Prostaglandin E2 mediates proliferation and chloride secretion in ADPKD cystic renal epithelia

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by a hyperproliferative cystic renal epithelium and clinically by polyuria (32). Recent observations suggest that altered prostanooid production contributes to these pathologic and clinical features. In kidneys of rodent PKD models and in renal cyst fluid from PKD patients, prostanoids, and more specifically PGE2, are present at higher concentrations than in controls (2, 5, 28, 35, 44). The molecular mechanisms by which mutations of cystogenic genes induce renal PGE2 production remain unknown, but the levels of renal PGE2 significantly affect the phenotype of PKD. Pharmacologic inhibition of PGE2 synthesis in nonorthologous rodent models of ADPKD reduces water excretion, tubular epithelial cell proliferation, tubulointerstitial fibrosis, and macrophage infiltration (28, 35). However, the animal models studied to date have not been genetically orthologous to human ADPKD. Moreover, the direct relationship between these pathologic events and polycystic genes [polycystin-1 (PC-1), in particular] has not been determined.

PGE2 functions through the activation of four subtypes of EP receptors: EP1, EP2, EP3, and EP4 (8). EP2 and EP4 receptors are classic Gαs-coupled receptors that increase intracellular cyclic AMP (cAMP) concentration. EP1 receptor increases intracellular Ca2+ concentration and the abundance of active β-catenin in PC-1-deficient cells, suggesting a mechanism for PGE2-mediated proliferation. Consistent with this hypothesis, antagonizing EP4 receptors reverted the growth advantage of PC-1-deficient cells, implicating a central role for the EP4 receptor in proliferation. To test whether PGE2-dependent Cl− secretion is also enhanced in PC-1-deficient cells, we used an Ussing chamber to measure short-circuit current (Isc). Addition of PGE2 induced a fivefold higher increase in Isc in PC-1-deficient cells compared with PC-1-replete cells. This PGE2-induced increase in Isc in PC-1-deficient cells was blocked by CFTR-172 and flufenamic acid, indicating that PGE2 activates CFTR and calcium-activated Cl− channels. In conclusion, PGE2 activates aberrant signaling pathways in PC-1-deficient epithelia that contribute to the proliferative and secretory phenotype characteristic of ADPKD and suggests a therapeutic role for PGE2 inhibition and EP4 receptor antagonism.

Two biologic processes drive cystogenesis in ADPKD: epithelial tubular proliferation and chloride (Cl−)-dependent fluid secretion (5, 6, 10, 43, 46, 48). In particular, high levels of intracellular cAMP have been shown to induce the proliferative response and cystic fibrosis transmembrane conductance regulator (CFTR)-dependent Cl− secretion in ADPKD tubular epithelia (10, 31). While vasopressin type 2 receptor-dependent increases in intracellular cAMP have garnered much attention in the cystogenesis of ADPKD, Elberg et al. (17) showed that exposure of PGE2 to human ADPKD tubular epithelia significantly increased intracellular cAMP, cystogenesis, and proliferation, suggesting a role for PGE2 in the pathogenesis of ADPKD. However, much of the evidence for the effect of PGE2 on proliferation has been found in models of colon cancer where PGE2 signals through EP1 (27), EP2 (12), or EP4 (13) receptors to induce cell growth. Conversely, cyclooxygenase (COX) inhibition prevents tumorigenesis in humans (34), and similarly, inhibition of COX reduced proliferation and cystogenesis in genetically nonorthologous models of ADPKD (35). The parallels between epithelial proliferation in models of colon cancer and PKD suggest that PGE2, an important renal prostanoid; secretory renal epithelia; cystic epithelia; cyclooxygenase

THE IMPORTANCE OF PRECISE regulation of prostaglandin (PG) synthetic and signaling pathways is emphasized by the essential role of PGs in normal physiologic functions, such as the control of sodium (Na+) and water excretion in the renal collecting duct (CD), as well as in the contribution of PGs to pathologic conditions, such as colon cancer (7, 12, 38). Autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive PKD are characterized histopathologically with hyperplasia of renal epithelial tubules, and with the development of cysts, macrophage infiltration, and cystic fibrosis (28, 35). Recent evidence suggests a role for PGE2 in the pathogenesis of ADPKD (35).

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epithelial metabolite, contributes to the proliferative phenotype of PKD epithelia.

Under normal physiologic conditions, renal PGE2 activates either EP1 or EP2 receptors to inhibit Na\(^+\) and water transport in the CD and induce diuresis (7, 14). In the early stages of ADPKD, before the development of chronic kidney disease, patients are polyuric and unable to maximally concentrate their urine, suggesting the possibility PGE2 may contribute to polyuria in these patients. In addition, ligand-dependent Cl\(^-\) secretion has been shown to be an important contributing factor for cyst enlargement in ADPKD (10). Several paracrine factors such as PGE2 (5), DDAVP (5, 6), vasopressin (5), and epinephrine (5) bind to G protein-coupled receptors that signal through intracellular cAMP and activate CFTR-dependent Cl\(^-\) secretion. Direct inhibition of CFTR (48) reduces cystogenesis and renal failure in murine models of ADPKD, supporting the clinical importance of Cl\(^-\) secretion into kidney cysts in ADPKD.

The involvement of PGE2 in dysregulated cellular proliferation and Cl\(^-\) secretion led us to hypothesize that PGE2 contributes to cystogenesis in ADPKD. To test this hypothesis, we assessed the role of PGE2-induced proliferation and Cl\(^-\) secretion in an established murine renal epithelial cell culture model deficient in PC-1 (protein name); PKD1 (gene name) (4). Our findings demonstrate that exogenous and endogenous PGE2 contributes to the classic proliferative and secretory features of PC-1-defective cystic epithelia and indicate that inhibition of PGE2 and its downstream signaling may be another strategy for the treatment of ADPKD.

**MATERIALS AND METHODS**

**Reagents**

*Inhibitors.* The inhibitors were as follows: 3 \(\mu\)M AH6809 (24) (Cayman Chemical; IC\(_{50}\) = 350 nM), 10 \(\mu\)M L161,982 (15) (Cayman Chemical), 100 nM SC560 (Cayman Chemical; IC\(_{50}\) = 9 nM), 1 \(\mu\)M CAY 10404 (Cayman Chemical; IC\(_{50}\) < 1 nM), 30 \(\mu\)M indomethacin (Sigma), 10\(^{-5}\) M CFTR inhibitor 172 (Calbiochem; EC\(_{50}\) ~ 300 nM) (30), and 2 \(\times\) 10\(^{-4}\) M flufenamic acid (FFA) (29) (Sigma). The agonists include 50 or 77 nM PGE2 (17) (Cayman Chemical), 1 \(\mu\)M TCS 2510 (Tocris Bioscience; EC\(_{50}\) = 2.5 nM), 1 \(\mu\)M (R) butaprost free acid (17) (Cayman Chemical), and 10 \(\mu\)M forskolin (Sigma) (5, 46). The antibodies were as follows: rabbit polyclonal anti-phospho (Thr202/Tyr204)-ERK antibody (Cell Signaling), rabbit polyclonal anti-total-ERK antibody (Cell Signaling), murine monoclonal active diphosphorylated Ser37, Thr41 \(\beta\)-catenin antibody (Millipore), and murine monoclonal \(\beta\)-actin antibody (Sigma).

**Cell Culture**

All cells were grown in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (complete medium) at 37°C in 5% CO\(_2\). Two sets of cell populations were generated upon transduction of murine inner medullary CD (IMCD)3 cells with lentiviral vectors stably expressing either a control anti-luciferase (siLuc) siRNA or a siRNA against PKD1 gene (siPKD1), as previously described (4). Parental wild-type IMCD3 cells were also used as a reference control. In siPKD1 knockdown cells, the expression of PC-1 protein has been previously shown to be reduced to ~20–30% of the siLuc or the parental controls (4). Cells were plated on plastic plates for proliferation studies or on permeable supports for electrophysiologic studies.

**Proliferation Studies**

*Manual counting.* Equal numbers of cells were plated in six-well plastic plates by counting manually with a hemocytometer. At 0, 24, 48, and 72 h after the cells adhered to the plastic wells, cells were dissociated with trypsin, resuspended in media containing trypsin blue, and viable cells were counted by hemocytometer. Indomethacin (30 \(\mu\)M) was added after cells were seeded and adhered. The cell number at each time point was normalized to the number of cells plated at time 0 h and expressed as a ratio of cells at time 0 h.

*Cyquant.* About ten thousand cells per well were plated into a 96-well plate. A standard curve was designed according to the sensitivity and limitations identified in the Cyquant protocol. Cells were incubated in DMEM/F12 with 10% FBS (for 18 h) to ensure attachment of cells to the plate. After 18 h, one plate of cells was frozen, whereas the media in the other plates were then changed to DMEM/F12 with 1% serum and growth was allowed to resume up to 48, 72, and 96 h. COX inhibitors and EP receptor antagonists were only added after adherence of cells at 18 h. To quantitate the cell number, 200 \(\mu\)l of GR dye/cell lysis buffer were added to each well, gently mixed, and incubated for 2 h to maximize and stabilize the fluorescense. A plate reader was used to measure fluorescence with an excitation at ~480 nm and emission at ~520 nm. The measured fluorescence was converted to a cell number based on a standard curve, and then this was expressed as a ratio comparing the cell number at a specified time point with the calculated cell count at the 18-h time point.

**PGE2 Measurement**

In media bathing the cells. One milliliter of serum and phenol red-free DMEM/F12 were incubated with siPKD1 or IMCD3 cells for 3 h (26). The conditioned media were collected and frozen at ~80°C for measurement of PGE2; at a later time, PGE2 concentration (pg/ml) was measured with PGE2 enzyme immunoassay (EIA) kit from Cayman Chemical, following the standard protocol enclosed with the kit, and PGE2 concentration was normalized to the number of cells to which the conditioned media were exposed.

**Intracellular cAMP Measurement**

Serum-starved cells were incubated with indomethacin, PGE2, vasopressin, (R)-butaprost acid free, TCS 2510, and forskolin for 2 h. The cells were collected in 0.1 mM HCL, incubated at room temperature, and assayed as per protocol for cAMP EIA Kit (Cayman Chemical). The data were normalized to cAMP concentration measured in the forskolin-treated cells (5).

**Immunoblotting**

Western blot analysis was performed as previously described (20). Forty micrograms of cellular protein were isolated, electrophoretically resolved, and transferred to Immobilon filters (Millipore, Billerica, MA). Filters were blocked in nonfat dried milk and Tween and immunoblotted with an anti-phospho antibody (see Reagents). After being washed, blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO) and bands were visualized by the West Pico chemiluminescence kit (Pierce, Rockford, IL). After the membrane was stripped and blocked, the blot was incubated with an anti-total protein-specific antibody or anti-actin antibody and visualized using the same methods as the primary antibody. The phosphorylated protein was expressed as a ratio to the total amount of that specific protein present or to actin.

**PGE2-Induced Current**

IMCD3 cells were expanded and plated on permeable polycarbonate Snapwell filters that were coated with human placental collagen. Both apical and basolateral medium was replaced every day and
transepithelial resistance ($R_{te}$) was measured with an EVOM (“chopstick” voltmeter; World Precision Instruments) before media change. When $R_{te} > 50 \, \Omega \cdot \text{cm}^2$, filters were mounted between the Lucite half chambers of an Ussing chamber apparatus (Physiological Instruments, San Diego, CA) as described previously (29). Briefly, cell sheets were bathed in Krebs Henseleit solution (in mM: 140 NaCl, 25 NaHCO$_3$, 5 KCl, 5 glucose, 2 CaCl$_2$, and 1 MgCl$_2$) and gassed with a mixture of 95% O$_2$-5% CO$_2$. Transepithelial voltage ($V_{te}$) across the cell sheets was clamped at 0 mV and a set voltage pulse of 1 mV was applied across the cell sheets for 200 ms every 20 s. The short-circuit current ($I_{sc}$) and $R_{te}$ across cell sheets were continuously recorded using Acquire and Analyze software (Physiological Instruments).

Once $I_{sc}$ stabilized, cell sheets were exposed to a series of pharmacological agents. First, amiloride ($10^{-5}$ M; Sigma) was added to the apical bath to block Na$^+$ reabsorption. Then, $7.7 \times 10^{-8}$ M of PGE$_2$ was added to both sides of the cell sheet. We then added CFTR inhibitor 172 ($10^{-5}$ M) and $2 \times 10^{-8}$ M FFA to the apical side to inhibit CFTR and calcium-activated Cl$^-$ channels (CaCCs), respectively.

Statistics

Data are given as means ± SE ($n = \text{number of wells or filters}$). Statistical analyses were performed using paired or unpaired t-tests (SigmaStat version 2.03; SPSS, San Rafael, CA).

RESULTS

Proliferation in PC-1-Deficient Cells is COX Dependent

Utilizing a previously characterized cell culture model of ADPKD (4), in which the expression of PC-1 was stably knocked down to 20–30% of that of parental cells, we sought to test whether PGE$_2$ contributed to the hyperproliferative and secretory phenotype of ADPKD renal epithelia that is characteristic of cyst formation. Both parental IMCD3 cells and cells transduced with a lentivector expressing a control siRNA specific for the luciferase gene (siLuc) (4) were used as references in these initial studies.

We first sought to test whether siPKD1 cells, deficient in PC-1 protein (4), increase in cell number (proliferate) faster than wild-type IMCD3 and siLuc-transduced control cells. For this experiment, cells were manually counted at each time point, and proliferation was expressed as a ratio of cells at each time point relative to cells present at time 0 h. Untreated (0 µM indomethacin) IMCD3 and siLuc cells grew at similar rates; however, siPKD1 cells proliferated significantly faster than IMCD3 and siLuc cells (Fig. 1; *$P < 0.05$, $n = 3$ wells for each cell type and condition). To determine whether prostanoids mediate the increase in cell proliferation in PC-1-deficient cells, we treated each cell type with indomethacin, a COX-1 and COX-2 inhibitor. At 72 h in culture, indomethacin (30 µM) reduced siPKD1 cell number by 50.3 ± 4.2% and IMCD3 cell number by 31.1 ± 2.9% of untreated siPKD1 and IMCD3 cells (Fig. 1; $SP < 0.05$, $n = 3$ wells for each cell type and condition), respectively. Moreover, indomethacin reduced cell proliferation in siPKD1 cells to levels observed in untreated control cells (IMCD3 and siLuc), implications a role for endogenous prostanoids stimulating proliferation in PC-1-deficient cells (Fig. 1).

These data were confirmed using the Cyquant (Invitrogen) cell proliferation assay, which measures DNA content through binding of a fluorescent dye to DNA (36). This method/assay permits a faster and simpler assessment of cell number and, since it provides comparable results, we used it in place of manual counting for the following experiments. siPKD1 cell number [white bar, $n = 6$ (wells)] increased faster than transduced (siLuc) control cells (gray bar, $n = 6$, *$P < 0.05$) at all time points and faster than parental IMCD3 cells (black bar, $n = 6$, *$P < 0.05$) at 72 and 96 h (Fig. 2A). Proliferation was similar between siLuc and IMCD3 cells. Because siLuc and parental IMCD3 cells express equal amounts of PC-1 protein (4) and grow at comparable rates (Fig. 2A), we chose to only include parental IMCD3 cells as the control in the remaining experiments. To extend our earlier findings and identify the contribution of specific COX isoforms on growth changes, cells were treated with inhibitors of COX-1 (100 nM SC560), COX-2 (1 µM CAY10404), or COX-1 and -2 (30 µM indomethacin), and cell counts were measured at different time points with Cyquant. Inhibition of COX-1 (SC560 bar, $n = 6$), COX-2 (Cay 10404 bar, $n = 6$), and COX-1 and -2 (INDO bar, $n = 6$) in siPKD1 cells markedly reduced proliferation at all time points compared with untreated siPKD1 (white bar; $@P < 0.05$, Fig. 2B). Inhibition of COX-1/-2 by indomethacin reduced proliferation of siPKD1 cells to a greater extent than inhibition of COX-1 or COX-2 individually ($SP < 0.05$) at 72 and 96 h. At 48 h COX-2 and COX-1/-2 inhibition reduced proliferation to a greater extent than did COX-1 inhibition in siPKD1 cells ($SP < 0.05$). As seen in prior experiments, siPKD1 cells proliferated faster (white bar, *$P < 0.05$, Fig. 2B) than did parental IMCD3 (black bar, Fig. 2B) cells, replicating our earlier findings and serving as an internal control.
PGE₂, a COX-Dependent Prostanoid, Regulates Proliferation

The relationship between COX activity and proliferation in PC-1-deficient cells suggests that endogenous PGE₂ production by renal epithelial cells may contribute to the hyperproliferative phenotype of cystic epithelia. Moreover, the PGE₂ concentration in renal tissue isolated from the Han:SPRD PKD1 model of ADPKD is greater than that measured in littermate controls (35, 44), suggesting that excessive autocrine production of PGE₂ may also contribute to the proliferative phenotype. To test whether PGE₂ concentrations are greater in PC-1-deficient cells, we measured PGE₂ in the conditioned media bathing the PC-1-deficient and PC-1-replete IMCD3 cells. However, PGE₂ concentration (pg/ml) did not differ between PC-1-deficient and parental control cells at any time point (Fig. 3A). When PGE₂ concentration in the media was normalized to the number of cells in culture, no difference was found at any time points (Fig. 3B), suggesting that exaggerated tubular PGE₂ secretion and subsequent autocrine/paracrine signaling were not inducing proliferation in PC-1-deficient cells. We speculated that local PGE₂ secretion may activate dysregulated EP receptor-mediated signaling in PC-1-deficient cells, contributing to the proliferative phenotype. The COX inhibition experiments only suggested that prostanoids (and possibly other eicosanoids) were involved in proliferation, but they did not specify PGE₂ as the culprit prostanoid. To address these issues, IMCD3 and PC-1-deficient cells were treated with 50 nM PGE₂ to investigate whether exogenous PGE₂ enhances cellular proliferation of siPKD1 cells. Wild-type IMCD3 cell number decreased significantly (dark gray bars, *P < 0.05, n = 6 wells) after treatment with 50 nM PGE₂ compared with untreated controls (black bars, n = 6 wells); however, siPKD1 cell number increased after treatment with PGE₂ (white bars, $P < 0.05, n = 6$ wells) compared with untreated siPKD1 cells (Fig. 4, light gray bars, n = 6 wells). PGE₂ induced prolifer-
Exogenous PGE2 reduces proliferation in human wild-type renal epithelia, which are similar to those reported by Yamaguchi et al. (46) in which PGE2 reduced proliferation in human wild-type renal epithelia, but it increased proliferation in human cystic renal epithelia. Thus, we conclude that dysregulated EP receptor signaling, rather than an increase in PGE2 secretion or concentration, is responsible for the proliferation in ADPKD epithelia.

**PGE2 Induces Intracellular cAMP Via EP4 Receptor**

To evaluate the signaling mechanisms underlying the proliferative response to PGE2, siPKD1 cells were treated with indomethacin (30 μM) to reduce endogenous levels of PGE2, and then incubated with various agonists, including vasopressin (1 μM, vasopressin receptor agonist), PGE2 (50 nM), (R)-butaprost free acid (1 μM, EP2 receptor agonist), TCS 2510 (1 μM, EP4 receptor agonist), and forskolin (10 μM, adenylyl cyclase activator), to measure their effects on intracellular cAMP concentration, the principal inducer of proliferation in cystic epithelia. We chose to characterize the PGE2 proliferative response with specific EP receptor agonists because of our concern that receptor antagonism could lead to cross-activation of other available EP receptors. PGE2 (12.9 ± 2.6%) and the EP4 agonist (TCS 2510; 12.3 ± 1.9%) stimulated an increase in intracellular cAMP concentration compared with indomethacin-treated cells (3.6 ± 0.5%, #P < 0.05, Fig. 5), suggesting that EP4 is the principal receptor regulating PGE2-mediated, cAMP-dependent proliferation in PC-1-deficient cells. Moreover, indomethacin treatment decreased intracellular cAMP concentration compared with untreated cells (7.2 ± 1.2%; *P < 0.05), suggesting that PGE2 acts in a paracrine fashion to maintain intracellular cAMP concentrations under basal conditions. In addition, PGE2 and EP4 receptor agonist induced greater intracellular cAMP concentration compared with either EP2 receptor agonist (@#P < 0.05) or vasopressin administration ($P < 0.05$). Notably, the cAMP response profile in siPKD1 cells is similar to that in wild-type IMCD3 (Fig. 5), suggesting that alternative downstream signaling mechanisms contribute to the proliferative response observed in PC-1-deficient cells.

**PGE2 and EP4 Receptor Activation Induce pERK and β-Catenin Signaling in PC-1-Deficient Cells**

The PGE2 signaling pathways contributing to tubular proliferation remain obscure in ADPKD; however, published papers suggest that PGE2 activates extracellular regulated kinase (ERK) in human ADPKD cystic epithelial cells to stimulate proliferation (5, 46). To test whether PGE2 stimulates ERK in our murine model, IMCD3 and PC-1-deficient cells were treated with 50 nM PGE2 or 1 μM TCS 2510, and phospho-ERK and total-ERK protein expression was evaluated in both groups of cells (Fig. 6A). PGE2 and TCS 2510 (EP4 agonist) reduced the steady-state abundance of phospho-ERK compared with untreated IMCD3 cells; in contrast, PGE2 and TCS 2510 raised the steady-state expression of phospho-ERK in PC-1-deficient cells compared with untreated PC-1-deficient cells (Fig. 6A). Densitometric analysis of three Western blots is summarized in Fig. 6B. However, because some studies suggest that phospho-ERK does not necessarily correlate with proliferation, and ERK inhibition may not inhibit proliferation/cystogenesis (39), we chose to evaluate the signaling pathways that operate in PGE2-mediated proliferation/cancer models, specifically PGE2-mediated β-catenin pathway.

Because PGE2 is a potent stimulator of proliferation in models of colon cancer, we hypothesized that a similar mechanism contributes to proliferation in ADPKD. In colon cancer PGE2 stimulates EP2- or EP4-dependent cAMP accumulation, which activates protein kinase A (PKA) (22). PKA, in turn, inhibits glycogen synthase kinase-3-dependent phosphorylation of β-catenin (19) which, ultimately, stabilizes β-catenin so that it translocates into the nucleus and leads to proliferation.
Ah6805, an EP2 receptor antagonist (AH bar, n = 6 wells), reduced proliferation (##P < 0.05 compared with untreated) by 25.2 ± 2.8% of untreated siPKD1 cells at 96 h, while L161,982 (L161 bar, @ P < 0.05, n = 6 wells) reduced proliferation at all time points by 25.1 ± 8.0, 29.6 ± 4.8, and 36.5 ± 4.0% of untreated siPKD1 cells (Fig. 8). EP4 and dual EP2/4 receptor antagonism diminished the change in cell number to a greater extent than did individual EP2 receptor antagonism (##P < 0.05), suggesting siPKD1 proliferation is principally dependent on EP4 receptor activation. Overall, these findings suggest that PGE2-mediated EP4 receptor activation, possibly through β-catenin stimulation, contributes to the proliferative phenotype classically observed in ADPKD cystic renal epithelia.

**PGE2 Stimulates CFTR and Calcium-Activated Cl− Secretion in PC-1-Deficient Cells**

Another classic feature of ADPKD epithelial cells is that they respond to ligand-dependent activation of Gs-coupled receptors and secrete Cl− into encapsulated cysts. To test the

### EP4 Receptor Antagonism Inhibits Proliferation

We next tested whether treatment with EP2 and/or EP4 receptor antagonists reduced proliferation in siPKD1 cells.
role of PGE2 on Cl− secretion, parental and siPKD1 cells were grown on permeable supports and placed in an Ussing chamber to evaluate PGE2-mediated transepithelial Cl− transport (10, 31). Amiloride was first added to cells to inhibit Na+ transport through the epithelial Na+ channel (ENaC) and to isolate Cl− transport pathways. The Vte and Rte did not differ between parental wild-type (Vte = −0.006 ± 0.08 mV, Rte = 52.8 ± 10.1 Ω·cm2; n = 10) and PC-1-deficient (Vte = 0.11 ± 0.5 mV, Rte = 64.3 ± 5.3 Ω·cm2; n = 12) cells. Addition of PGE2 (77 nM) to both sides of polarized siPKD1 and wild-type IMCD3 cells induced a rapid increase in PGE2-mediated short-circuit current (IscPGE2) in siPKD1 cells, but not in wild-type IMCD3 cells (Fig. 9A). Addition of a specific CFTR inhibitor, CFTRinh-172 (CI), to the apical side of polarized siPKD1 cells led to a ~50% decrease in IscPGE2. Sequential addition of a nonselective CaCC inhibitor FFA reduced the IscPGE2 in siPKD1 cells by an additional ~20–30%. No change in IscPGE2 was observed in wild-type IMCD3 (WT) cells after incubation of these inhibitors. Our collective electrophysiologic data showed that IscPGE2 was about fivefold greater in PC-1-deficient (siPKD1) cells than in wild-type IMCD3 cells (Fig. 9B, n = 16–18, *P < 0.05). Approximately 45% of IscPGE2 was sensitive to CFTR inhibition (n = 8, *P < 0.05); 30–40% of IscPGE2 was sensitive to FFA inhibition (n = 7, *P < 0.05, Fig. 9C).

To further confirm that IscPGE2 involves Ca2+ signaling pathways and CaCC-mediated Cl− secretion, we used [Ca2+]i chelator BAPTA-AM to decrease [Ca2+]i in siPKD1 cells. Pretreatment of cells with BAPTA-AM inhibited IscPGE2 by 40% (n = 8, *P < 0.05, Fig. 9C), indicating that CaCC participates in IscPGE2 in siPKD1 cells. We also treated IMCD3 and siPKD1 cells with forskolin and found that it induced an increase in Isc of 1.48 ± 0.2 and 3.48 ± 0.6 µA/cm2, respectively, illustrating that, although both wild-type and PC-1-deficient cells have the cAMP signaling machinery to secrete Cl−, only PC-1-deficient cells have an exaggerated and dysregulated cAMP response.

**DISCUSSION**

The purpose of this paper was to test whether PGE2 influences cell number and ion transport in a well-characterized cell culture model of ADPKD such that these dysregulated cellular characteristics may contribute to cyst formation and growth. Classically, ligand-dependent activation of cAMP pathways induces epithelial cell proliferation and Cl− secretion in ADPKD cell culture models (5, 10, 31, 46, 47), which, presumably, represent pathogenic pathways to cystogenesis. Inhibition of the cAMP pathway or downstream effectors in murine models of ADPKD retards cyst formation and progression to renal failure (41, 48). To this end, we show here that, in PC-1-deficient collecting duct cells, PGE2 1) induces proliferation through autocrine secretion of PGE2 and activation of EP2/EP4 Gs-coupled receptors, 2) principally stimulates EP4 receptors to increase intracellular cAMP concentration, 3) increases the abundance of activated β-catenin, and 4) induces a fivefold increase in Cl− secretion through CFTR and CaCCs. Cell proliferation and Cl− secretion are not only classic phenotypic features of ADPKD tubular epithelia, but they are also pathologic features of kidney cysts, and, therefore, implicate autocrine/paracrine renal epithelial synthesis of PGE2 as potentiators of cystogenesis.

Our findings corroborate the basic conclusions of Yamaguchi et al. (46) in which they found that administration of exogenous PGE2 induces proliferation and Cl− secretion in human ADPKD cystic epithelial cells. More specifically, we found that PGE2 induced disparate effects on the number of wild-type vs. PC-1-deficient cells (Fig. 4). These results are similar to those of Yamaguchi et al. (46) where PGE2 inhibits proliferation in control human kidney cells by ~40%, but it induces proliferation by ~50% in human ADPKD renal epithelia. Because Yamaguchi et al. did not characterize the PKD alleles affected in their human ADPKD and control cells, it is difficult to ascertain precisely the role of PGE2 signaling in ADPKD. Our defined model of ADPKD, where PC-1 protein expression is reduced by 70–80% compared with the parental cell line, lends strong support to the contention that reduction of functional PC-1 protein induces aberrant phenotypic responses to PGE2.

Yamaguchi et al. further demonstrated that administration of exogenous PGE2 activated an Ik (statistical significance not mentioned) that likely represents Cl− secretion in human ADPKD renal epithelial cells (46). Our studies corroborate these findings by showing that PGE2 induces a significantly greater increase in IscPGE2 in PC-1-deficient cells than in control cells (Fig. 9B). Furthermore, the increase in IscPGE2 in PC-1-deficient cells is dependent on activity of both CFTR and CaCC (Fig. 9C), which likely mediate Cl− secretion. Although not directly addressed in this paper, CFTR-dependent IscPGE2 is likely due to activation of EP4 receptor, which stimulates an increase in intracellular cAMP concentration (Fig. 5) and downstream PKA activity. This is the classic pathway leading to CFTR activation (5, 10, 30, 31, 42).

To our knowledge, this is the first demonstration of PGE2-mediated, CaCC-dependent Cl− secretion; only purinergic receptor activation has been shown to induce CaCC-mediated Cl− secretion in ADPKD epithelia (37). EP1, a Gq-coupled receptor, raises [Ca2+]i through activation of the inositol triphosphate-dependent pathway and, thus, is a plausible mechanism by which PGE2 activates CaCCs (9, 21). The molecular

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**Fig. 8.** EP2 and EP4, Gs-coupled receptors, contribute to the proliferative capacity of siPKD1 cells. After cells were plated for 18 h, siPKD1 cells were treated with EP2, EP4, and EP2 and ER receptor antagonists, cells were counted by Cyquant, and cell counts were expressed as a ratio to the 18-h time point. EP2 receptor antagonism (AH6908, 3 µM) reduced cell number only at 96 h compared with siPKD1-untreated cells (P < 0.05) while EP4 receptor antagonism (L161982, 10 µM) and dual receptor antagonism reduced proliferation at all time points compared with siPKD1-untreated cells (P < 0.05). Dual EP2/EP4 and EP4 antagonism prevented proliferation to a greater extent than EP2 receptor antagonism alone (P < 0.05). Cell counts for siPKD1-untreated cells were greater than untreated IMCD3 cells (P < 0.05) from 48 to 96 h.
identity of the CaCCs is not entirely clear; however, recent studies suggest the bestrophin and TMEM16 family of channels are possible candidates (3, 25). Interestingly, bestrophin-1 is expressed in kidney and has been linked to proliferation of renal epithelial cells in culture and in vivo, thus, indicating that this channel is a plausible candidate for mediating CaCC in ADPKD epithelial cells (1, 29). Moreover, transcription of bestrophin-1 is regulated by the transcription factor OTX2 which is, in turn, induced by β-catenin. Since we observed that PGE2 increases β-catenin signaling in ADPKD cells, this finding could implicate a mechanism by which bestrophin-1 might be upregulated in ADPKD (18, 45).
Our findings also indicate that endogenous synthesis of PGE₂ and autocrine/paracrine activation of EP receptors are also important contributors to the proliferative phenotype of PC-1-deficient cells. Inhibition of cyclooxygenase and selective antagonism of EP2 and EP4 receptors reduced proliferation in PC-1-deficient cells. Specifically, EP4 receptor antagonism inhibited proliferation with greater speed and effect than did EP2 receptor antagonism, implicating the greater importance of EP4 receptors in proliferation. The robust increase in intracellular cAMP in response to EP4 vs. EP2 agonism also supports the notion that EP4 is the principal receptor mediating cAMP-dependent proliferation. Moreover, PGE₂ and an EP4 agonist stimulated similar levels of intracellular cAMP concentration, suggesting that the principal mechanism of PGE₂-induced, cAMP-dependent proliferation is through EP4 receptor activation. This is not surprising in light of the fact that the Kᵦₐ of PGE₂ for the EP4 receptor is 100-fold lower than that for the EP2 receptor (16). Belibi et al. (5) similarly showed that treatment of human ADPKD renal epithelial cells with L161,982, an EP4 receptor antagonist, inhibited PGE₂-mediated intracellular cAMP generation by ~50%. On the other hand, Elberg et al. (17) showed that EP2 receptor-dependent cellular proliferation was the predominant mechanism underlying proliferation. Because the PKD alleles affected were not identified in the above studies, it is plausible that the different findings between the studies of Belibi et al. (5) and Elberg et al. (17) relate to different PKD alleles and/or mutations.

Because PGE₂ and EP4 receptor agonism stimulated intracellular cAMP concentration to a similar extent in IMCD3 and PC-1-deficient cells, we wanted to test whether downstream signaling differed between wild-type and PC-1-deficient cells. As expected, PGE₂ and TCS 2510 increased phospho-ERK expression in PC-1-deficient cells, but they generally decreased phospho-ERK expression in IMCD3 cells (5, 46). Because PGE₂ induces proliferation through PKA-dependent β-catenin activation in colon cancer and because ADPKD is associated with hyperactivation of β-catenin, we tested whether PGE₂ also induces β-catenin signaling in PC-1-deficient cells. PGE₂ induced active β-catenin expression only in PC-1-deficient cells, implicating alternative (non-ERK dependent) mechanisms for PGE₂-mediated proliferation (39).

In sum, our data reinforce earlier evidence in uncharacterized ADPKD renal epithelial cells that PGE₂ activates cellular proliferation and Cl⁻ secretion in a PC-1-deficient renal epithelial cell model. We advance these findings by demonstrating that local PGE₂ principally activates EP4 receptors to induce cell proliferation. Our data suggest that PGE₂ activates the ERK and β-catenin pathway through an EP4/cAMP-dependent mechanism, although further studies are required to confirm this pathway and the effect of ERK and β-catenin on proliferation. PGE₂ also stimulates Cl⁻ secretion through CFTR channels and CaCCs, indicating that PGE₂ may contribute to cyst formation and enlargement through dual effects on proliferation and secretion. These findings suggest that inhibition of PGE₂-dependent pathways could be targeted for therapeutic interventions in ADPKD patients. Specifically, these findings suggest that EP4 receptor antagonism represents a clinically appealing therapeutic target for ADPKD.

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