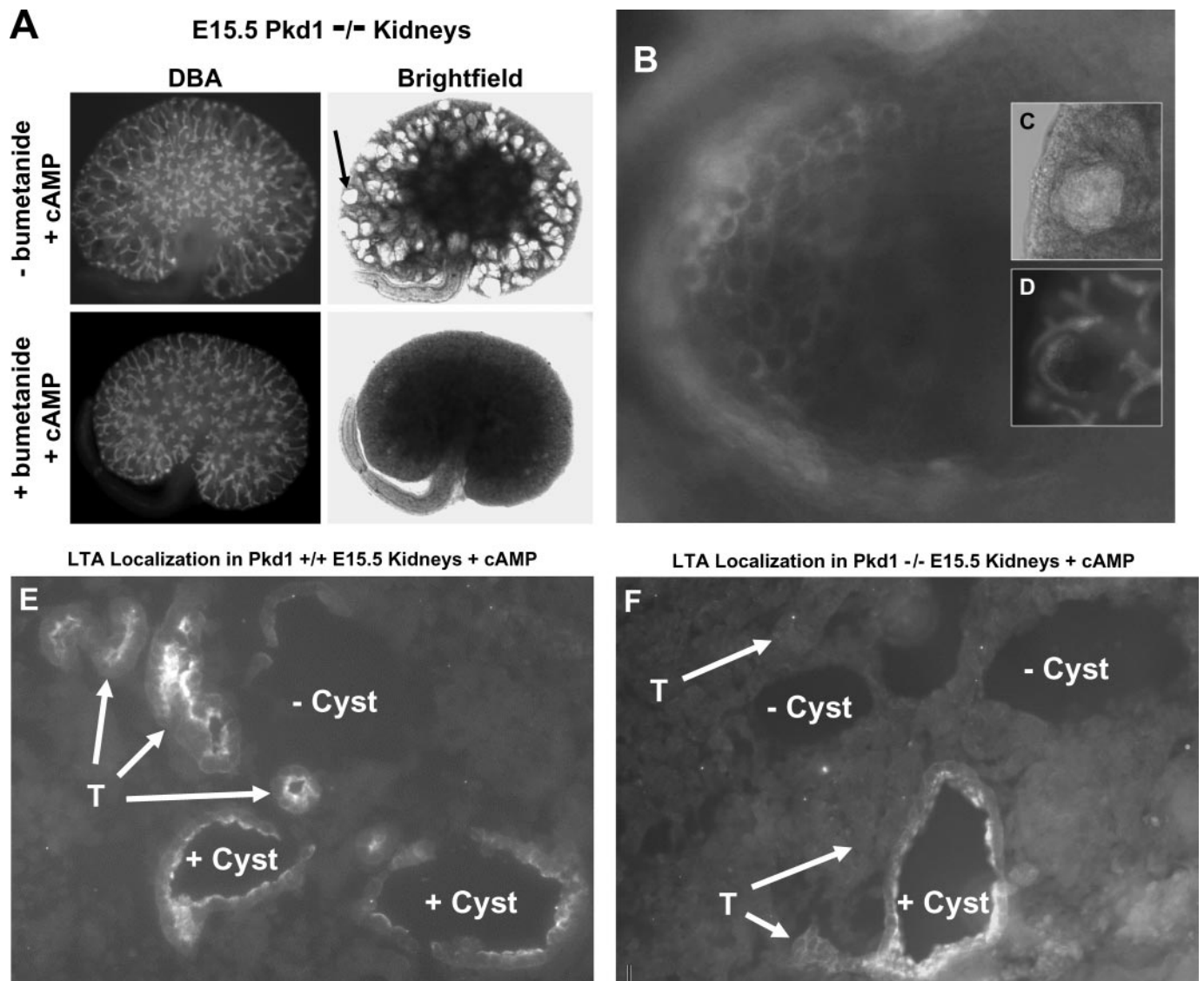
ADPKD and co-inheriting CF are not completely protected from cystic disease (27,28,68), suggesting that non-CFTR-based mechanisms may be present to allow cyst growth in ADPKD.

Although we found that the *Cftr*  $-/-$  genotype completely suppressed cyst formation in *Pkd1*  $-/-$  embryonic kidneys in response to cAMP (Figure 10, A and B), the *Cftr*  $-/-$  genotype did not rescue *Pkd1*  $-/-$  embryos from lethality. At E15.5, *Pkd1*  $-/-$ :*Cftr*  $-/-$  embryos are edematous and hemorrhagic and cannot be distinguished from *Pkd1*  $-/-$  embryos that carry one or two wild-type *Cftr* alleles. Also, in 147 mice that were generated in our colony from *Pkd1*  $+/-$ :*Cftr*  $+/-$  matings, we had not seen a *Pkd1*  $-/-$  mouse at the time when we genotype the pups (approximately 10 postnatal days; we would have expected approximately nine surviving *Pkd1*  $-/-$  mice if *Cftr*  $-/-$  were protective). This outcome is consistent with evidence that was obtained by others suggesting that lethality of *Pkd1*  $-/-$  mice is associated with cardiovascular (63,64) or placental (69) failure, rather than kidney failure.

In one study that was carried out in the *Bpk* recessive PKD mouse model, it was determined that the *Cftr*  $-/-$  genotype did not result in improved renal function or renal cystic disease (70). The authors discussed a number of possible explanations, including that BPK cysts are continuous with glomeruli, thereby retaining their afferent connections, and as such may not depend on fluid secretion (70). It also is possible that CFTR-based secretory mechanisms are not responsible for cyst enlargement in autosomal recessive PKD (ARPKD). In fact, to the contrary, there is evidence for  $Na^+$  hyperabsorption in cultured ARPKD cyst-lining cells (71) and in epithelial cells of the *Tg737* mouse model of recessive PKD (72).

The process of fluid secretion in metanephric kidneys was confirmed by showing that the organic anion fluorescein accumulated in the tubular dilations. Fluorescein is taken up readily by basolateral transport and concentrated within proximal tubule cells (40) and then is transported across the apical membrane in the cyst-like lumens, where it accumulates to high levels, rendering the cystic structures highly fluorescence. It is apparent in Figures 5 and 8B that some cysts accumulated high levels of fluorescein, whereas others did not. The lower fluorescein levels in some dilated tubules may indicate that some of the dilated structures were derived from nonproximal tubules, which would not be expected to transport fluorescein. It also is possible that these fluorescein-negative structures were derived from the most immature proximal tubules, those that had not yet developed organic anion transport systems. Indeed, a recent study showed that expression of the fluorescein transporters OAT1 and OAT3 is very low in early rat embryonic kidneys, increasing to much higher levels through fetal development (43).

It is apparent from our experiments that not all tubules formed cystic dilations. One explanation that partially accounts for this observation is that dilations may form more readily in tubules that have not yet made outflow connections to the collecting system. This would allow secretion to expand rapidly these enclosed tubules but not the more mature tubules that have patent connections for fluid release. A possibly related observation is that when more mature, E15.5 kidneys are placed



**Figure 13.** Dolichos biflorus agglutinin (DBA) and Lotus tetragonobolus agglutinin (LTA) staining of *Pkd1*  $+/+$  and *Pkd1*  $-/-$  metanephric kidneys. Embryonic kidneys from matings of *Pkd1*  $+/-$  mice were placed in culture at E15.5 and treated with 100  $\mu$ M 8-Br-cAMP (+ cAMP) with or without 100  $\mu$ M bumetanide for 4 d (M19.5) (DMSO was used in the  $-$ bumetanide cultures). (A) The kidneys were harvested, DBA-FITC labeled, and photographed (left, fluorescence; right, bright-field) to visualize UB branching and cysts, respectively. Whereas bumetanide completely blocked cyst formation, it did not seem to affect UB branching. Arrow indicates cyst shown at higher magnification in B through D. (B) Higher magnification, whole-mount fluorescence image of the cyst indicated by the arrow in A, showing DBA-stained individual cells. (C) Bright-field image of the same cyst. (D) Fluorescence image of the same cyst surrounded by UB. B and D show that this cyst is DBA positive and thus seems to be of CD origin. (E and F) LTA staining of a *Pkd1*  $+/+$  kidney (E) and a *Pkd1*  $-/-$  kidney (F). Both LTA-positive and LTA-negative tubules (T) and LTA-positive (+ Cyst) and LTA-negative ( $-$  Cyst) cysts are shown.

in metanephric culture, they do not respond to cAMP with as dramatic cystic dilation as seen in E13.5 kidneys (Figure 3), suggesting that more tubule connections have been established at this later developmental stage to allow outflow of the cAMP-driven, secreted fluid. Lectin staining showed both LTA-positive cysts and DBA-positive cysts, suggesting that multiple tubule segments are capable of cystic dilation in response to cAMP.

## Conclusion

We have demonstrated that developing metanephric kidney tubules are capable of cAMP-mediated, CFTR- and

NKCC1-dependent fluid secretion, raising the question of the physiologic importance of such a mechanism in kidney development. One can speculate that fluid secretion may help to form the lumens of the developing tubules. However, the absence of a renal phenotype in patients with CF would argue that such a mechanism may not be essential. These results also suggest that *in utero*, PKD-deficient kidneys may be predisposed to cystic dilation but that cyst formation *per se* requires elevated cAMP. As such, it may be possible that localized, intermittent increases in cAMP levels in develop-

ing ADPKD kidneys actually trigger the early stages of cyst formation. If so, then a combination of therapies to inhibit both cell proliferation and fluid secretion, including CFTR and NKCC1 inhibitors, may be effective in reducing cyst formation early in the PKD disease process.

## Acknowledgments

This research was supported by DK057301 (J.P.C., D.P.W., R.L.M., and J.J.G.), RR017686 (D.P.W.), DK056791 (R.C.D.), and DK052483 and DK065123 (D.R.A.), and by a grant from the PKD Foundation (J.P.C.).

This work was based in part on previous work (*J Am Soc Nephrol* 15, 651A, 2004).

We thank Terry Peterson and Monica Johnson for expert assistance and Greg Vanden Heuvel for helpful discussions.

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