The positive effect of selective prostaglandin E2 EP2 and EP4 receptor blockade on cystogenesis *in vitro* is counteracted by increased kidney inflammation *in vivo*

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ABSTRACT

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a major cause of end-stage renal disease in man. The central role of cyclic adenosine monophosphate (cAMP) in ADPKD pathogenesis has been confirmed by numerous studies including positive clinical trial data. In this study, we investigated the potential role of another major regulator of renal cAMP, prostaglandin E_2 (PGE_2), in modifying disease progression in ADPKD models using selective receptor modulators to all four PGE_2 receptor subtypes (EP1-4). In 3D-culture model systems utilising dog (MDCK) and human-derived (UCL93, OX161-C1) kidney cell lines, PGE_2 strikingly promoted cystogenesis and inhibited tubulogenesis by stimulating proliferation and reducing apoptosis. The effect of PGE_2 on tubulogenesis and cystogenesis in 3D-culture was mimicked or abolished by selective EP2 and EP4 agonists or antagonists but not those specific to EP1 or EP3. In a *Pkd1* mouse model (*Pkd1$^{nl/nl}$*), renal PGE_2 and COX-2 expression were increased by ~2-fold at the peak of disease (week 4). However, *Pkd1$^{nl/nl}$* mice treated with selective EP2 (PF-04418948) or EP4 (ONO-AE3-208) antagonists from birth for 3 weeks had more severe cystic disease and fibrosis associated with increased cell proliferation and macrophage infiltration. A similar effect was observed for the EP4 antagonist ONO-AE3-208 in a second *Pkd1* model (*Pax8^{rtTA}-TetO-Cre-Pkd1$^{f/f}$*). In conclusion, despite the positive effects of slowing cyst growth *in vitro*, the more complex effects of inhibiting EP2 or EP4 *in vivo* resulted in a worse outcome, possibly related to unexpected pro-inflammatory effects.

KEYWORDS: ADPKD, cystogenesis, prostaglandin E2, inflammation, cyclic AMP, macrophages

TRANSLATIONAL STATEMENT

ADPKD is the major genetic cause of kidney failure in man with currently limited treatment options. Our study sought to investigate whether selective receptor blockade of PGE2 action via the EP2 and EP4 receptors which signal mainly via cAMP, could inhibit cyst formation in experimental disease models. Despite positive effects in cyst assays, an unexpected increase in the cyst burden was observed in two *Pkd1* mouse models after *in vivo* dosing, associated with an increase in inflammation. Our results do not support the use of EP2 or EP4 antagonists as therapeutic options in ADPKD.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common kidney genetic disorder, accounting for 7–10% of patients with end-stage renal disease, and is caused by mutations in two genes, *PKD1* (~85%) or *PKD2* (~15%) [1]. It is characterized by the formation of fluid-filled cysts which arise from tubular epithelial cells that exhibit a hyperproliferative and pro-apoptotic phenotype [2, 3]. Recently, the vasopressin V2 receptor (VPV2R) antagonist, tolvaptan, has been approved for the treatment for ADPKD patients with evidence of rapid disease progression [4, 5]. However, the use of tolvaptan is associated with poorly tolerated side-effects and a rare but unpredictable incidence of liver toxicity [4]. Hence, the discovery of safer and more effective alternative drugs to slow disease progression in ADPKD is of major clinical interest.

Prostaglandin E_2 (PGE_2) is a lipid mediator synthesized from arachidonic acid through several enzymatic steps including cyclooxygenases (COX-1 and COX-2) and Prostaglandin E synthase [6]. Several studies suggest that COX-2 is the major cyclooxygenase responsible for PGE_2 production [7, 8]. PGE_2 binds to four different G-protein coupled receptors (GPCR), EP1-4, which are differentially expressed in different tissues and which signal through different G-proteins thus resulting in complex outputs [9]. In particular, EP2 and EP4 are known to couple to Gs which stimulates cyclic adenosine monophosphate (cAMP) formation by activating adenylyl cyclase [10]. Cyclic AMP is a central player in cyst formation and expansion [11-14]. Several GPCR ligands (VPV2R, Endothelin B and somatostatin receptors) have been shown to modify disease severity in ADPKD models though modulating renal cAMP concentrations [15-18].

PGE_2 has been isolated in cyst fluid [19] and its urinary concentration found to be increased in patients with reduced kidney function [20]. *In vitro*, the effect of PGE_2 on stimulating cyst formation has been shown on IMCD-3 and human epithelial cells [21, 22]. These early observations suggested that PGE_2 could be a major modifier of cyst growth. However the study

of PGE$_2$ signalling has been hampered by the lack of potent and selective receptor modulators (agonists and antagonists). Conversely, the more recent availability of these compounds has led to a resurgence of preclinical and clinical investigation of different EP subtypes as selective targets to treat a variety of orphan diseases [23].

In this paper, we report the effects of modifying PGE$_2$ signalling on cyst growth by utilising new potent and selective receptor modulators to all four EP receptors (EP1-4) in cellular and animal models of ADPKD. Our results suggest a striking effect of EP2 and EP4 agonists in inhibiting tubulogenesis and promoting cyst formation and expansion *in vitro*. However the administration of EP2 and EP4 antagonists to a neonatal model of ADPKD, the hypomophic *Pkd1* mouse (*Pkd1$^{nl/nl}$*) and a post-natal Cre-inducible *Pkd1* model (*Pax8^{rtTA}-TetO-Cre-Pkd1$^{f/f}$*) led surprisingly to more severe cystic disease. These results suggest a more complex effect of EP2 and EP4 antagonists *in vivo* resulting in a worse outcome in this model, possibly related to unexpected pro-inflammatory effects.

RESULTS

EP2 or EP4 receptor activation inhibit tubulogenesis and promote cystogenesis

To study the effect of PGE$_2$ on spontaneous tubulogenesis, the normal human renal epithelial cell line UCL93 was grown in a Type I collagen matrix. After 20 days, prominent tubule structures had formed with evidence of branching (Figure 1A). The addition of different concentrations of PGE2 (1-100 nM) after 6 days of culture significantly increased the percentage of cystic structures with a 2-fold increase at 10 nM PGE$_2$ (Figure 1B). All four EP receptor subtypes are expressed in this line although EP2 and EP4 were more highly expressed than EP1 and EP3 (data not shown). Importantly, the effect of PGE$_2$ was mimicked by an EP2 selective agonist (ONO-AE1-259-01) or an EP4 selective agonist (ONO-AE1-329) suggesting that stimulation of either receptor can initiate cyst formation (Figure 1C and D). Stimulation of

UCL93 with PGE_2, EP2 or EP4 selective agonists stimulated cAMP formation (Figure 1E and F) suggesting that cyst formation is cAMP-mediated and involves EP2 and EP4.

PGE_2 stimulates cyst growth in MDCK and OX161-C1 cell lines

The potential effect of PGE_2 and its receptors on cyst growth was next studied in 3D-cyst assays using the established Madin-Darby canine kidney cell II (MDCK II) and in a human *PKD1* cystic epithelial cell line, OX161-C1 (Figure 2A). The latter expresses all four EP subtypes in a similar pattern to UCL93 (data not shown). The addition of PGE_2 significantly enhanced cyst growth in both cell lines over time in a dose-dependent manner with OX161-C1 cells being more sensitive to the effect of PGE_2 than MDCK II cells (Figure 2B and C). Since MDCK II cysts responded in a clearer dosage-dependent manner than OX161-C1, we investigated the effects of PGE_2 on proliferation (Ki-67) or apoptotic (cleaved caspase-3) rates in MDCK II cysts. PGE_2 had a dual effect on MDCK II cysts by increasing cell proliferation and inhibiting apoptosis in a dose-dependent manner (Figure 2D-G). We did not measure apoptosis at earlier time points and it is possible that luminal apoptotic cells could have been removed. These changes were significantly correlated to changes in the average cyst area measured in the same wells (Supplementary figure S1A, B). As expected, PGE2 stimulated cAMP accumulation in both cell lines (Supplementary figure S1C, D).

EP2 and EP4 agonists mimic PGE_2-induced cyst growth

A differential array of EP gene expression revealed fold-changes of 1.55 (EP1), 5.05 (EP2), -1.19 (EP3) and 1.89 (EP4) between PKD1 cystic and normal cells although only EP2 was significantly increased [24]. We confirmed that OX161-C1 expresses all four EP receptor subtypes and MDCK II expresses EP2 and EP4 mRNA by PCR (data not shown). We next

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tested the effects of selective agonists to EP1-4 in both cell lines grown in 3D-culture for up to 20 days. The EP1 agonist ONO-DI-004 and the EP3 agonist ONO-AE-248 did not significantly increase cyst area of either MDCK II or OX161-C1 after 14 days incubation although a decrease was noted at the highest concentration of ONO-AE-248 in OX161-C1 (data not shown). By contrast, cyst growth in both lines was significantly enhanced by either EP2 (ONO-AE1-259-01) or EP4 (ONO-AE1-329) agonists in a dose-dependent manner (Figure 3A-D). Similar to PGE_2, the EP2 and EP4 agonists also enhanced cAMP formation in OX161-C1 and MDCK II (Figure 3E and F) and stimulated MDCKII cyst growth by increasing cell proliferation and decreasing cell apoptosis (Supplementary Figure S2). These results strongly suggested that PGE_2 stimulates cyst growth *in vitro* by activating EP2 and EP4.

EP2 and EP4 antagonists abolish PGE_2-induced cyst growth

To confirm that PGE_2-induced cyst growth is mediated by EP2 and EP4, we tested the effect of selective EP2 and EP4 antagonists on PGE_2-induced cyst growth in both cell lines. As shown in Figure 4, the EP2 antagonist ONO-AE8-111 or the EP4 antagonist ONO-AE8-11 both decreased the effect of PGE_2 on cyst growth. Similar effects were observed with the EP2 antagonist PF-04418948 and a different EP4 antagonist CJ-42794 (Data not shown). Taken together, these results confirm that PGE_2 induces cyst growth via EP2 and EP4 and that blockade of either receptor is sufficient to inhibit the effect of PGE_2.

The PGE_2 pathway is up-regulated in an early-onset *Pkd1* mouse model

To study the regulation of the PGE_2 pathway in an *in vivo* ADPKD model, we utilised the early onset hypomorphic *Pkd1*-neolox (*Pkd1^{nl/nl}*) mouse model [25]. In this model, *Pkd1^{nl/nl}* kidneys were already cystic and larger at birth compared to *Pkd1^{wt/wt}* at each time point (Supplementary

Figure S3A–E). Typically, peak kidney growth and cyst expansion occurred around week 3 followed by a gradual decrease up to week 10 (Figure 5A and B, Supplementary Figure S3F). Cysts developed from multiple nephron segments (distal and proximal tubules, collecting ducts and loop of Henle) during this growth phase (data not shown). At 3 weeks, $Pkd1^{nl/nl}$ kidneys were characterised by an increase in both proliferative (Ki-67 positive) and apoptotic (TUNEL positive) cells (Supplementary Figure S4), an accumulation of F4/80-positive cells and fibrotic tissue especially around cysts (Supplementary figure S5). Renal cAMP concentrations increased significantly from week 2 to 3 in $Pkd1^{nl/nl}$ kidneys but then decreased sharply after week 4, changing in parallel to fractional total kidney weights (Figure 5A and C).

We next analysed different components of the PGE_2 system in this model. Levels of $Ptgs2$ mRNA (encoding for COX-2) were increased in $Pkd1^{nl/nl}$ kidneys from weeks 2 to 6 followed by a decline up to week 10 (Figure 5D). In 4 week old animals, PGE_2 concentrations in whole kidney lysates was increased by at least two-fold: 537 pg/mg protein ($Pkd1^{wt/wt}$) vs 1174 pg/mg protein ($Pkd1^{nl/nl}$) (Figure 5E). The expression profile of the different PGE_2 receptors showed distinct expression patterns over time (Supplementary Figure S6). $Ptger1$ and $Ptger4$ mRNA were significantly up-regulated in $Pkd1^{nl/nl}$ kidney from week 4 to 10 with a peak at week 6 while $Ptger2$ was significantly up-regulated at week 2 and also from week 6 to 10. $Ptger3$ expression was lower in $Pkd1^{nl/nl}$ kidneys between weeks 2 to 6 though this did not reach significance. In 2 week-old $Pkd1^{wt/wt}$ kidneys, EP4 was strongly expressed by distal tubules and the loops of Henle, co-localising with calbindin and Tamm-Horsfall protein respectively in serial sections (Supplementary Figure S7A). In 2week-old $Pkd1^{nl/nl}$ kidneys, EP4 was detected in small cysts and dilated tubules but was not expressed in larger cysts (Supplementary Figure S7B). Unfortunately we did not find an EP2 antibody suitable for immunohistochemistry.

EP2 and EP4 receptor blockade increases disease severity *in vivo* in an early-onset *Pkd1* mouse model

Since cyst formation occurred *in utero* and was obvious in newborn (PN1) *Pkd1$^{nl/nl}$* mice (Supplementary Figure S3), we decided to commence drug treatment shortly after birth. To achieve this, compounds were initially administered through the drinking water (PN1-7) of lactating mothers and after the first week of life, by daily IP injection (PN7–20) to the pups. Four treatment groups each received either DMSO (vehicle), the EP2 antagonist PF-04418948, the EP4 antagonist ONO-AE3-208 or both compounds in combination (Figure 6A). Animals from all four groups grew normally and tolerated the compounds with normal increases in body weight (data not shown). Unexpectedly, the average kidney weights and kidney-to-body weight ratio from PN21 mice treated with either EP2 or EP4 antagonists alone did not decrease but were higher (Figure 6B–D) with a significant increase for the EP4 antagonist treated animals. Histological analysis of kidney sections showed that the cystic index in both EP2 and EP4 treated groups were significantly higher than those of vehicle treated animals (Figure 6E and F) with a non-significant trend (p=0.056) towards increased fibrosis (Supplementary figure S8D). Kidney function was not significantly altered between the groups nor was there a significant change in total kidney cAMP content or apoptosis (Supplementary figure 8A-C). However, the percentage of Ki-67-positive cells and F4/80-positive cells was significantly increased in both EP2 and EP4 groups with a significant correlation between the percentage of F4/80-positive cells and kidney-to-body weight ratio (Figure 6G-I). Compared to vehicle treated animals, mice treated with EP2 and EP4 antagonists in combination had a less severe phenotype than those treated with either compound alone associated with a trend towards reduced fibrosis, cell proliferation and macrophage number. Treated animals did not show any significant change between AQP1 and AQP2 positive tubules excluding a differential effect on segment-specific cysts (Supplementary figure S9).

EP4 receptor blockade *in vivo* increases disease severity in an inducible kidney-specific *Pkd1* model

To confirm these findings, we utilised a second *Pkd1* mouse model (*Pax8^{rtTA}-TetO-Cre-Pkd1^{f/f}*) [26] to induce kidney-specific post-natal *Pkd1* deletion (PN13-15) by doxycycline (DOX) IP injection which generates a less severe model than *Pkd1^{nl/nl}* mice. Post-induction, mice were treated by daily IP injection for 14d (PN16–29) prior to sacrifice at PN30. The four treatment groups received either DMSO (vehicle), the EP2 antagonist PF-04418948, the EP4 antagonist ONOAE3-208 or both compounds in combination (Figure 7A). Average kidney weights and kidney-to-body weight ratios treated with the EP4 antagonist were higher than vehicle or EP2 treated mice with a non-significant increase in the EP4 antagonist treated animals (Figure 7B–D). Although there was no significant change in cystic index, cyst number in the EP4 treated group was higher (p=0.053) than vehicle treated animals (Figure 7E-G) and was associated with a significant increase in the percentage of Ki-67-positive cells and F4/80-positive cells (Figure 7H-I). As in the first model (*Pkd1^{nl/nl}*), the percentage of F4/80-positive cells and kidney-to-body weight ratio showed a highly significant correlation (Figure 7J).

EP4 receptor blockade *in vivo* increases accumulation of M2 macrophages in both *Pkd1* models.

The observed increase in macrophage number particularly following EP4 receptor blockade led us to investigate whether there was a shift in macrophage polarisation between M1 (Nos2 positive) and M2 (Ym1 or Mrc1 positive) subtypes by immunohistochemistry and immunofluorescence labelling. In both models, M2 macrophages (Ym1 or Mrc1) were significantly increased in the EP4 group (Figure 8) whereas the percentage of M1 macrophages did not change (Supplementary figures S10).

DISCUSSION

In this study, we present clear evidence that PGE_2 promotes cystogenesis in cellular models of ADPKD via the EP2 and EP4 receptors. Since EP2 and EP4 are known to couple to Gs which stimulates cAMP formation by activating adenylyl cyclase [27, 28], this confirms that the pro-cystogenic action of PGE_2 is *via* cAMP formation. Using selective receptor agonists, we exclude a major role for EP3, which inhibits cAMP generation *via* Gi [29] and EP1, which activates Gq, leading to intracellular Ca^{2+} elevation through phospholipase C [30]. The effect of PGE_2 acting via EP2 and EP4 was particularly striking on inhibiting normal tubulogenesis and promoting cystogenesis in a non-cystic human cell line (UCL93). The action of EP2 and EP4 appeared to be independent and non-redundant in both MDCK and OX161-C1. We did not observe a change in the pattern of EP receptor expression between normal (UCL93) or PKD1 cystic cells. Our results differ from previous reports suggesting a more restricted role for EP2 or EP4 in mediating the action of PGE_2 on cyst formation in primary human cystic cells or murine IMCD-3 cells respectively [21, 22]. These differences could reflect cell type differences in EP expression.

The promising effects observed using EP2 and EP4 antagonists on PGE_2-induced cyst growth *in vitro* were however not reproduced *in vivo*. We chose to study a well-established *Pkd1* mouse model (*Pkd1^{nl/nl}*) which has an early-onset phenotype but whose phenotype has been shown to respond to several therapeutic drugs [31-34]. Both PGE_2 concentration and *Ptgs2* expression were increased at the peak of cystic disease (3-4 weeks) in parallel to the increase in renal cAMP concentrations. Since cyst formation was already significant at birth, we decided to administer EP2 or EP4 antagonists from PN1-21 to cover the peak period of cyst formation. Unexpectedly, blockade of either receptor led to an increase in the severity of disease and was associated with an increase in cell proliferation and macrophage infiltration. Similar results were observed for EP4 blockade in a second *Pkd1* model (*Pax8^{rtTA}-TetO-Cre-Pkd1^{f/f}*) with

post-natal disease onset although in this model, the effect of EP2 antagonism was neutral. These could represent differences relating to the timing of disease onset in both models ie neonatal (hypomorphic) and post-natal (inducible).

The simplest explanation for our findings is that EP2 and EP4 antagonism led to a pro-inflammatory phenotype *in vivo*. Indeed, the role of PGE_2 in inflammation is complex as it has been reported to exert both pro- and anti-inflammatory responses, depending on the tissue and receptor expression [35, 36]. The role of EP4 in kidney inflammation has been investigated more extensively than EP2. In the Unilateral Ureteral Obstruction (UUO) model which is characterized by increases in PGE_2, COX-2, EP2 and EP4 mRNA, EP4-null mice developed more interstitial fibrosis and macrophage infiltration compared to wild-type UUO mice [37]. Conversely, treating UUO-wild type mice with an EP4 agonist (ONO-4819) reduced interstitial fibrosis and macrophages number [37]. A similar effect of a different EP4 agonist (CP-044,519-02) on fibrosis and macrophage infiltration was noted in the subtotal (5/6) nephrectomy model of chronic kidney failure [38]. These observations are not restricted to kidney disease models as EP4-null mice develop greater airway inflammation than their wild-type controls in three models of airway inflammation [39]. The anti-inflammatory effect of EP4 on macrophages could relate to alternative non-Gs/cAMP pathways such as EP4 receptor-associated protein/β-arrestin signalling in these cells [40].

By contrast, the role of EP2 in kidney disease models has not been studied in detail. However, a likely role in the regulation of macrophage maturation has emerged. EP2 expression is upregulated in activated peritoneal macrophages [41] but EP2-null macrophages or macrophages treated with EP2 antagonist exhibit enhanced maturation *in vitro*; EP2-null mice had higher numbers of circulating mature macrophages and in the peritoneal cavity [42]. The removal of this restraint on systemic macrophage maturation could have contributed to increased macrophage recruitment in EP2-treated *Pkd1* hypomorphic mice.

Several recent papers have highlighted the role that macrophages could play in modifying disease severity in other murine models of ADPKD [43, 44]. Macrophages have been shown to promote cyst growth by both proliferation-dependent and proliferation-independent mechanisms[45]. Typically, alternatively activated (M2-like) macrophages have been associated with tubular proliferation whereas pro-inflammatory (M1-like) macrophages can lead to cyst expansion through tubular injury [45, 46]. Conversely, the cystic epithelium can play a direct role in stimulating macrophage infiltration (MCP-1 secretion) or polarisation to M2 phenotypes (L-lactic acid secretion) [46]. Our results add cystic-derived PGE_2 to the emerging epithelial-macrophage axis in ADPKD as both a pro-proliferative and anti-inflammatory mediator. Our *in vivo* results were more complex than anticipated due to the opposing effects of EP2 and EP4 on epithelial proliferation and macrophage infiltration or activation. Of interest, mice treated with EP2 and EP4 antagonists in combination had similar proliferative rates to vehicle-treated mice but still had more macrophages and a higher cystic index. These results suggest that the direct effects of EP2 and EP4 antagonists in blocking PGE_2-stimulated epithelial cell proliferation (that we observed *in vitro*) was unable to counterbalance the negative effect of increased macrophage infiltration or activation that led to both proliferation and injury. A consistent effect in both models was the increase in F4/80 positive macrophage number (especially M2-like) following EP4 receptor blockade. Our data therefore add to the accumulating evidence that macrophages play a key role in modifying cystogenesis in ADPKD. The complexity of different macrophage subpopulations in healthy and diseased kidney has been reported recently by several groups. Of potential relevance to our findings was the reaccumulation of a 'juvenile-like' resident macrophage population (R2b) in pre-cystic kidneys of a cilia mouse mutant (*Ift88*): R2b macrophages express CD206/Mrc1 and Ym1, classical 'M2' markers and appeared to stimulate cystogenesis in this model [47]. The role of PGE2 and particularly EP4 in regulating the accumulation and/or activation of M2 and R2b macrophages

in cystic kidneys merits further investigation. It would be interesting to test the ability of EP4 (or EP2) agonists to induce anti-inflammatory effects in this and other PKD models as has been reported elsewhere [37, 38]. However we predict that this is likely to induce direct proliferative effects on cystic epithelial cells.

Our study has some limitations. We only tested the effects of blocking EP2 and EP4 in the earliest phase of disease (PN1-21) coinciding with the peak of cyst formation. Our results therefore do not exclude a role for EP2 or EP4 in the subsequent stages of disease e.g. in the late development of fibrosis in this model. Second, the $Pkd1^{nl/nl}$ model is an early-onset model with significant cystic disease detectable at birth. We did not test the role of blocking EP2 or EP4 *in utero* as this could have interfered with embryonic development. A genetic approach would be necessary to test an earlier role for EP2 or EP4 deficiency in the cystic phenotype.

In conclusion, we have shown that that PGE_2 induces cyst formation and expansion via activation of EP2 and EP4 receptors through stimulating cell proliferation and decreasing cell apoptosis. Despite the positive effects of EP2 and EP4 antagonists on slowing down cyst growth *in vitro*, the more complex effects of inhibiting EP2 or EP4 *in vivo* resulted in a more severe cystic phenotype *in vivo* due to unexpected pro-inflammatory effects (Figure 9).

METHODS

Cell lines

Madin-Darby Canine Kidney II (MDCK II) cells were cultured in DMEM (GIBCO) supplemented with 10 % fetal calf serum (Sigma-Aldrich), 50 U/ml Penicillin/Streptomycin (Lonza), 2 mmol/l L-glutamine (Lonza) and maintained at 37 °C in a humidified atmosphere of 5 % CO_2. The generation of conditionally immortalized human normal (UCL93) and *PKD1* cyst-lining epithelial cells (OX161) has been previously described [24, 48]. In 3D culture, OX161 cells gave rise to predominant cystic (60%) but also simple tubular structures or complex

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tubulocystic structures. We recloned this line by single cell dilution to identify clones with a higher ratio (90%) of cysts in 3D culture and chose clone 1 (C1) for further study. Immortalized human cells were cultured in DMEM (GIBCO) supplemented with 5 % Nu-Serum (Corning), 50 U/ml Penicillin/Streptomycin (Lonza), 2 mmol/l L-glutamine (Lonza) and maintained at 33 °C in a humidified atmosphere of 5 % CO2.

Experimental animals and study design

All animal experiments were performed under the authority of a UK Home Office license. Hypomorphic *Pkd1*$^{nl/nl}$ harboring an intronic neomycin-cassette in *Pkd1* gene and their *Pkd1*$^{wt/wt}$ and *Pkd1*$^{wt/nl}$ littermates were sacrificed at different ages from PN1 to PN70 to study the evolution of the disease (n=2-11 per group) [25]. *Pax8*^{rtTA}-*TetO-Cre-Pkd1*$^{f/f}$ mice (*Pkd1*$^{fl/fl}$) were induced using doxycycline (DOX) by daily IP injections for three days (5mg/kg/day, PN13–15) [26]. To study the effect of EP2 and EP4 antagonists on disease progression, *Pkd1*$^{nl/nl}$ (n=6-7) and *Pkd1*$^{fl/fl}$ (n=8-12) were divided into 4 groups: one vehicle-treated control group, one group treated with 5mg/kg/day PF-04418948, a selective EP2 antagonist, one group treated with 10mg/kg/day ONO-AE3-208, a selective EP4 antagonist and one group treated with 5mg/kg/day PF-04418948 and 10mg/kg/day ONOAE3-208 in combination. *Pkd1*$^{nl/nl}$ mice: from PN1 to PN7, drugs were dissolved in the drinking water and delivered to the pups through the milk and from PN7 to PN20, drugs were administered by daily intraperitoneal injections. Mice from the vehicle group mice received daily intraperitoneal injections of DMSO in Kolliphor® EL (Sigma-Aldrich). Mice were sacrificed at PN21, corresponding to the peak of the disease. *Pkd1*$^{fl/fl}$ mice: from PN16 to PN29, drugs were administered by daily intraperitoneal injections. Mice from the vehicle group mice received daily intraperitoneal injections of DMSO in Kolliphor® EL (Sigma-Aldrich). Mice were sacrificed at PN30, corresponding to the peak of the disease.

Cyclic AMP Enzyme-Linked Immunosorbent Assays

Kidneys were mechanically homogenized in 0.1 M HCl (Acros Organics) using pre-filled Triple-pure zirconium beads (Benchmark Scientific) and a microtube homogenizer (Benchmark Scientific) at 4 °C. After centrifugation at 10000 x g for 10 minutes, cAMP from the supernatant were quantified using an enzyme immunoassay kit (Enzo) following the manufacturer's instructions without acetylation.

Cyclic AMP extracted from cells was quantified using an in-house ELISA we have developed and validated against the commercial ELISA (Supplemental Figure S8E). After 24 hours of starvation, confluent cells were incubated for 30 min with 0.25 mmol/l 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 5 mg/ml lactalbumin hydrolysate (Sigma-Aldrich) prior to incubation with drugs to be tested (Sigma-Aldrich) for 1 hour. Cells were lysed in 0.1 mol/l HCl and cAMP was measured as followed. ELISA 96 well-EIA/RIA Plates plates (Costar) were coated overnight with 5 µg/ml Goat Anti-Rabbit IgG Antibody (Millipore) in PBS. After washing the plate with 0.05 % Tween® 20 in PBS, pH 7.2-7.4, the plates were blocked with 1 % BSA (Acros-Organics) in PBS pH 7.2-7.4 for 1 hour at RT followed by additional washes with washing buffer. Competitive reactions to measure cAMP were done by pipetting into wells the same volume of cAMP lysate, neutralizing reagent (Tris-Base 0.1 mol/l, Sigma-Aldrich), rabbit anti-cAMP Antibody (Genscript), cAMP-HRP (Genscript) and incubating the plate for 2 hours at RT on a plate shaker. Known concentrations of cAMP (Sigma-Aldrich) diluted in 0.1 mol/l HCl were used to generate a standard curve covering the concentration range 3 to 729 pmol/ml. After extensive washes, the plate was incubated with TMB substrate (ThermoFischer) at room temperature for ~20 min after which stop solution (2 M sulfuric acid) was added and the absorbance was measured at 450 nm using ELISA plate reader. Protein

concentration was quantified using a DC protein assay kit (Bio-rad) and used to normalise cAMP concentrations.

See the Online Supplement for description of other methods used in this study.

Statistical analysis

Data are presented as means values ± SEM. Statistical analysis were performed with GraphPad Prism software. The degree of significance is denoted as following: *P <0.05; **P <0.01; ***P< 0.001; ****P< 0.0001. The test performed for statistical analysis is indicated in the graph's legend.

DISCLOSURE

None

SUPPLEMENTARY MATERIAL

Supplementary Methods.

Supplementary Figure S1: PGE_2 stimulates cell proliferation, decreases cell apoptosis and induces cAMP formation in MDCK II and OX161-C1 cells.

Supplementary Figure S2: EP2 and EP4 activation increase cell proliferation and inhibit cell apoptosis in MDCK II cysts.

Supplementary Figure S3: Early-onset cyst formation in $Pkd1^{nl/nl}$ mice.

Supplementary Figure S4: $Pkd1^{nl,nl}$ kidneys are characterised by an increase in proliferating and apoptotic cells.

Supplementary Figure S5: $Pkd1^{nl,nl}$ kidneys are characterised by an increase in interstitial macrophages and fibrosis.

Supplementary Figure S6: EP1, EP2, EP3 and EP4 gene expression in $Pkd1^{wt,wt}$, $Pkd1^{wt,nl}$ and $Pkd1^{nl,nl}$ kidneys from post-natal weeks 2 to 10.

Supplementary Figure S7: EP4 immuno-localisation in 2 week $Pkd1^{wt,wt}$ and $Pkd1^{nl,nl}$ kidneys.

Supplementary Figure S8: Additional analysis of $Pkd1^{nl,nl}$ mice treated with EP2 and EP4 antagonists.

Supplementary Figure S9: Segmental origin of cysts in $Pkd1^{nl,nl}$ mice treated with EP2 and EP4 antagonists.

Supplementary Figure S10: Macrophage subtypes in $Pkd1^{nl,nl}$ and $Pkd1^{fl,fl}$ mice treated with EP4 antagonists.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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FIGURE LEGENDS

Figure 1: PGE$_2$ inhibits tubulogenesis and promotes cystogenesis through EP2 and EP4 receptors.

PGE$_2$ (A, B), ONO-AE1-259-01, an EP2 receptor agonist, or ONO-AE1-329, an EP4 receptor agonist (C, D) inhibit spontaneous tubulogenesis in UCL93, a non-cystic human renal epithelial cell line, grown initially for 6 d to promote tubular formation and then incubated for a further 14 days with compounds. Scale bar, 500 μm. Values are expressed as means ±SEM, n>100 structures per condition. PGE$_2$ (E), ONO-AE1-259-01,or ONO-AE1-329 (F) stimulate cAMP accumulation in UCL93 cells after 60 min incubation Values are expressed as means ±SEM, n=6. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared with the control group. Statistical significances were determined using a one-way ANOVA followed by Dunnett's multiple comparison test or an Unpaired t-test.

Figure 2: PGE$_2$ induces cyst growth by increasing proliferation and decreasing apoptosis.

PGE2 stimulates cyst growth in MDCK II and OX161-C1 cells in a dose and time-dependent manner (A-C). Scale bar, 50 μm. Values are expressed as means ±SEM, n=30–78 cysts per condition for MDCK II and n=84–113 cysts per condition for OX161-C1. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared with the control group. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test. PGE2 stimulates cell proliferation (D, E) and inhibits apoptosis (F, G) in a dose dependent manner in MDCK II cysts. The proliferative rate at day 10 was quantified as the percentage of Ki-67 positive cells per cyst. The apoptotic rate was quantified as the percentage of cysts with >5 cleaved caspase-3 positive nuclei. Values are expressed as means ±SEM, n=30 cyst per condition. *P<0.05; **P<0.01; ***P<0.001 compared with the control group. Statistical

significances were determined using a one-way ANOVA followed by Dunnett's multiple comparison test.

Figure 3: EP2 and EP4 activation induce cyst growth of MDCK II and OX161-C1 cells

The EP2 receptor agonist ONO-AE1-259-01 (A) or the EP4 receptor agonist ONO-AE1-329 (B) stimulated cyst growth in MDCK II and OX161-C1 cells. Values are expressed as means ±SEM, $n=45–72$ cysts per condition for MDCK II and $n=73–162$ cysts per condition for OX161-C1. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$ compared with the control group. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test. (C) Example of MDCKII cysts after 20 d of ONO-AE1-259-01 or ONO-AE1-329. Scale bar, 100 μm. (D) Example of OX161-C1 cysts after 10, 14 and 20 d incubation with ONO-AE1-259-01 or ONO-AE1-329. Scale bar, 50 μm. ONO-AE1-259-01 or ONO-AE1-329 stimulate cyclic AMP accumulation in MDCK II (E) and a OX161-C1 cells (F) after 60 min. Values are expressed as means ±SEM, $n=6$. *$P<0.05$; **$P<0.01$ compared with the control group. Statistical significances were determined using a one-way ANOVA followed by Dunnett's multiple comparison test or an Unpaired t-test.

Figure 4: EP2 and EP4 antagonists abolish PGE2-induced cyst growth *in vitro*.

The effect of PGE2 (10nM) on cyst growth in MDCK II (A, B) and OX161-C1 (C, D) cells is blocked by EP2 (ONO-AE8-111) or EP4 (ONO-AE3-208) selective antagonists after 14 d and 10 d respectively Values are expressed as means ±SEM, $n=64–75$ cysts per condition for MDCK II and $n=101–144$ cysts per condition for OX161-C1. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$ compared with the PGE$_2$ group (▪). Statistical significance was

determined using the non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparisons test.

Figure 5: The evolution of cystic disease in *Pkd1*$^{nl/nl}$ mice is associated with an up-regulation of PGE$_2$ synthesis.

Two Kidney-to-body weight ratio (A), cystic index (B), renal cAMP content (C) and *Ptgs2* mRNA (D) were measured in *Pkd1*^{wt,wt} and *Pkd1*^{nl,nl} kidneys from PN14 to PN70. Values are expressed as means ±SEM (n=4–11 per group). **$P<0.01$; ***$P<0.001$; ****$P<0.0001$ compared to *Pkd1*^{wt,wt} at the same age. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test. (E) PGE$_2$ concentrations were measured on whole kidney lysates from 4-week-old *Pkd1*^{wt,wt} and *Pkd1*^{nl,nl}. Values are expressed as means ±SEM (n=6 per group). **$P<0.01$ compared to *Pkd1*^{wt,wt}. Statistical significance was determined using an Unpaired t-test.

Figure 6: Administration of EP2 and EP4 antagonists worsened the cystic phenotype of *Pkd1*^{nl,nl} mice.

(A) Outline of the experimental design of the *in vivo* experiments. (B) Kidney weight of PN21 *Pkd1*^{nl,nl} mice treated with EP2 or EP4 antagonists. (C) Gross morphology of *Pkd1*^{nl,nl} kidneys from mice treated with EP2 or EP4 antagonists. Two kidney-to-body weight ratio (2KW/BW, D), cystic index (E) and (F) representative images of PAS-stained kidney sections from *Pkd1*^{nl,nl} kidneys from mice treated with EP2 or EP4 antagonists. (G) Percentage of Ki-67-positive cells or (H) F4/80 positive cells in kidney sections from mice treated with EP2 or EP4 antagonists. (I) Correlation between the percentage of F4/80-positive cells and fractional kidney weights. Values are expressed as means ±SEM (n=6–7 per group). *$P<0.05$; **$P<0.01$;

***$P<0.001$ compared to vehicle group. Statistical significance was determined using an unpaired t-test.

Figure 7: Administration of an EP4 antagonist increased the cystic phenotype of _Pkd1^{fl,fl}_ mice.

(A) Outline of experimental strategy. Kidney weight (g, B) and Kidney-to-body weight ratio (2KW/BW, C) of 30-day-old _Pkd1^{fl,fl}_ mice treated with EP2 or EP4 antagonists. (D) Gross morphology of _Pkd1^{fl,fl}_ kidneys from mice treated with EP2 or EP4 antagonists. Cystic index (%, E) and Kidney cysts (Number of cysts, F) of _Pkd1^{fl,fl}_ kidneys from mice treated with EP2 or EP4 antagonists. (G) Representative images of H&E stained kidney sections from _Pkd1^{fl,fl}_ kidneys from mice treated with EP2 or EP4 antagonists. (H) Percentage of Ki-67-positive cells on kidneys sections from mice treated with EP2 or EP4 antagonists. (I) Percentage of F4/80-positive cells on kidneys sections from mice treated with EP2 or EP4 antagonists. (J) Correlation between the percentage of F4/80-positive cells and kidney-to-body weight ratio. Values are expressed as means ±SEM, _n_=8–12 mice per group. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to vehicle group. Statistical significance was determined using an unpaired t-test.

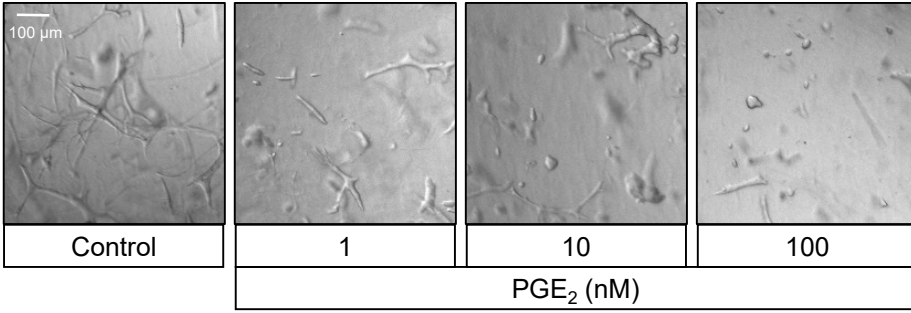**Figure 8: Macrophage subtypes in EP4 antagonist treated mice.**

(A) Fraction of Nos2-F4/80 and (B) Ym1-F4/80 macrophaes from _Pkd1^{nl,nl}_ (left) or _Pkd1^{fl,fl}_ mice (right) in vehicle and EP4 antagonist groups. Values are expressed as means ±SEM, n=4 mice per group with five randomly selected regions used for counting. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to vehicle group. Statistical significance was determined using an unpaired t-test. (C) Fluorescent labelling of kidney sections from _Pkd1^{nl,nl}_ mice stained with

Mrc1 (Red) and DAPI (Grey). The boxed area is expanded in the right image to show the presence of multiple Mrc1 positive macrophages surrounding a medium sized cyst. (D) Mrc1 positive cells were quantified in $Pkd1^{fl,fl}$ mice treated with vehicle and EP4 antagonist. (F) Correlation between the percentage of F4/80-positive cells and MRC1-positive cells present in serial sections from the same animals. Values are expressed as means ±SEM, n=11-12 mice per group. *P<0.05; **P<0.01 compared to vehicle group. Statistical significance was determined using Welch's t-test.

Figure 9: The likely roles of EP2 and EP4 in ADPKD pathogenesis.

(A) *In vitro*, PGE$_2$ enhances cyst growth *in vitro* by stimulating epithelial cell (EC) proliferation, decreasing epithelial cell apoptosis and stimulating chloride (Cl$^-$) secretion through EP2 and EP4 mediated cAMP production. (B) *In vivo*, EP2 and EP4 antagonists also induce an increase in macrophage number which itself leads to an increase in EC injury and proliferation, counteracting the direct effects of EP2 and EP4 on cyst epithelia. The graphical illustration was drawn by using the images from Servier Medical Art by Les Laboratoires Servier, with slight modifications (https://smart.servier.com/).
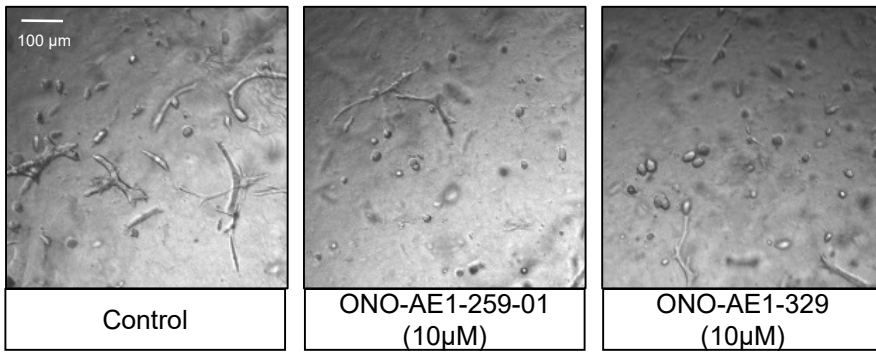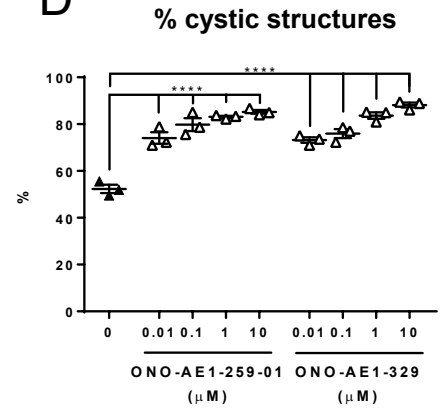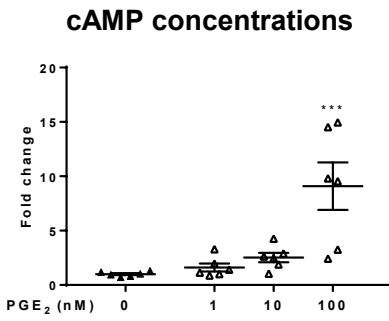
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

Both Kidney Weight / Body Weight

B

Cystic index

C

Renal cAMP level

D

Ptgs2

E

PGE2

Figure 6

A

- Neutralized NaOH (Vehicle)
- 5mg/kg/day PF-04418948
- 10mg/kg/day ONO-AE3-208
- 5mg/kg/day PF-04418948 + 10mg/kg/day ONO-AE3-208

D-0   D-1   D-7   D-21

Daily I.P. injections
- DMSO (Vehicle)
- 5mg/kg/day PF-04418948
- 10mg/kg/day ONO-AE3-208
- 5mg/kg/day PF-04418948 + 10mg/kg/day ONO-AE3-208

B

Kidney weight

g

*

Vehicle   EP2   EP4   EP2 and EP4

C

Pkd1nl,nl

| Vehicle | EP2 antagonist | EP4 antagonist | EP2+4 |

1 cm

RK=0.27, LK=0.23
2KW/BW=8.48

RK=0.41, LK=0.32
2KW/BW=10.73

RK=0.41, LK=0.32
2KW/BW=10.73

RK=0.37, LK=0.35
2KW/BW=11.21

D

2KW/BW

%

**

Vehicle   EP2   EP4   EP2 and EP4

E

Cystic Index

%

*
*

Vehicle   EP2   EP4   EP2 and EP4

F

Pkd1nl,nl

| Vehicle | EP2 antagonist | EP4 antagonist | EP2+4 |

1000 µm

Cyst Number=292
Av. cyst Area=34049 µm^2
CI=46.59 %

Cyst Number=341
Av. cyst Area=39642 µm^2
CI=57.89 %

Cyst Number=424
Av. cyst Area=38576 µm^2
CI=60.1 %

Cyst Number=375
Av. cyst Area=40278 µm^2
CI=58 %

G

Proliferation

% Ki-67 positive cells

***
*

Vehicle   EP2   EP4   EP2 and EP4

H

Inflammation

% F4/80 positive area

*
*

Vehicle   EP2   EP4   EP2 and EP4

I

Treated Pkd1nl,nl

% F4/80 positive area

R=0.4112
P=0.0361

2KW/BW

Figure 7

A

D-0 → D-13 → D-16 → D-30

Daily I.P. injections
- 5mg/kg/day DOX

Daily I.P. injections
- DMSO (Vehicle)
- 5mg/kg/day PF-04418948
- 10mg/kg/day ONO-AE3-208
- 5mg/kg/day PF-04418948 + 10mg/kg/day ONO-AE3-208

Termination

B Kidney Weight

C 2KW/BW

D

Pkd1$^{fl/fl}$

Vehicle	EP2 antagonist	EP4 antagonist	EP2+4
RK=0.20, LK=0.21 2KW/BW=3.05	RK=0.23, LK=0.21 2KW/BW=3.31	RK=0.26, LK=0.32 2KW/BW=4.09	RK=0.39, LK=0.41 2KW/BW=5.90

1 cm

E Cystic Index

F Kidney Cysts

P=0.053

G

Pkd1$^{fl/fl}$

Vehicle	EP2 antagonist	EP4 antagonist	EP2+4
Cyst Number=1215 CI=49.1 %	Cyst Number=1274 CI=46.4 %	Cyst Number=1734 CI=55.3 %	Cyst Number=1720 CI=57.1 %

2mm

H Proliferation

**

I Inflammation

*

J Treated *Pkd1$^{fl/fl}$*

R=0.6143
P=0.00001

Figure 8

A

Pkd1^{nl,nl}

Pkd1^{fl,fl}

B

Pkd1^{nl,nl}

Pkd1^{fl,fl}

C

DAPI MRC1

DAPI MRC1

300μm

50μm

D

Pkd1^{fl,fl}

E

R=0.6036
P=0.002290