Prostaglandin E_2 mediates proliferation and chloride secretion in ADPKD cystic renal epithelia

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[1]*Department of Medicine, The Mount Sinai School of Medicine, New York, New York;* [2]*Department of Medicine, The James J. Peters Veterans Affairs Medical Center, Bronx, New York;* [3]*Department of Medicine, Stanford University School of Medicine, Stanford, California; and* [4]*Department of Pediatrics, The Mount Sinai School of Medicine, New York, New York*

Liu Y, Rajagopal M, Lee K, Battini L, Flores D, Gusella GL, Pao AC, Rohatgi R. Prostaglandin E_2 mediates proliferation and chloride secretion in ADPKD cystic renal epithelia. *Am J Physiol Renal Physiol* 303: F1425–F1434, 2012. First published August 29, 2012; doi:10.1152/ajprenal.00010.2012.—Prostaglandin E_2 (PGE_2) contributes to cystogenesis in genetically nonorthologous models of autosomal dominant polycystic kidney disease (ADPKD). However, it remains unknown whether PGE_2 induces the classic features of cystic epithelia in genetically orthologous models of ADPKD. We hypothesized that, in ADPKD epithelia, PGE_2 induces proliferation and chloride (Cl^-) secretion, two archetypal phenotypic features of ADPKD. To test this hypothesis, proliferation and Cl^- secretion were measured in renal epithelial cells deficient in polycystin-1 (PC-1). PC-1-deficient cells increased in cell number (proliferated) faster than PC-1-replete cells, and this proliferative advantage was abrogated by cyclooxygenase inhibition, indicating a role for PGE_2 in cell proliferation. Exogenous administration of PGE_2 increased proliferation of PC-1-deficient cells by $38.8 \pm 5.2\%$ ($P < 0.05$) but inhibited the growth of PC-1-replete control cells by $49.4 \pm 1.9\%$ ($P < 0.05$). Next, we tested whether PGE_2-specific E prostanoid (EP) receptor agonists induce intracellular cAMP and downstream β-catenin activation. PGE_2 and EP4 receptor agonism (TCS 2510) increased intracellular cAMP concentration and the abundance of active β-catenin in PC-1-deficient cells, suggesting a mechanism for PGE_2-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 PGE_2-dependent Cl^- secretion is also enhanced in PC-1-deficient cells, we used an Ussing chamber to measure short-circuit current (I_{sc}). Addition of PGE_2 induced a fivefold higher increase in I_{sc} in PC-1-deficient cells compared with PC-1-replete cells. This PGE_2-induced increase in I_{sc} in PC-1-deficient cells was blocked by CFTR-172 and flufenamic acid, indicating that PGE_2 activates CFTR and calcium-activated Cl^- channels. In conclusion, PGE_2 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 PGE_2 inhibition and EP4 receptor antagonism.

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 by a hyperproliferative cystic renal epithelium and clinically by polyuria (32). Recent observations suggest that altered prostanoid 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 PGE_2, are present at higher concentrations than in controls (2, 5, 28, 35, 44). The molecular mechanisms by which mutations of cystogenic genes induce renal PGE_2 production remain unknown, but the levels of renal PGE_2 significantly affect the phenotype of PKD. Pharmacologic inhibition of PGE_2 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.

PGE_2 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 Ca^{2+} concentration ($[Ca^{2+}]_i$) through a Gq-IP_3-dependent pathway (8, 23). EP3 receptor has multiple splice variants that, depending on the interacting G protein, activate either Gα, Gi, or Gq proteins (8). Thus, PGE_2 elicits a specific cellular response based on the activation of a specific repertoire of EP receptors.

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 PGE_2 to human ADPKD tubular epithelia significantly increased intracellular cAMP, cystogenesis, and proliferation, suggesting a role for PGE_2 in the pathogenesis of ADPKD. However, much of the evidence for the effect of PGE_2 on proliferation has been found in models of colon cancer where PGE_2 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 PGE_2, an important renal

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

Under normal physiologic conditions, renal PGE$_2$ 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 PGE$_2$ 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 PGE$_2$ (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 PGE$_2$ in dysregulated cellular proliferation and Cl$^-$ secretion led us to hypothesize that PGE$_2$ contributes to cystogenesis in ADPKD. To test this hypothesis, we assessed the role of PGE$_2$-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 PGE$_2$ contributes to the classic proliferative and secretory features of PC-1-defective cystic epithelia and indicate that inhibition of PGE$_2$ and its downstream signaling may be another strategy for the treatment of ADPKD.

MATERIALS AND METHODS

Reagents

Inhibitors. The inhibitors were as follows: 3 μM AH6809 (24) (Cayman Chemical; IC$_{50}$ = 350 nM), 10 μM L161,982 (15) (Cayman Chemical), 100 nM SC560 (Cayman Chemical; IC$_{50}$ = 9 nM), 1 μM CAY 10404 (Cayman Chemical; IC$_{50}$ < 1 nM), 30 μM indomethacin (Sigma), 10^{-5} M CFTR inhibitor 172 (Calbiochem; EC$_{50}$ ~300 nM) (30), and 2 × 10^{-4} M flufenamic acid (FFA) (29) (Sigma). The agonists include 50 or 77 nM PGE$_2$ (17) (Cayman Chemical), 1 μM TCS 2510 (Tocris Bioscience; EC$_{50}$ = 2.5 nM), 1 μM (R) butaprost free acid (17) (Cayman Chemical), and 10 μM 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 dephosphorylated Ser37, Thr41 β-catenin antibody (Millipore), and murine monoclonal β-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 (si*PKD1*), as previously described (4). Parental wild-type IMCD3 cells were also used as a reference control. In si*PKD1* 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 trypan blue, and viable cells were counted by hemocytometer. Indomethacin (30 μM) 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 μl of GR dye/cell lysis buffer were added to each well, gently mixed, and incubated for 2 h to maximize and stabilize the fluorescence. 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.

PGE$_2$ Measurement

In media bathing the cells. One milliliter of serum and phenol red-free DMEM/F12 were incubated with si*PKD1* or IMCD3 cells for 3 h (26). The conditioned media were collected and frozen at −80°C for measurement of PGE$_2$ at a later time. PGE$_2$ concentration (pg/ml) was measured with PGE$_2$ enzyme immunoassay (EIA) kit from Cayman Chemical, following the standard protocol enclosed with the kit, and PGE$_2$ 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, PGE$_2$, 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.

PGE$_2$-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 $R_{te} > 50$ $\Omega \cdot cm^2$, 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, 7.7×10^{-8} M of PGE$_2$ was added to both sides of the cell sheet. We then added CFTR inhibitor 172 (10^{-5} M) and 2×10^{-4} M FFA to the apical side to inhibit CFTR and calcium-activated Cl$^-$ channels (CaCCs), respectively.

Statistics

Data are given as means $\pm$ SE (n = 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 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 si*PKD1* 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, si*PKD1* 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 si*PKD1* cell number by $50.3 \pm 4.2\%$ and IMCD3 cell number by $31.1 \pm 2.9\%$ of untreated si*PKD1* and IMCD3 cells (Fig. 1; $P < 0.05$, $n = 3$ wells for each cell type and condition), respectively. Moreover, indomethacin reduced cell proliferation in si*PKD1* cells to levels observed in untreated control cells (IMCD3 and siLuc), implicating 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,
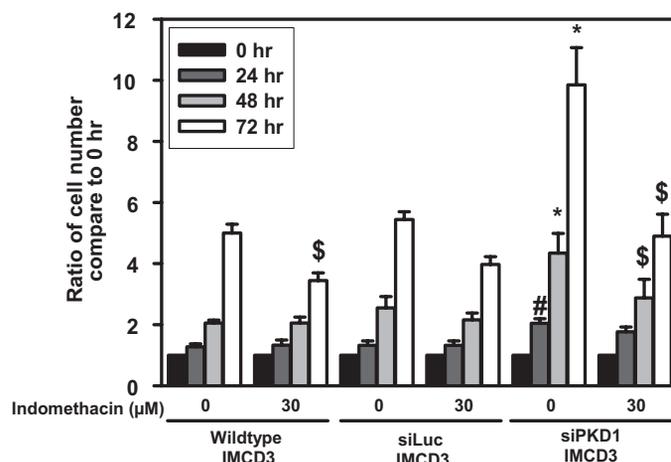

Fig. 1. si*PKD1* cells, deficient in polycystin-1 (PC-1), increase in number faster than wild-type and transfection control cells through a cyclooxygenase (COX)-dependent mechanism. si*PKD1* and control cells [wild-type and siLuc-transfected inner medullary collecting duct (IMCD)3 cells] were plated in wells and counted manually at *time 0 h* (day of plating), 24 h, 48 h, and 72 h after plating. The cell count data are presented as a ratio of the number of cells at 24, 48, and 72 h to the number of cells counted at 0 h. Cell counts were performed on untreated and cells treated with 30 μM indomethacin, a COX-1 and COX-2 inhibitor. In untreated cells, the change in cell counts from *time 0* was not different between wild-type and siLuc-transfected IMCD3 cells; however, si*PKD1* cell number increased faster at 48 and 72 h compared with wild-type and siLuc-transfected IMCD3 (*$P < 0.05$, $n = 3$ wells) and faster at 24 h than wild-type IMCD3 (#$P < 0.05$, $n = 3$) cells. Indomethacin reduced the change in cell number in si*PKD1* cells compared with untreated si*PKD1* cells ($P < 0.05$, $n = 3$) at 48 and 72 h, such that it was comparable to change in cell number of untreated wild-type and siLuc-transfected IMCD3 cells. Finally, indomethacin also inhibited proliferation in wild-type IMCD3 cells at 72 h compared with their untreated controls.

since it provides comparable results, we used it in place of manual counting for the following experiments. si*PKD1* 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. 2*A*). 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. 2*A*), 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 si*PKD1* cells markedly reduced proliferation at all time points compared with untreated si*PKD1* (white bar; @$P < 0.05$, Fig. 2*B*). Inhibition of COX-1/-2 by indomethacin reduced proliferation of si*PKD1* cells to a greater extent than inhibition of COX-1 or COX-2 individually ($P < 0.05$) 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 si*PKD1* cells (#$P < 0.05$). As seen in prior experiments, si*PKD1* cells proliferated faster (white bar, *$P < 0.05$, Fig. 2*B*) than did parental IMCD3 (black bar, Fig. 2*B*) cells, replicating our earlier findings and serving as an internal control.
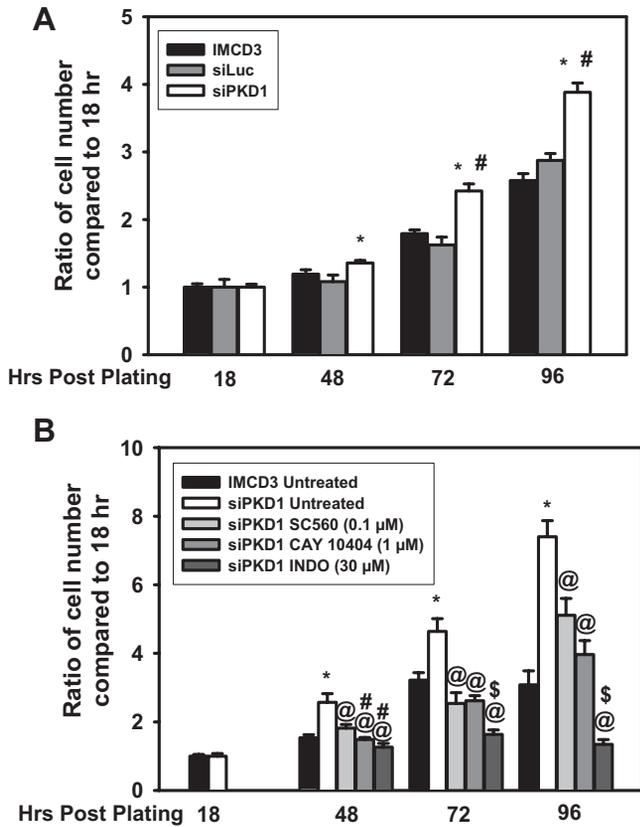
Fig. 2. Cell number quantification using Cyquant illustrates a similar COX-dependent hyperproliferative phenotype as manual cell counting. Cells were plated overnight for 18 h to ensure that enough cells adhere to the plate to measure fluorescence. At each time point, cells were frozen, thawed, incubated with Cyquant, and fluorescence was measured at 485 nm. Utilizing a standard curve, we counted the number of cells at each time point and presented the data as a ratio compared with the 18-h cell count. *A*: change in cell number was greater in the si*PKD1* than in siLuc-transfected cells (*$P < 0.05$) at all time points (48–96 h), while si*PKD1* was only greater than wild-type IMCD3 cells at 72 and 96 h (#$P < 0.05$). IMCD3 and siLuc cells proliferated at similar rates. *B*: treatment of si*PKD1* cells with COX-1 (SC560), COX-2 (CAY10404), and COX-1 and -2 (indomethacin) inhibitors reduced the change in cell number compared with untreated si*PKD1* cells (@$P < 0.05$), whereas untreated si*PKD1* cells proliferated faster than wild-type IMCD3 cells (*$P < 0.05$). At 48 h, COX-2 and dual COX-1/-2 inhibition reduced the change in cell number to a greater extent than COX-1 inhibition alone (#$P < 0.05$). Moreover, dual COX-1/-2 inhibition reduced proliferation to a greater extent than either COX-1 or COX-2 inhibition alone ($$P < 0.05$).

PGE$_2$, a COX-Dependent Prostanoid, Regulates Proliferation

The relationship between COX activity and proliferation in PC-1-deficient cells suggests that endogenous PGE$_2$ production by renal epithelial cells may contribute to the hyperproliferative phenotype of cystic epithelia. Moreover, the PGE$_2$ concentration in renal tissue isolated from the Han:SPRD model of ADPKD is greater than that measured in littermate controls (35, 44), suggesting that excessive autocrine production of PGE$_2$ may also contribute to the proliferative phenotype. To test whether PGE$_2$ concentrations are greater in PC-1-deficient cells, we measured PGE$_2$ in the conditioned media bathing the PC-1-deficient and PC-1-replete IMCD3 cells. However, PGE$_2$ concentration (pg/ml) did not differ between PC-1-deficient and parental control cells at any time

point (Fig. 3*A*). When PGE$_2$ concentration in the media was normalized to the number of cells in culture, no difference was found at any time points (Fig. 3*B*), suggesting that exaggerated tubular PGE$_2$ secretion and subsequent autocrine/paracrine signaling were not inducing proliferation in PC-1-deficient cells. We speculated that local PGE$_2$ 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 PGE$_2$ as the culprit prostanoid. To address these issues, IMCD3 and PC-1-deficient cells were treated with 50 nM PGE$_2$ to investigate whether exogenous PGE$_2$ enhances cellular proliferation of si*PKD1* cells. Wild-type IMCD3 cell number decreased significantly (dark gray bars, *$P < 0.05$, $n = 6$ wells) after treatment with 50 nM PGE$_2$ compared with untreated controls (black bars, $n = 6$ wells); however, si*PKD1* cell number increased after treatment with PGE$_2$ (white bars, $$P < 0.05$, $n = 6$ wells) compared with untreated si*PKD1* cells (Fig. 4, light gray bars, $n = 6$ wells). PGE$_2$ induced prolifer-
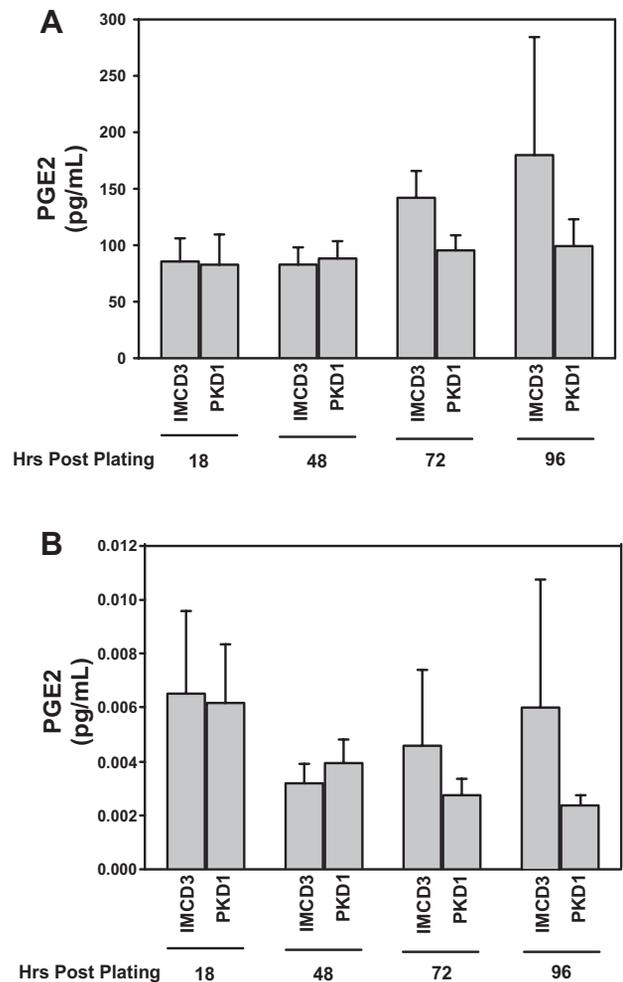

Fig. 3. Absolute prostaglandin E$_2$ (PGE$_2$) concentration in the media (*A*) or after normalization to cell number (*B*) was similar between wild-type IMCD3 and si*PKD1* cells. PGE$_2$ in the media bathing the cells was measured at each time point. *A*: no difference in absolute PGE$_2$ concentration (pg/ml) in the media was observed. *B*: no difference in PGE$_2$ concentration was observed after normalizing to the number of cells (pg·ml^{-1}·cell^{-1}).
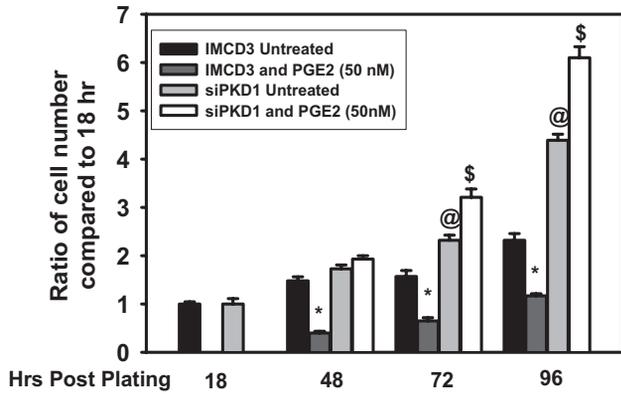
Fig. 4. Exogenous PGE$_2$ inhibits proliferation in wild-type IMCD3 cells and induces proliferation in si*PKD1* cells. Exogenous supplementation of PGE$_2$ to the media after the 18-h time point reduced IMCD3 cell numbers compared with the untreated IMCD3 at all time points (*$P < 0.05$). On the other hand, PGE$_2$ induced greater increase in si*PKD1* cell number compared with untreated si*PKD1* cells ($P < 0.05$). si*PKD1* cells proliferate faster than IMCD3 cells (@$P < 0.05$), which acts as an internal control.

ation by $38.8 \pm 5.2\%$ in si*PKD1* cells while inhibiting proliferation by $49.4 \pm 1.9\%$ in IMCD3 cells at 96 h. These findings are similar to those reported by Yamaguchi et al. (46) in which PGE$_2$ 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 PGE$_2$ secretion or concentration, is responsible for the proliferation in ADPKD epithelia.

PGE$_2$ Induces Intracellular cAMP Via EP4 Receptor

To evaluate the signaling mechanisms underlying the proliferative response to PGE$_2$, si*PKD1* cells were treated with indomethacin (30 μM) to reduce endogenous levels of PGE$_2$, and then incubated with various agonists, including vasopressin (1 μM, vasopressin receptor agonist), PGE$_2$ (50 nM), (R)-butaprost free acid (1 μM, EP2 receptor agonist), TCS 2510 (1 μM, EP4 receptor agonist), and forskolin (10 μM, adenylate cyclase activator), to measure their effects on intracellular cAMP concentration, the principal inducer of proliferation in cystic epithelia. We chose to characterize the PGE$_2$ proliferative response with specific EP receptor agonists because of our concern that receptor antagonism could lead to cross activation of other available EP receptors. PGE$_2$ (12.9 $\pm$ 2.6%) and the EP4 agonist (TCS 2510; 12.3 $\pm$ 1.9%) stimulated an increase in intracellular cAMP concentration compared with indomethacin-treated cells (3.6 $\pm$ 0.5%, #$P < 0.05$, Fig. 5), suggesting that EP4 is the principal receptor regulating PGE$_2$-mediated, cAMP-dependent proliferation in PC-1-deficient cells. Moreover, indomethacin treatment decreased intracellular cAMP concentration compared with untreated cells (7.2 $\pm$ 1.2%; *$P < 0.05$), suggesting that PGE$_2$ acts in a paracrine fashion to maintain intracellular cAMP concentrations under basal conditions. In addition, PGE$_2$ 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 si*PKD1* 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.

PGE$_2$ and EP4 Receptor Activation Induce pERK and β-Catenin Signaling in PC-1-Deficient Cells

The PGE$_2$ signaling pathways contributing to tubular proliferation remain obscure in ADPKD; however, published papers suggest that PGE$_2$ activates extracellular regulated kinase (ERK) in human ADPKD cystic epithelial cells to stimulate proliferation (5, 46). To test whether PGE$_2$ stimulates ERK in our murine model, IMCD3 and PC-1-deficient cells were treated with 50 nM PGE$_2$ or 1 μM TCS 2510, and phospho-ERK and total-ERK protein expression was evaluated in both groups of cells (Fig. 6A). PGE$_2$ and TCS 2510 (EP4 agonist) reduced the steady-state abundance of phospho-ERK compared with untreated IMCD3 cells; in contrast, PGE$_2$ 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 PGE$_2$-mediated proliferation/cancer models, specifically PGE$_2$-mediated β-catenin pathway.

Because PGE$_2$ 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 PGE$_2$ 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
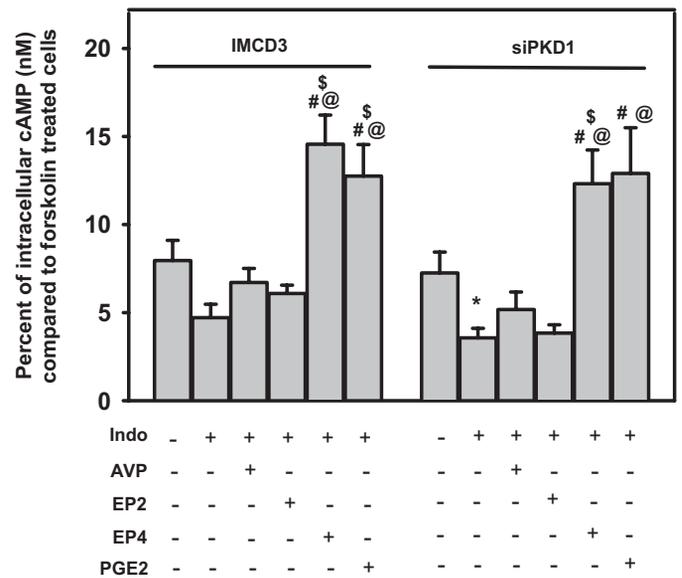

Fig. 5. PGE$_2$ and EP4 agonist induce a robust increase in intracellular cAMP in wild-type and PC-1-deficient cells. Intracellular cAMP concentration was measured in each condition and presented as a percentage of the forskolin-induced cAMP concentration. Indomethacin (30 μM) significantly reduced intracellular cAMP (*$P < 0.05$, $n = 3$) compared with untreated PC-1-deficient cells, whereas EP4 receptor agonism ($n = 3$) and PGE$_2$ ($n = 4$) increased cAMP (#$P < 0.05$) compared with indomethacin-treated cells in both cells. PGE$_2$ and EP4 receptor stimulated a greater cAMP response than EP2 receptor agonist (@$P < 0.05$, $n = 3$) and vasopressin ($P < 0.05$, $n = 3$). PGE$_2$-mediated cAMP concentration tended to be greater ($P = 0.064$) than vasopressin treatment in PC-1-deficient cells, but it did not reach statistical significance.
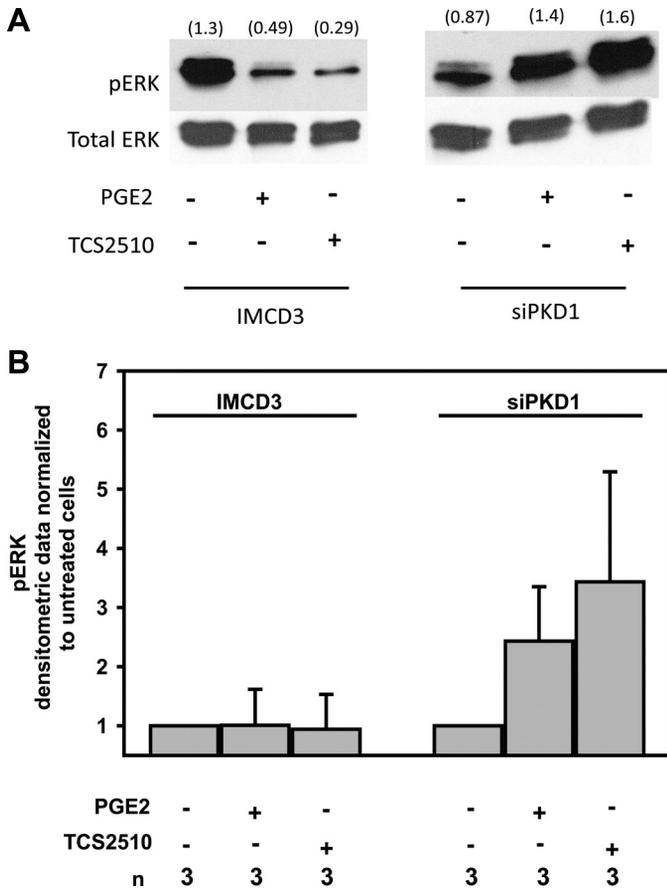
A

B

Fig. 6. PGE$_2$ and EP4 receptor-dependent activation of phospho-ERK in PC-1-deficient cells. IMCD3 and PC-1-deficient cells were treated with PGE$_2$ (50 nM) and TCS 2510 (1 μM) for 30 min, cellular protein lysate was harvested, and immunoblotted with anti-phospho-ERK and anti-total-ERK antibodies. The densitometric data were presented as a ratio of phospho-ERK to total-ERK as represented by the numbers in the parentheses. A: phospho-ERK expression was reduced in IMCD3 cells treated with PGE$_2$ or TCS 2510 (EP4 receptor agonist), whereas phospho-ERK expression was increased in PC-1-deficient cells treated with PGE$_2$ or TCS 2510. All cells were pretreated with indomethacin to reduce basal expression of PGE$_2$ before exposure to different agonists or no treatment (control). B: densitometric data normalized to untreated controls demonstrate that in 2 of 3 experiments there was decreased phospho-ERK expression in IMCD3 cells treated with PGE$_2$ and EP4 agonist and an increase in phospho-ERK abundance in 3 of 3 experiments performed in PC-1-deficient cells treated identically.

(11, 12, 38). Moreover, human and murine models of ADPKD suggest that hyperactivation of the β-catenin pathway contributes to cystogenesis (33, 40). Therefore, we tested whether PGE$_2$ and EP4 agonism (TCS 2510) induce activated β-catenin (dephosphorylated at Ser37 and Thr41) after 30 min of stimulation. PGE$_2$ decreased expression of active dephosphorylated β-catenin, whereas TCS 2510 did not alter the expression of active β-catenin compared with untreated (Fig. 7A) IMCD3 cells. However, both PGE$_2$ and TCS 2510 increased the abundance of active dephosphorylated β-catenin in PC-1-deficient cells compared with untreated PC-1-deficient cells (Fig. 7A). Densitometric data from four IMCD3 experiments and three si*PKD1* experiments are summarized in Fig. 7B.

EP4 Receptor Antagonism Inhibits Proliferation

We next tested whether treatment with EP2 and/or EP4 receptor antagonists reduced proliferation in si*PKD1* cells.

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 si*PKD1* 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 si*PKD1* 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 si*PKD1* proliferation is principally dependent on EP4 receptor activation. Overall, these findings suggest that PGE$_2$-mediated EP4 receptor activation, possibly through β-catenin stimulation, contributes to the proliferative phenotype classically observed in ADPKD cystic renal epithelia.

PGE$_2$ 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 Gαs-coupled receptors and secrete Cl$^-$ into encapsulated cysts. To test the
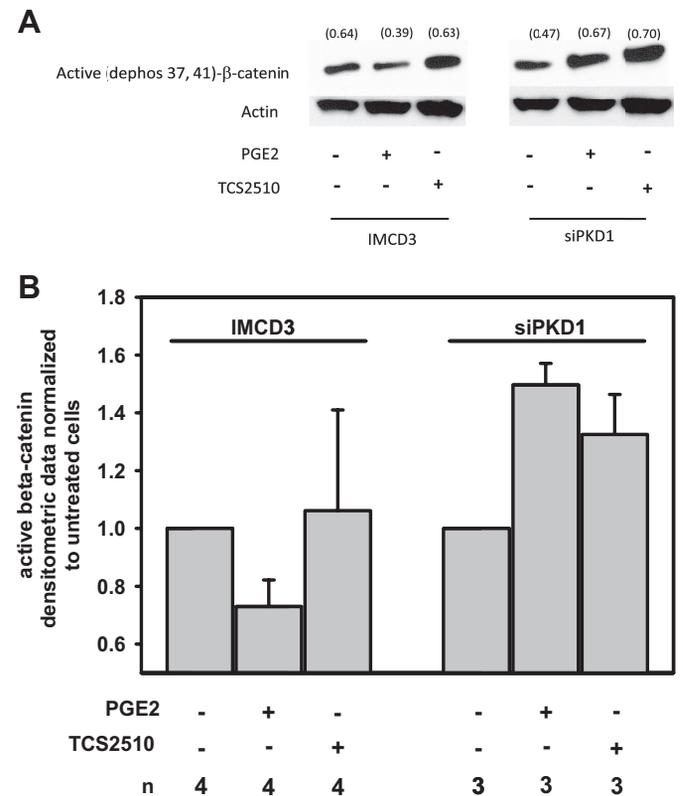
A

B

Fig. 7. PGE$_2$ and EP4 receptor-dependent activation induces dephosphorylated Ser37, Thr41 β-catenin in PC-1-deficient cells. In a similar experiment, active dephosphorylated Ser37, Thr41 β-catenin was measured in wild-type and PC-1-deficient IMCD3 cells and normalized to actin. The ratio of active β-catenin to actin is presented in parentheses above the band. All cells were pretreated with indomethacin to reduce basal expression of PGE$_2$ before exposure to different agonists or no treatment (control). A: in a single Western blot, PGE$_2$ and TCS 2510 induced active β-catenin in PC-1-deficient cells but not in wild-type cells. B: densitometric data normalized to untreated controls demonstrate there was a trend for decreased active β-catenin expression in IMCD3 cells ($n = 4$) treated with PGE$_2$ (3 of 4 experiments), whereas an EP4 agonist had a variable effect. On the other hand, active β-catenin protein abundance increased in PC-1-deficient cells treated with PGE$_2$ (3 of 3 experiments) and increased with EP4 agonism in 2 of 3 experiments compared with PC-1-deficient control cells.
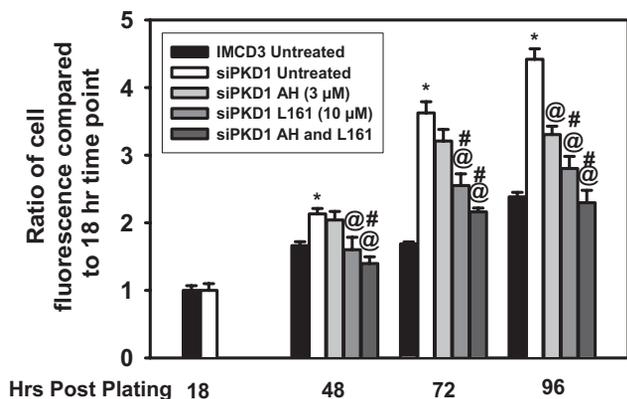
Fig. 8. EP2 and EP4, $G\alpha s$-coupled receptors, contribute to the proliferative capacity of si*PKD1* cells. After cells were plated for 18 h, si*PKD1* cells were treated with EP2, EP4, and EP2 and 4 receptor antagonists, cells were counted by Cyquant, and cell counts were expressed as a ratio to the 18-h time point. EP2 receptor antagonism (AH6908, 3 μM) reduced cell number only at 96 h compared with si*PKD1*-untreated cells (@$P < 0.05$) while EP4 receptor antagonism (L161982, 10 μM) and dual receptor antagonism reduced proliferation at all time points compared with si*PKD1*-untreated cells (@$P < 0.05$). Dual EP2/4 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.

role of PGE_2 on Cl^- secretion, parental and si*PKD1* cells were grown on permeable supports and placed in an Ussing chamber to evaluate PGE_2-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 V_{te} and R_{te} did not differ between parental wild-type ($V_{te} = -0.006 \pm 0.08$ mV, $R_{te} = 52.8 \pm 10.1$ $\Omega \cdot cm^2$; $n = 10$) and PC-1-deficient ($V_{te} = 0.11 \pm 0.5$ mV, $R_{te} = 64.3 \pm 5.3$ $\Omega \cdot cm^2$; $n = 12$) cells. Addition of PGE_2 (77 nM) to both sides of polarized si*PKD1* and wild-type IMCD3 cells induced a rapid increase in PGE_2-mediated short-circuit current (I_{sc}^{PGE2}) in si*PKD1* cells, but not in wild-type IMCD3 cells (Fig. 9A). Addition of a specific CFTR inhibitor, $CFTR_{inh}$-172 (Cl), to the apical side of polarized si*PKD1* cells led to a $\sim$50% decrease in I_{sc}^{PGE2}. Sequential addition of a nonselective CaCC inhibitor FFA reduced the I_{sc}^{PGE2} in si*PKD1* cells by an additional $\sim$20–30%. No change in I_{sc}^{PGE2} was observed in wild-type IMCD3 (WT) cells after incubation of these inhibitors. Our collective electrophysiologic data showed that I_{sc}^{PGE2} was about fivefold greater in PC-1-deficient (si*PKD1*) cells than in wild-type IMCD3 cells (Fig. 9B, $n = 16$–18, *$P < 0.05$). Approximately 45% of I_{sc}^{PGE2} was sensitive to CFTR inhibition ($n = 8$, *$P < 0.05$); 30–40% of I_{sc}^{PGE2} was sensitive to FFA inhibition ($n = 7$, *$P < 0.05$, Fig. 9C).

To further confirm that I_{sc}^{PGE2} involves Ca^{2+} signaling pathways and CaCC-mediated Cl^- secretion, we used $[Ca^{2+}]_i$ chelator BAPTA-AM to decrease $[Ca^{2+}]_i$ in si*PKD1* cells. Pretreatment of cells with BAPTA-AM inhibited I_{sc}^{PGE2} by 40% ($n = 8$, *$P < 0.05$, Fig. 9C), indicating that CaCC participates in I_{sc}^{PGE2} in si*PKD1* cells. We also treated IMCD3 and si*PKD1* cells with forskolin and found that it induced an increase in I_{sc} of 1.48 ± 0.2 and 3.48 ± 0.6 μA/cm^2, 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 PGE_2 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, PGE_2 *1*) induces proliferation through autocrine secretion of PGE_2 and activation of EP2/EP4 $G\alpha s$-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 PGE_2 as potentiators of cystogenesis.

Our findings corroborate the basic conclusions of Yamaguchi et al. (46) in which they found that administration of exogenous PGE_2 induces proliferation and Cl^- secretion in human ADPKD cystic epithelial cells. More specifically, we found that PGE_2 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 PGE_2 inhibits proliferation in control human kidney cells by $\sim$40%, but it induces proliferation by $\sim$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 PGE_2 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 PGE_2.

Yamaguchi et al. further demonstrated that administration of exogenous PGE_2 activated an I_{sc} (statistical significance not mentioned) that likely represents Cl^- secretion in human ADPKD renal epithelial cells (46). Our studies corroborate these findings by showing that PGE_2 induces a significantly greater increase in I_{sc}^{PGE2} in PC-1-deficient cells than in control cells (Fig. 9B). Furthermore, the increase in I_{sc}^{PGE2} 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 I_{sc}^{PGE2} 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 PGE_2-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 $[Ca^{2+}]_i$ through activation of the inositol triphosphate-dependent pathway and, thus, is a plausible mechanism by which PGE_2 activates CaCCs (9, 21). The molecular
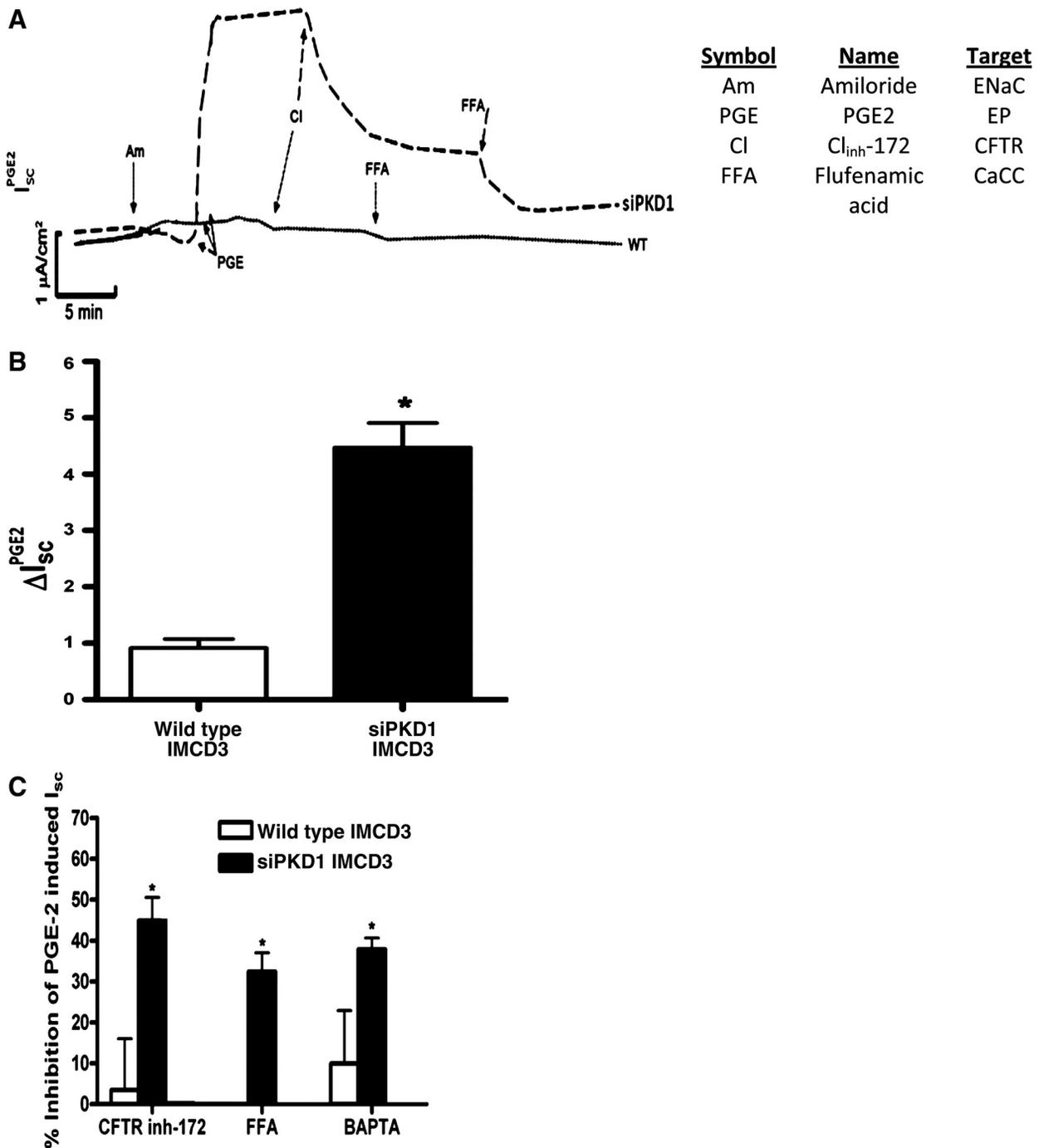
Fig. 9. PGE$_2$ stimulates a CFTR- and calcium-dependent Cl$^-$ short-circuit current (I_{sc}^{PGE2}) in si*PKD1* cells, but not in parental wild-type cells. *A*: IMCD3 and si*PKD1* cells were grown to confluence on permeable supports, placed in an Ussing chamber, amiloride (Am) was added apically, and transepithelial current was measured before and after addition of apical and basolateral PGE$_2$ (PGE, 77 nM). The addition of PGE$_2$ induced a robust current in si*PKD1* (- - -) cells but had little effect on IMCD3 (• • • •) cells. Apical application of CFTR$_{inh}$-172 (10^{-5} M, Cl, CFTR inhibitor) and flufenamic acid (FFA; 2×10^{-4} M, CaCC inhibitor) inhibited the current in si*PKD1* cells by ~50 and ~30%, respectively. *B*: I_{sc}^{PGE2} was ~5-fold greater in si*PKD1* cells than wild-type IMCD3 cells (*$P < 0.05$, $n = 16$–18 filters). *C*: ~45% of I_{sc}^{PGE2} was attributable to CFTR (*$P < 0.05$, $n = 8$) while 30–40% of the current was inhibitable by FFA (*$P < 0.05$, $n = 7$) and BATPA-AM ([Ca^{2+}]$_i$ chelator; *$P < 0.05$, $n = 8$).

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 PGE$_2$ 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 PGE_2 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, PGE_2 and an EP4 agonist stimulated similar levels of intracellular cAMP concentration, suggesting that the principal mechanism of PGE_2-induced, cAMP-dependent proliferation is through EP4 receptor activation. This is not surprising in light of the fact that the K_d of PGE_2 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 PGE_2-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 PGE_2 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, PGE_2 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 PGE_2 induces proliferation through PKA-dependent β-catenin activation in colon cancer and because ADPKD is associated with hyperactivation of β-catenin, we tested whether PGE_2 also induces β-catenin signaling in PC-1-deficient cells. PGE_2 induced active β-catenin expression only in PC-1-deficient cells, implicating alternative (non-ERK dependent) mechanisms for PGE_2-mediated proliferation (39).

In sum, our data reinforce earlier evidence in uncharacterized ADPKD renal epithelial cells that PGE_2 activates cellular proliferation and Cl^- secretion in a PC-1-deficient renal epithelial cell model. We advance these findings by demonstrating that local PGE_2 principally activates EP4 receptors to induce cell proliferation. Our data suggest that PGE_2 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. PGE_2 also stimulates Cl^- secretion through CFTR channels and CaCCs, indicating that PGE_2 may contribute to cyst formation and enlargement through dual effects on proliferation and secretion. These findings suggest that inhibition of PGE_2-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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