Pannexin-1 mediates fluid shear stress-sensitive purinergic signaling and cyst growth in polycystic kidney disease

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Abstract
Tubular ATP release is regulated by mechanosensation of fluid shear stress (FSS). Polycystin-1/polycystin-2 (PC1/PC2) functions as a mechanosensory complex in the kidney. Extracellular ATP is implicated in polycystic kidney disease (PKD), where PC1/PC2 is dysfunctional. This study aims to provide new insights into the ATP signaling under physiological conditions and PKD. Microfluidics, pharmacologic inhibition, and loss-of-function approaches were combined to assess the ATP release in mouse distal convoluted tubule 15 (mDCT15) cells. Kidney-specific Pkd1 knockout mice (iKsp-Pkd1−/−) and zebrafish pkd2 morphants (pkd2-MO) were as models for PKD. FSS-exposed mDCT15 cells displayed increased ATP release. Pannexin-1 inhibition and knockout decreased FSS-modulated ATP release. In iKsp-Pkd1−/− mice, elevated renal pannexin-1 mRNA expression and urinary ATP were observed. In Pkd1−/− mDCT15 cells, elevated ATP release was observed upon the FSS mechanosensation. In these cells, increased pannexin-1 mRNA expression was observed. Importantly, pannexin-1 inhibition in pkd2-MO decreased the renal cyst growth. Our results demonstrate that pannexin-1 channels mediate ATP release into the tubular lumen due to pro-urinary flow. We present pannexin-1 as novel therapeutic target to prevent the renal cyst growth in PKD.

KEYWORDS
ATP, fluid shear stress, pannexin-1, polycystin-1, purinergic signaling

Abbreviations: Abcc6, ATP binding cassette subfamily c member 6; BB-FCF, brilliant blue-FCF; Cx30, connexin30; Cx30.3, connexin30.3; Cx37, connexin37; Entpd2, ectonucleoside triphosphate diphosphohydrolase 2; Entpd3, ectonucleoside triphosphate diphosphohydrolase 3; FBS, fetal bovine serum; FSS, fluid shear stress; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; MO, translation blocking morpholino; Nr5e, ecto-5′-nucleotidase; Panx1, pannexin-1; PC1, polycystin-1; PC2, polycystin-2; PKD, polycystic kidney disease; Pkd1, polycystic kidney disease 1; Pkd2, polycystic kidney disease 2; Pgs2, prostaglandin-endoperoxide synthase 2.
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1 | INTRODUCTION

Mutations in the genes polycystic kidney disease 1 (PKD1) and polycystic kidney disease 2 (PKD2) lead to polycystic kidney disease (PKD). PKD is characterized by increased cell proliferation, fluid accumulation, and altered extracellular matrix synthesis in the kidney. These characteristics lead to the renal cyst formation and growth, predominantly in the distal tubules and collecting duct (CD), and ultimately to end-stage kidney disease.1–4 PKD is one of the most common inherited renal diseases and accounts for ~10% of all patients on renal replacement therapy worldwide.5,6 The Pkd1 gene encodes for the protein polycystin-1 (PC1), which locates to the primary cilium and plasma membrane of renal tubular epithelial cells. PC1 forms a complex with polycystin-2 (PC2, encoded by Pkd2), which is a nonsel ective cation channel.7–11 PC1 is suggested to act as a mechanosensor of fluid shear stress (FSS) generated by pro-urine flow, regulating the physiological responses in renal tubular epithelial cells.12,13

In PKD, extracellular ATP plays an important role in disease progression,14,15 Under physiological conditions, extracellular ATP activates ionotropic P2X and metabotropic P2Y receptors at the luminal cell surface to regulate and maintain the kidney function.16 In recent years, autocrine and paracrine effects of the purinergic signaling have been suggested to provide a detrimental acceleration of cyst growth in PKD.17–20 It has been shown that the renal cyst fluid in PKD rats and PKD patients contains high ATP levels.21,22 Also, cystic PKD cell cultures display increased ATP release at basal conditions.23 Furthermore, previous studies have related purinergic receptors P2Y2 and P2X7 to cyst growth in an in vitro cystic model, and in vivo, in zebrafish and mouse PKD models.23–26

Thus far, translation of these findings into therapeutic applications for PKD related to purinergic signaling are limited.24 Conversely, approaches that did reach clinical research stages for PKD involve vasopressin antagonists, mTOR inhibitors, and somatostatin analogues. However, these approaches are not a definitive curative treatment option.1,25–29 An improved understanding of the molecular mechanisms behind ATP signaling in PKD may promote the development of novel therapeutic strategies for this disease.

ATP can be released from renal epithelial cells upon the mechanical stimulation triggered by variable pro-urine flow.30,31 Yet, the ATP extrusion mechanisms mediating flow-sensitive ATP release as well as their regulation are not fully elucidated.32,33 The extracellular ATP in the tubule lumen signals the apical membrane of renal cells to trigger an intracellular response which results in regulation of electrolyte and water transport.34,35

In this study, we aimed to disclose the mechanisms regulating the apical ATP release in renal cells upon flow sensing. To this end, the urine of healthy human volunteers and cellular and organismal kidney models were interrogated. The relevance of these mechanisms for disease progression in the context of PKD was investigated, where purinergic signaling was anticipated to play a pathologic role.

2 | MATERIALS AND METHODS

2.1 | Ethics approval

Urine samples of healthy human volunteers were collected after acute water loading experimentation in accordance with the principles expressed in the Declaration of Helsinki. All participants gave written informed consent, and the study protocol was approved by the Institutional Review Board of the Radboud University Medical Center (approval no. NL47178.091.13). The local animal experimental committee of the Leiden University Medical Center and the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture approved the animal procedures performed.

2.2 | Cell culture

To assess the role of FSS on ATP release, μ-slide I0.4 and VI0.4 channels (Ibidi GmbH, Planegg, Germany) were used for ATP and gene expression experiments, respectively. Static ATP release was assessed using 12-well plates. Mouse distal convoluted tubule 15 (mDCT15) cells (gift from Dr Robert Hoover, Emory University, Atlanta, GA, USA), a model of the distal convoluted epithelial cell, were used in all experiments.36 mDCT15 cells (either wild type, Ift140−/− or Pkd1−/−) were seeded in the channels (μ-slide I0.4: 1.2 × 10⁵ cells/cm² and μ-slide VI0.4: 5 × 10⁴ cells/cm²) with DMEM/F-12 1:1 nutrient mixed media supplemented with 5% v/v fetal bovine serum (FBS, GE Healthcare, Little Chalfont, United Kingdom) and 10 μg/mL ciprofloxacin, and maintained at 37°C and 5% (v/v) CO₂. The following day, cells were serum-starved for 24 hours to induce cell differentiation. Shortly before FSS experimentation, biocompatible silicone tubing (Ø0.8 mm, Ibidi GmbH, Planegg, Germany) was connected to a peristaltic pump (ISM931C, Ismatec, Wertheim, Germany) and the microfluidic channels. During microfluidic experiments serum-free DMEM/F-12 1:1 nutrient mixed media was used.

2.3 | ATP measurements

To assess the ATP release by mDCT15 cells due to FSS, cells were exposed during 1 minute to different physiologically relevant FSS levels (ie, 0.3, 0.6, and 1.2 dyn/cm²). Cells were also exposed to repetitive cycles of 0.3 dyn/cm² (1 min) followed by 1.2 dyn/cm² (1 min) with static intervals between
cycles of 1 to 25 minutes, thus simulating dynamic changes in the pro-urine flow.

To investigate the involvement of mTORC1 and pannexin-1 in FSS-modulated ATP release, cells were pre-incubated for 1 hour with rapamycin (100 nM, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and brilliant blue FCF (BB-FCF, 100 μM, Sigma-Aldrich, St. Louis, MO, USA), respectively. In this case, during microfluidic experimentation, media was supplemented with the corresponding inhibitor. Control cells were exposed to media supplemented with the corresponding vehicle (DMSO or MilliQ-water, for rapamycin or BB-FCF, respectively). Flow-through was collected after 1 minute of FSS exposure and used for the ATP measurement (Figure 1A). Cell morphology after exposure to different FSS conditions was monitored by brightfield microscopy (Figure 1B).

ATP levels were determined in the (flow-through) medium, total cell lysates, and urine using the ATPlite Luminescence Assay System (Perkin Elmer, Waltham, MA, USA) according to manufacturer’s instructions. A standard curve, ranging from 0.6 × 10⁶ to 2.5 × 10⁶ and 0.3 × 10⁶ to 10.0 × 10³ pmol/L ATP for intracellular and extracellular ATP, respectively, was used. All samples measured were within the confines of the standard curve used. ATP content in the medium and total cell lysates was corrected to the total volume and normalized to the total cell number in the corresponding channel or well. For in vitro experimentation, fold change of extracellular ATP for each condition to control is depicted. In vivo, ATP content in urine was normalized to urinary creatinine for human subjects and to urination to control is depicted. In vivo, ATP content in urine was corrected to the total volume and normalized to the total cell lysates, and urine using the ATPlite Luminescence Assay System (Perkin Elmer, Waltham, MA, USA) and brilliant blue FCF (BB-FCF, 100 μM, Sigma-Aldrich, St. Louis, MO, USA), respectively. All samples measured were within the confines of the standard curve used. ATP content in the medium and total cell lysates was corrected to the total volume and normalized to the total cell number in the corresponding channel or well. For in vitro experimentation, fold change of extracellular ATP for each condition to control is depicted. In vivo, ATP content in urine was normalized to urinary creatinine for human subjects and to urination volume collected in 24 hours for mice.

2.4 Acute water loading

Urine samples of healthy human volunteers were collected, for ATP measurement, after acute water loading experimentation. These samples were obtained from a previously performed study and supplemented with protease inhibitors at a final concentration of 50 μM PMSF, 20 μM aprotinin, 10 μM pepstatin A, and 20 μM leupeptin, and stored at −80°C. All volunteers were healthy males between the age of 25 and 35 years with a body weight lower than 100 kg. Subjects were asked to refrain from coffee, tea, and alcohol intake and exercise for 24 hours before and during the study. In 30 minutes, each subject ingested 20 ml/kg body weight of water. Midstream urine was collected before (t = 0) and after water ingestion in 30 minutes intervals for up to 150 minutes. Urine samples were immediately stored at −80°C.

2.5 Mouse experimentation

Inducible kidney-specific Pkd1 knockout mice (iKsp-Pkd1lox/lox) were used to assess the role of PC1, in vivo, in urinary ATP excretion. Tamoxifen was administered, via oral gavage, to iKsp-Pkd1lox/lox mice on postnatal days 18, 19, and 20 (PN18) to induce a kidney specific knockout of Pkd1 (iKsp-Pkd1del). iKsp-Pkd1lox/lox mice which received no tamoxifen were considered as age and genotype-matched controls. ATP measurements were performed as previously described. The expression of the following genes was assessed: Abcc6, Cx37, Entpd2, Entpd3, Nt5e, P2rx4, P2rx5, P2rx6, P2rx7, P2ry1, P2ry2, Panx1, and Ptgs2. Gapdh was used for each gene of interest as a reference gene and non-template controls were used during RTqPCR determinations as negative controls. Since all primers used had an efficiency of approximately 100% at the concentration (400 nM) used, the relative gene expression was analyzed using the Livak method (2−ΔΔCt) (Supporting Table S1). The RTqPCR procedures described here complied with the MIQE guidelines.

2.6 Gene expression analysis

To evaluate the effect of FSS on gene expression, cells in μ-slide VI channels were exposed during 3 hours to either no flow (=static) or 0.6 dyn/cm² FSS (Figure 4A). After experimentation, cells were lysed and RNA was isolated using the RNeasy mini kit according to manufacturer’s instructions (Qiagen, Hilden, Germany). cDNA synthesis using Molyne Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and RTqPCR, using SYBR green master mix (Bio-Rad Laboratories, Hercules, CA, USA) were performed as previously described. The expression of the following genes was assessed: Abcc6, Cx37, Entpd2, Entpd3, Nt5e, P2rx4, P2rx5, P2rx6, P2rx7, P2ry1, P2ry2, Panx1, and Ptgs2. Gapdh was used for each gene of interest as a reference gene and non-template controls were used during RTqPCR determinations as negative controls. Since all primers used had an efficiency of approximately 100% at the concentration (400 nM) used, the relative gene expression was analyzed using the Livak method (2−ΔΔCt) (Supporting Table S1). The RTqPCR procedures described here complied with the MIQE guidelines.

2.7 Fura-2-based Ca²⁺ imaging

Intracellular Ca²⁺ was measured using the ratiometric dye Fura-2. Press-to-Seal silicone isolators with adhesive 24 wells (Molecular Probes, Eugene, OR, USA) were attached to a 24 × 50 mm cover glass. The wells were coated with Poly-L-Lysine (Sigma-Aldrich, St. Louis, MO, USA) and washed with PBS. The procedures were performed in a Ca-HT buffer (4.2mM KCl, 132mM NaCl, 1mM MgCl₂, 1.4mM CaCl₂, 5.5mM glucose, and 10mM HEPES in MilliQ). Subsequent, 15 μL of cell suspension was added to each well and incubated for 1.5 hours. About 5 μL of medium was removed and 10 μL Fura-2-AM mixture (0.3% v/v Fura-2-AM, 0.015% v/v Pluronic acid (10% v/v in Ca-HT buffer) was added and incubated for 1 hr. For the conditions in which P2Y1 was
inhibited, MRS2500 (Tocris Bioscience, Bristol, United Kingdom) was added to the Fura-2 AM mixture at a concentration of 1 μM. One measurement per well was performed. The well was placed into a perfusion chamber mounted onto the stage of an inverted microscope (Zeiss Axiovert 200M, Carl Zeiss, Jena, Germany). Intracellular Ca$_{2+}$ was monitored...

**FIGURE 1**  Fluid shear stress increases ATP release in vitro and in vivo. A, Schematic overview of the in vitro microfluidic set-up used to measure cellular ATP release. B, Brightfield microscopy of mDCT15 cellular morphology before and after exposure to either 0.3 and 1.2 dyn/cm$^2$ FSS. C, Elevated FSS-modulated ATP release in mDCT15 cells exposed to 1 minute of 0.3, 0.6, and 1.2 dyn/cm$^2$ FSS (n = 4), significant difference (P < .05) denoted with symbol a. D-F, Attenuated FSS-modulated ATP release in mDCT15 cells after repeated 1 min sampling during exposure, for 5 min in total, to 0.3, 0.6, and 1.2 dyn/cm$^2$ FSS, respectively (n = 4). G, Recovery of FSS-modulated ATP release after static intervals of 1 to 25 min between 1 min FSS exposure periods (0.3 to 1.2 dyn/cm$^2$) (n = 4), significant differences between 0.3 and 1.2 dyn/cm$^2$ are denoted with symbols a, b and c. H, In vivo resemblance of FSS-modulated ATP release in healthy human subjects after acute water loading (n = 7), significant differences indicating elevated urinary ATP (from t = 0 to t = 60 and t = 90, respectively) are denoted with symbol a, whereas significant differences indicating a decreased urinary ATP (from t = 90 to t = 150) are denoted with symbol b. Values are presented as the mean ± SEM.
by exciting Fura-2 with monochromatic light of wavelength 340 and 380nm (Polychrome IV, TILL Photonics, Gräfelfing, Germany). Fluorescence emission light was directed by a 415DCLP dichroic mirror (Omega Optical, Brattleboro, VT, USA) through a 510WB40 emission filter (Omega Optical, Brattleboro, VT, USA) onto a CoolSNAP HQ monochrome CCD camera (Roper Scientific, Vianen, The Netherlands). The integration time of the CCD camera was set at 200ms with a sampling interval of 3 seconds. After 30 seconds of measuring, either ATP (100 μM) in Ca-HT, ATP (100 μM) + MRS2500 (1 μM) in Ca-HT or just Ca-HT was added to the mini-well. At each timepoint the 340/380nm ratio was calculated per region. To determine the maximal response, the delta peak was calculated by subtracting the baseline ratio from the maximum ratio for every cell. Only the responding cells were used for data analysis.

2.8 Immunocytchemistry

Primary cilia in mDCT15 cells were visualized as previously described.42 The following antibodies were used: primary antibody rabbit polyclonal anti-ARL13B (1:200, Proteintech, Rosemont, IL, USA) and secondary antibody Alexa Fluor 594-conjugated anti-rabbit IgG (1:250, Molecular Probes, Eugene, OR, USA). Nuclei were counterstained and cells mounted using DAPI-Fluoromount-G (ITK Diagnostics, Uithoorn, The Netherlands). Acquisition of images was performed using confocal laser scanning microscopy (FV1000, Olympus, Tokyo, Japan) equipped with a 60X oil-immersion objective. ARL13B-positive primary cilia (594 nm laser), identified as dots or small dashes on the xy-plane, and DAPI-positive nuclei (405 nm laser) were visualized. A z-stack with 0.25 μM distance between each focal plane was obtained. Images were processed using Fiji (https://fiji.sc/) software (National Institutes of Health, Bethesda, MD, USA).

2.9 sgRNA design

Guide RNA’s (sgRNA) targeting exon 9 of the *Mus musculus* gene *Pkd1* and exon 1 of the *Mus musculus* gene *Panx1* were designed using http://crispor.tefor.net/ (Supporting Table S1).43 The oligonucleotide pair for each sgRNA was phosphorylated and annealed using T4 Polynucleotide Kinase (New England Biolabs, Ipswich, MA, USA) in a thermocycler (37°C for 30 minutes, 95°C for 5 minutes and ramped down to 25°C with 5°C/ min). The plasmids PX458 (Addgene #48138)44 and PX333 (Addgene #64073)45 were linearized using BbsI, or BsaI, restriction enzymes (New England Biolabs, Ipswich, MA, USA) at 37°C for 1hr. The annealed oligonucleotides were ligated into the linearized PX458 or PX333 plasmid using T4 Ligase (New England Biolabs, Ipswich, MA, USA) overnight at 16°C. Next, the ligation mix was transformed by 42°C heat shock into TOP10F competent cells. The next day, colonies were picked and the PX458 or PX333 plasmid, containing the sgRNA, isolated. The PX333 was modified by inserting T2A-eGFP (derived from PX458) after NLS-Cas9-NLS resulting in the plasmid PX333-GFP, suitable for GFP-positive FACS sorting. The PX333 plasmid allows for the dual expression of sgRNA’s.

2.10 CRISPR/Cas9-mediated genome editing

mDCT15 cells were transfected with the PX458 (Addgene #48138,44 or PX333-GFP plasmid (modified from Addgene #64073),45 containing the sgRNA’s, using Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA, USA). GFP-positive cells were generated independently by either use of the PX458 or PX333-GFP plasmid. The targeted region of either *Ifi140* exon 8 or *Pkd1* exon 9 was amplified using the prepGEM DNA extraction kit (ZyGEM, Hamilton, New Zealand). The targeted region of either *Ifi140* exon 8 or *Pkd1* exon 9 was amplified using AmpliTaq GOLD (Thermo Fisher Scientific, Waltham, MA, USA) in a thermocycler (95°C for 5 min and 30 cycles of 95°C for 15 seconds, 58°C for 30 s, and 72°C for 30 s followed by a final elongation step of 72°C for 7 min). PCR products were run on a 2% (w/v) agarose gel to assess that one single amplicon was amplified for the PX458 transfected cells, as for the PX333-GFP transfected cells, multiple amplicons corresponding to wild type, hetero- or homozygous can be expected. Next, PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) using TA cloning and transformed into TOP10F competent cells. Plasmids containing an insert were sequenced, using sanger sequencing and SnapGene software (version 4.2.4, GSL Biotech LLC, Chicago, IL, USA), to assess each clone for homozygous frame shift mutations. The *Pkd1*”mDCT15 clones were validated by western blot (Figure 5A) using 4%-15% Criterion TGX Precast Gels (Bio-Rad Laboratories, Hercules, CA, USA), PVDF membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA), and the antibodies mouse anti-PC1 (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA) and peroxidase-conjugated sheep anti-mouse (1:10,000, Sigma-Aldrich, St. Louis, MO, USA). Blots were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA). Both *Pkd1*”mDCT15 clones used in this study were generated independently by either use of the PX458 or PX333-GFP plasmid. The *Ifi140*”mDCT15 cell line was
previously generated and validated by sanger sequencing and immunocytochemistry. 46

2.11 | Zebrafish experimentation

The effect of BB-FCF on cyst growth was studied in an established zebrafish model of PKD, where PKD is induced by the use of a translation blocking morpholino (MO) targeting the zebrafish ortholog of human PKD2 (pkd2). 47 Wild-type Tupfel long-fin zebrafish were used for experimentation. In brief, PKD was induced in zebrafish larvae by microinjecting one- to two-cell stage embryos with the following *pkd2*-MO: 5′-AGGACGAACGCActggagctc-3′. This MO has been previously validated as *pkd2*-morphants phenocopy *pkd2* mutants. 48 In parallel, control embryos were injected with a standard mismatch control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′), directed against a human β-globin intron mutation, at the amount of 100 μM. Non-treated fish were also included in our study. In this case, only the vehicle (MilliQ water) was added (same volume as *pkd2*-MO). In total, four experimental groups were realized: two groups microinjected with the *pkd2*-MO, exposed and non-exposed to BB-FCF; and two groups microinjected with the mismatch control MO, exposed and non-exposed to BB-FCF. At 5 days postfertilization (dpf) larvae were anesthetized with tricaine/Tris pH 7.0 solution. Using brightfield microscopy (20x magnification) mortality, pronephros cystic phenotype, curved tail and pericardial edema were assessed, and high-resolution images of larvae were obtained. Images were processed using Fiji (https://fiji.sc/) software (National Institutes of Health, Bethesda, MD, USA) (Supporting Figure S1A,B). All animal procedures detailed here were performed in accordance with national and international legislations and were approved by the ethical review committee of the Radboud University Nijmegen.

2.12 | Statistical analyses

Differences between groups were analyzed using an unpaired Student’s t test when two experimental groups were compared and a factor of variance was considered. When more than two experimental groups were considered, a one-way (one factor of variance) or a two-way ANOVA (two factors of variance) was applied, followed by the Tukey’s test for multiple comparisons. Data in the figures are expressed as mean ± SEM. In the case of the experiments performed in vitro, the mean is the average value of independent experiments (n = 3-4).

Each independent experiment consisted of at least two (in the case of ATP measurements) or three (in the case of gene expression measurements) replicates for each of the conditions tested. For in vivo experiments, the sample size is depicted in the figure legend. Differences in survival of zebrafish larvae were analyzed with the logrank (Mantel-Cox) test. *P < .05* was considered statistically significant. All statistical analyses were performed with Prism 8 (GraphPad, La Jolla, CA, USA).

3 | RESULTS

3.1 | Flow modulates ATP extrusion in vitro and in vivo

To assess whether FSS influences the cellular ATP release from renal cells, mDCT15 cells were exposed to physiological FSS (Figure 1A). Cell morphology and monolayer integrity were not affected by the culture conditions (Figure 1B). A significant elevated ATP release was observed in cells exposed to 1.2 dyn/cm² FSS for 1 minute compared to 0.3 dyn/cm² FSS (100 ± 75 vs 481 ± 127%, *P < .05*) (Figure 1C). ATP release attenuated when cells were exposed up to 5 minutes to these rates (Figure 1D-F). Cells exposed to cycles of alternating 1 minute exposures to 0.3 and 1.2 dyn/cm² followed by 1 minute rest displayed episodic peaks of ATP release when switching from 0.3 to 1.2 dyn/cm² FSS (100 ± 31 vs 220 ± 23% and 5 ± 1 vs 19 ± 4%, respectively, *P < .05*). Increasing the static interval to 25 minutes resulted in higher ATP release after applying FSS compared to a static interval of 1 minute (7 ± 5 vs 69 ± 11%, *P < .05*) (Figure 1G). To assess whether ATP extrusion is FSS-modulated in vivo, an acute water loading test in healthy human subjects was performed. Acute water loading significantly increased the urinary output, indicative of elevated urinary flow, resulting in higher urinary ATP excretion (27 ± 12 vs 2,426 ± 530 and 27 ± 12 vs 2,951 ± 696 pmol ATP/mmol creatinine for *t = 0* vs *t = 60* and *t = 0* vs *t = 90* minutes, respectively, *P < .05*) (Figure 1H). Overtime, urinary output decreased, resulting in lower urinary ATP excretion (2,951 ± 696 vs 901 ± 266 pmol ATP/mmol creatinine for *t = 90* vs *t = 150* minutes, respectively, *P < .05*).

3.2 | ATP extrusion not dependent on primary cilia

Studies have shown that FSS-modulated ATP release is primary cilia-dependent. 49 Therefore, *Ift140Δ/c* mDCT15 cells were used, where ciliogenesis is inhibited, to study the ATP extrusion upon FSS mechanosensation. 46 After staining for
the primary cilia-specific protein ARL13B, no primary cilia were detected in \textit{Ifi140}^{-/-} cells. Wild-type cells displayed primary cilia (Figure 2A). Cells exposed to 0.3 and 1.2 dyn/cm\textsuperscript{2} FSS exhibited similar FSS-modulated ATP release between wild-type and \textit{Ift140}^{-/-} cells (100 ± 34 vs 894 ± 107 and 100 ± 44 vs 770 ± 274%, respectively, \(P < .05\)) (Figure 2B).

### 3.3 mTORC1 inhibition amplifies flow-modulated ATP release

Because mTORC1 is inhibited in cells upon flow sensing, the role of mTORC1 in FSS-modulated ATP release was investigated.\textsuperscript{50} In detail, mDCT15 cells pre-incubated with 100nM rapamycin for 1hr and untreated cells were exposed to either 0.3 or 1.2 dyn/cm\textsuperscript{2} FSS. The FSS-modulated ATP release was significantly increased in rapamycin-treated cells as compared to untreated cells (963 ± 123 vs 147 ± 19 and 445 ± 60 vs 100 ± 34%, respectively, \(P < .05\)) (Figure 3A). Furthermore, the intracellular ATP levels in rapamycin-treated cells were significantly higher than untreated cells (144 ± 16 vs 100 ± 5 and 153 ± 7 vs 99 ± 7%, \(P < .05\)) (Figure 3B).

### 3.4 Flow regulates \(P2ry1\), \(P2rx5\), \(Entpd2\), and \(Entpd3\) expression

In order to investigate whether FSS affects the purinergic signaling in mDCT15 cells, wild-type cells were exposed to static fluid and 0.6 dyn/cm\textsuperscript{2} FSS for 3 hours. A significant increase in \(P2rx5\) and \(P2ry1\) mRNA was observed after exposure to 0.6 dyn/cm\textsuperscript{2} FSS when compared to static controls (267 ± 58 vs 101 ± 10 and 195 ± 26 vs 101 ± 10%, respectively, \(P < .05\)). No changes in mRNA expression of \(P2rx4\), \(P2rx6\), \(P2rx7\), and \(P2ry2\) were observed (101 ± 9 vs 119 ± 5, \(P = .16\); 100 ± 5 vs 119 ± 10, \(P = .16\); 102 ± 12 vs 95 ± 17, \(P = .77\); and 103 ± 18 vs 116 ± 15%, \(P = .61\), respectively) (Supporting Figure S2B-G). Expression of FSS-sensitive gene \(Ptgs2\) (Cox-2) was measured as positive control indicating the mRNA expression under FSS (131 ± 69 vs 660 ± 220%, \(P = .08\)) (Supporting Figure S2A).\textsuperscript{42} Significantly higher mRNA expression of \(Entpd2\) and \(Entpd3\) was detected after exposure to FSS, whereas the mRNA expression of \(Nt5e\) was not altered (105 ± 24 vs 533 ± 132%, \(P < .05\), 105 ± 24 vs 503 ± 104%, \(P < .05\) and 101 ± 9 vs 79 ± 10%, \(P = .20\), respectively) (Supporting Figure S2H-J). Since extracellular ATP is known to affect intracellular Ca\textsuperscript{2+} levels via purinergic receptors, mDCT15 cells were exposed to 100 \(\mu\)M extracellular ATP. Consequently, increased intracellular Ca\textsuperscript{2+} was observed 10 seconds after the ATP exposure. This response was significantly attenuated when cells were incubated with 10 \(\mu\)M MRS2500, a specific P2Y1 inhibitor (0.79 ± 0.02 vs 0.59 ± 0.03ΔPeak, \(P < .05\)) (Supporting Figure S3).\textsuperscript{51}

### 3.5 Pannexin-1 contributes to flow-modulated ATP extrusion

In order to identify the extrusion mechanisms gating FSS-modulated ATP release, mRNA expression of candidates mediating the ATP release was assessed after mDCT15 cells were exposed to either static or 0.6 dyn/cm\textsuperscript{2} FSS (Figure 4A). In virtue of their expression in kidney, pannexin-1, connexin-37,

![FIGURE 2](image-url) FSS-modulated ATP release is not dependent on the presence of primary cilia. A. Immunocytochemical observation of primary cilia, detected through ARL13B staining, in wild-type mDCT15 cells. In \textit{Ifi140}^{-/-} mDCT15 cells no primary cilia were observed. B. Elevated ATP release in both wild-type and \textit{Ift140}^{-/-} mDCT15 cells exposed to 1 min of 1.2 dyn/cm\textsuperscript{2} FSS as compared to 1 min of 0.3 dyn/cm\textsuperscript{2} FSS (n = 4). Values are presented as the mean ± SEM, significant differences (\(P < .05\)) are denoted with symbol a.
and Abcc6 were considered to regulate FSS-sensitive ATP excretion.\textsuperscript{52-54} A significant increase in \textit{Panx1} mRNA, but not \textit{Cx37} and \textit{Abcc6}, was observed in wild-type cells after exposure to 0.6 dyn/cm\textsuperscript{2} FSS when compared to static (297 ± 28 vs 111 ± 37\%, \textit{P} < .05, 112 ± 8 vs 101 ± 8\%, \textit{P} = .37 and 100 ± 4 vs 107 ± 6\%, \textit{P} = .42, respectively) (Figure 4B-D). To further investigate whether pannexin-1 channels mediate the FSS-rate sensitive ATP release, wild-type and \textit{Panx1}\textsuperscript{-/-} mDCT15 cells were exposed to 0.3 and 1.2 dyn/cm\textsuperscript{2} with or without 100 \textmu M brilliant blue FCF (BB-FCF). Via sanger sequencing a homozygous deletion starting in 5′UTR and ending in exon 1 (g.15,045,106_15,044,905del) was observed in \textit{Panx1}\textsuperscript{-/-} cells, effectively removing the start codon for Pannexin-1 (Supporting Figure S4). An increased ATP release was observed when wild-type cells were subjected to 1.2 dyn/cm\textsuperscript{2} FSS compared to non-exposed cells (586 ± 143 vs 100 ± 34\%, \textit{P} < .05) (Figure 5C). When \textit{Panx1}\textsuperscript{-/-} cells were exposed to 1.2 dyn/cm\textsuperscript{2} FSS, similar increased ATP release was observed when compared to wild-type cells (526 ± 302 vs 100 ± 42\%, \textit{P} = .13) (Figure 5E). Intracellularly, ATP levels were significantly increased in \textit{Panx1}\textsuperscript{-/-} vs wild-type cells (255 ± 56 vs 100 ± 5 and 211 ± 18 vs 100 ± 7\%, \textit{P} < .05) (Figure 5D,F). ATP release measured in \textit{Panx1}\textsuperscript{-/-} mDCT15 cells was not sensitive to varying FSS. mTOR inhibition, using 100 nM rapamycin, did not amplify FSS-modulated ATP release in these cells (100 ± 52 vs 120 ± 65, \textit{P} = .99 and 235 ± 113 vs 331 ± 150\%, \textit{P} = .91, respectively) (Figure 5G). In contrast with wild-type cells, treatment of \textit{Panx1}\textsuperscript{-/-} cells with 100 nM rapamycin had no significant effect on intracellular ATP (100 ± 24 vs 96 ± 22, \textit{P} = .99 and 92 ± 16 vs 100 ± 18\%, \textit{P} = .99, respectively) (Figure 5H). These data were reproduced in a second, independently generated, \textit{Panx1}\textsuperscript{-/-} clone (Supporting Figure S5).

3.7 | Pannexin-1 mediates increased basal ATP extrusion when polycystin-1 is dysfunctional

There is growing evidence indicating extracellular ATP may contribute to PKD pathogenesis.\textsuperscript{24} Therefore, we investigated the role of ATP in \textit{Pkd1}\textsuperscript{-/-} mDCT15 cells and \textit{iKsp-Pkd1}\textsuperscript{del} mice. Wild-type and \textit{Pkd1}\textsuperscript{-/-} mDCT15 cells were exposed to renal FSS-modulated ATP release. To this end, \textit{Pkd1}\textsuperscript{-/-} mDCT15 cells were generated and validated (Figure 5A). ARL13B-positive primary cilia were detected in \textit{Pkd1}\textsuperscript{-/-} cells (Figure 5B). ATP extrusion was observed when \textit{Pkd1}\textsuperscript{-/-} cells were exposed to 0.3 and 1.2 dyn/cm\textsuperscript{2} FSS. Significantly higher ATP release after 0.3 dyn/cm\textsuperscript{2} exposure in \textit{Pkd1}\textsuperscript{-/-} cells was observed vs wild-type cells (775 ± 250 vs 100 ± 34\%, \textit{P} < .05) (Figure 5C). When \textit{Pkd1}\textsuperscript{-/-} cells were exposed to 1.2 dyn/cm\textsuperscript{2} FSS, similar increased ATP release was observed when compared to wild-type cells (526 ± 302 vs 100 ± 42\%, \textit{P} = .13) (Figure 5E). Intracellularly, ATP levels were significantly increased in \textit{Pkd1}\textsuperscript{-/-} vs wild-type cells (256 ± 302 vs 100 ± 42\%, \textit{P} < .05) (Figure 5D,F). ATP release measured in \textit{Pkd1}\textsuperscript{-/-} mDCT15 cells was not sensitive to varying FSS. mTOR inhibition, using 100 nM rapamycin, did not amplify FSS-modulated ATP release in these cells (100 ± 52 vs 120 ± 65, \textit{P} = .99 and 235 ± 113 vs 331 ± 150\%, \textit{P} = .91, respectively) (Figure 5G). In contrast with wild-type cells, treatment of \textit{Pkd1}\textsuperscript{-/-} cells with 100 nM rapamycin had no significant effect on intracellular ATP (100 ± 24 vs 96 ± 22, \textit{P} = .99 and 92 ± 16 vs 100 ± 18\%, \textit{P} = .99, respectively) (Figure 5H). These data were reproduced in a second, independently generated, \textit{Pkd1}\textsuperscript{-/-} clone (Supporting Figure S5).
0.6 dyn/cm² FSS or a static environment. In line with elevated ATP release of \( \text{Pkd1}^{-/-} \) cells, significantly increased \( \text{Panx1} \) mRNA was observed in \( \text{Pkd1}^{-/-} \) vs wild-type cells under static conditions (776 ± 91 vs 101 ± 9%, \( P < .05 \)) (Figure 6A). mRNA of \( \text{Cx37} \) and \( \text{Abcc6} \) were not altered (105 ± 21 vs 79 ± 18 and 100 ± 4 vs 128 ± 25%, respectively, \( P > .05 \)) (Figure 6B-C). Similar results were obtained when cells were exposed to 0.6 dyn/cm² FSS (Supporting Figure S6). ATP release of \( \text{Pkd1}^{-/-} \) cells exposed to 1.2 dyn/cm² FSS was reduced when incubated with 100 μM BB-FCF as compared to non-incubated cells (48 ± 14 vs 105 ± 28%, \( P < .05 \)) (Figure 6D). Under static, ATP release was significantly increased in \( \text{Pkd1}^{-/-} \) vs wild-type cells (202 ± 22 vs 100 ± 28%, \( P < .05 \)). Incubation of \( \text{Pkd1}^{-/-} \) cells with 100 μM BB-FCF inhibited ATP extrusion in static conditions (114 ± 15 vs 202 ± 22%, \( P < .05 \)), whereas in wild-type cells no decrease in static ATP release was observed when exposed to 100 μM BB-FCF (79 ± 11 vs 100 ± 28%, \( P = .87 \)) (Figure 6E). In line with the in vitro findings, a significant higher urinary ATP excretion was observed in \( \text{iKsp-Pkd1del} \) mice compared to non-induced age and genotype-matched controls (2.05 ± 0.24 vs 0.81 ± 0.22 pmol/24 h, \( P < .05 \)) (Figure 6F). Furthermore, \( \text{iKsp-Pkd1del} \) mice showed increased renal \( \text{Panx1} \) mRNA compared to controls (141 ± 18 vs 100 ± 4%, \( P < .05 \)) (Figure 6G).
Our data indicate that, in PKD, purinergic signaling is dysregulated with respect to the healthy context. Therefore, inhibition of ATP release through pannexin-1 could attenuate the disease progression. This hypothesis was tested in a zebrafish PKD model. Renal cyst formation, the hallmark of PKD, was observed in zebrafish after inhibition of the ortholog of human PKD2, *pkd2*, one of the genes mutated in PKD. Typical *pkd2* morphant phenotype (cystic pronephros and curved tail) was observed in larvae injected with *pkd2*-MO at 5 dpf, while control fish (injected with control MO) did not display any morphological abnormality (Figure 7A-C). Conspicuously,
cyst growth in pkd2 morphants was attenuated in morphants exposed to 100 μM BB-FCF from 4hpf to 5dpf (Figure 7D), a treatment with no effects on control fish morphology (Figure 7B). Additionally, in pkd2 morphants treated with BB-FCF, increased survival was observed at 5dpf, compared to non-exposed pkd2 morphants (82% and 24%, respectively) (Figure 7E). pkd2 morphants exposed to BB-FCF displayed similar degree of cyst formation as untreated pkd2 morphants (Figure 7F), but cysts were significantly smaller in BB-FCF-exposed pkd2 morphants (319 ± 28 vs 119 ± 13%, P < .05) (Figure 7G). No changes in pericardial edema area or the number of curly tails, characteristic features of pkd2 morphants,47 were observed upon BB-FCF treatment (5 ± 1 vs 6 ± 1%, P = .49 for the pericardial edema area) (Figure 7H, I).
FIGURE 7  Inhibition of Pannexin-1 channels in a zebrafish PKD model results in decreased cyst growth. A and B, Phenotypic brightfield images of representative zebrafish larvae (5 days postfertilization) after treatment with control MO (A) or with control MO plus 100 μM BB-FCF (B) (20x magnification). C and D, Phenotypic brightfield images of representative zebrafish larvae (5 days postfertilization) after treatment with pkd2 MO (C) or with pkd2 MO plus 100 μM BB-FCF (D) (20x magnification). Zoomed-in image of pronephros cyst is depicted in image-box in left upper corner. (E) Survival percentage of zebrafish larvae (5 days postfertilization) upon treatment with control MO, with (100% survival) or without (100% survival) 100 μM BB-FCF administration, or upon treatment with pkd2 MO, with (82% survival) or without (24% survival) 100 μM BB-FCF. (F) Percentage of zebrafish larvae displaying a pronephros cyst after treatment of control MO with (n = 20) or without (n = 20) 100 μM BB-FCF administration and pkd2 MO with (n = 24) or without 100 μM BB-FCF (n = 14). (G) Relative pronephros cyst area in zebrafish larvae (5 days postfertilization) after treatment of pkd2 MO with (n = 15) or without (n = 10) 100 μM BB-FCF. (H) Pericard-sack area (pericardial edema) relative to the eye area of pkd2 morphants (n = 10) and pkd2 morphants treated with 100 μM BB-FCF (n = 15). (I) Percentage of zebrafish larvae displaying curled tail phenotype (black bars). (J) Degree of tail bending (curved tail) of pkd2 morphants (n = 10) and pkd2 morphants treated with 100 μM BB-FCF (n = 15). Values are presented as the mean ± SEM, significant differences (P < .05) are denoted with symbol a.
However, degree of tail bending was significantly reduced in *pkd2* morphants treated with BB-FCF (41.3 ± 3.2 vs 62.5 ± 4.7°, *P* < .05) (Figure 7J).

### DISCUSSION

This study demonstrates that the renal ATP-channel pannexin-1 is key for release of ATP from tubular epithelial cells toward the urinary compartment upon flow mechanosensation in physiological conditions and disease models of PKD. Moreover, our data show that inhibition of pannexin-1 attenuates the renal cyst growth, pointing to involvement of ATP excretion in PKD progression. These conclusions are substantiated by the following findings: i) *Panx1* expression is upregulated upon flow exposure in renal cells, and correlates with apical ATP release that is reduced when these cells are exposed to the specific pannexin-1 inhibitor BB-FCF; ii) *Panx1* expression and urinary ATP levels are higher in iKsp-*Pkd1*del vs control mice, correlating with increased ATP extrusion and *Panx1* expression in *Pkd1*−/− vs wild-type cells; iii) cyst growth is attenuated in a zebrafish model of PKD (*pkd2* morphants) exposed to pannexin-1 inhibitor BB-FCF.

Tubular flow stimulates the ATP secretion by mDCT15 cells. Importantly, the FSS magnitudes employed in our study (0.3 to 1.2 dyn/cm²) cover the expected physiological FSS for the nephron.55-58 We aimed to translate this in vitro phenomenon to the in vivo situation. In this sense, urinary ATP excretion was higher with increased urinary output, as showed by the acute water loading test performed in human healthy subjects. Conversely, when urinary output decreases, urinary ATP excretion was also reduced. In turn, variations in extracellular ATP will have effect on purinergic signaling.59 Purinergic ligands, including ATP, will interact with P2X/P2Y or P1 receptors, affecting kidney function, among others maintenance of electrolyte homeostasis.16,34,60 In line with this, our findings indicate FSS-dependent expression of several purinergic components such as *P2ry1*, *P2rx5*, *Entpd2* and *Entpd3*. Exposing mDCT15 cells to extracellular ATP elicited an intracellular Ca²⁺ response, indicating that these cells are purinergically active. Furthermore, mDCT15 cells are a relevant model to use in this study since the cyst formation in PKD originates mainly from CD but also the distal tubule, including the thick ascending limb of Henle’s loop and DCT.1,4 In addition, ATP released from DCT can have a profound effect, via purinergic signaling, on the CD.

A link between FSS-modulated ATP release and primary cilia was not observed in the mDCT15 cells used in this study. Previously, flow-stimulated ATP release has been shown to be primary cilia-dependent.33,49 However, primary cilia-independent ATP release has also been reported.61 These discrepancies may be attributed to differences in the mechanical stimulation by flow such as the presence or absence of a variable pressure on the cell surface.49 Our findings showed that FSS-modulated ATP release can occur without primary cilia, at least in mDCT15 cells.26

While FSS-modulated ATP release has effects on purinergic signaling and therefore on renal function, little is known about the molecular mechanism that regulates FSS-modulated ATP release. A link between mTORC1 and FSS was previously established in MDCK cells, in which primary cilia sensing of flow was implicated.50 Interestingly, when rapamycin-incubated mDCT15 cells were exposed to 0.3 or 1.2 dyn/cm² FSS, cellular ATP release was amplified. This finding suggests a regulatory role of mTORC1 in FSS-modulated ATP release. In addition, we observed an FSS-insensitive increase in intracellular ATP levels by rapamycin. This observation might be attributed to the stimulation of glycolysis by rapamycin, as previously observed in other cell models.52-64 We also observed an increase in intracellular ATP in *Pkd1*−/− cells compared to wild type. These observations are in line with a previous publication demonstrating enhanced glycolysis and increased intracellular ATP levels in *Pkd1*−/− cells and cystic kidney.55 In this regard, the absence of a further increase in intracellular ATP in *Pkd1*−/− cells exposed to rapamycin might be attributed to a saturated glycolytic capacity or a negative regulation mechanism absent in wild-type cells.

Among the plethora of cellular ATP extrusion mechanisms, pannexin-1, connexin-30 (Cx30), connexin-30.3 (Cx30.3), and connexin-37 (Cx37) can potentially mediate the urinary ATP excretion.32,52,53 Pannexins and connexins form transmembrane channels with strong apical expression in renal tubular epithelial cells.52,66 Previous studies showed that perfusion of tubules expressing Cx30, causes ATP release into the tubular lumen.67,68 Renal Cx30 expression is restricted to the connecting tubule and CD.53,69 In contrast, pannexin-1 and Cx37 are expressed ubiquitously along the nephron, whereas Cx30.3 is confined to CD.52,53 *Abcc6* mediates ATP release in liver and is expressed in kidney.54,70 In our study, only the expression of *Panx1* was significantly increased when cells were exposed to 0.6 dyn/cm². Though the duration of FSS exposure was different between gene expression and ATP experiments, these findings suggest that pannexin-1 could be the primary regulator of FSS-modulated ATP extrusion. Indeed, inhibition and knockout of pannexin-1 reduced FSS-modulated ATP release.

PC1 and PC2, have been postulated to play a key role in mechanosensation.12,71 We show that in a PKD mouse model, urinary ATP levels were increased compared to control mice, which has never been reported before.15 Interestingly, *Panx1* expression levels were higher in iKsp-*Pkd1*del vs control mice and these results were confirmed in *Pkd1*−/− cells. The increase in basal ATP release observed in *Pkd1*−/− cells was largely mediated via pannexin-1 since the pannexin-1 inhibitor BB-FCF significantly reduced this response. Importantly,
FSS did not enhance the ATP release in Pkd1−/− cells, corroborating the lack of mechanosensitive ATP extrusion in these cells. Dysfunctional PC1 also critically affects the transcriptome of kidney cells.⁷² In addition, increased expression of pannexin-1 in cyst lining cells of a mouse and rat PKD model has recently been reported.⁷⁰,⁷³ Under given stimuli (e.g., mechanostimulation, intracellular concentrations of certain ions, etc.), higher pannexin-1 expression may result in higher transport activity. Our findings suggest that higher urinary ATP levels in PKD could be explained by an upregulation of Panx1 expression, a feature that may be related to the PKD transcriptome.

Our findings are also in line with other studies showing elevated ATP release in primary PKD cells.¹⁴,²² Furthermore, mTORC1 inhibition in Pkd1−/− cells did not enhance FSS-modulated ATP release, suggesting that PC1 is required for mTORC1 to regulate FSS-modulated ATP release. Conspicuously, intracellular ATP was higher in Pkd1−/− cells, however inhibition of mTORC1 did not increase intracellular ATP. These findings indicate that without PC1, ATP dynamics, both intra- and extracellular, are altered.

Elevated extracellular ATP levels are likely independent of flow sensing in PKD. This is highlighted by our in vivo and in vitro PKD models (iKsp-Pkd1del mice and Pkd1−/− mDCT15 cells, respectively) where increased ATP extrusion was observed. In early PKD, hyperfiltration is common.⁷⁴ However, based on data displayed in the present study, this would not cause elevated ATP release since PC1 is required for FSS-modulated ATP extrusion. Strikingly, basal ATP extrusion is increased in Pkd1−/− cells and supported by elevated urinary ATP excretion in iKsp-Pkd1del mice. The elevated extracellular ATP can be detrimental to PKD progression.⁷⁵ This is indicated by studies showing elevated expression of P2Y2, P2Y6 and P2X7 in a rat model of PKD, and that expression of both P2X4 and P2X7 increases during progression of PKD.¹⁸,²¹ In addition, inhibition of P2X7 in a zebrafish PKD model reduced cyst formation.⁷⁶ Extracellular ATP has been shown to mediate chloride (Cl−) secretion through Ca²⁺ signaling.¹⁴ It is hypothesized that an elevated Cl− secretion contributes to cyst growth. In fact, it was shown that activation of Cl− channel TMEM16A, via P2Y2, promotes renal cyst growth.⁷⁷,⁷⁹ In PKD, increased extracellular ATP may be available via pannexin-1, but also the expression profile of purinergic receptors is shifted in cystic epithelia toward increased P2X4 and P2X7 prevalence, enlarging detrimental effects of ATP on disease progression. Reducing extracellular ATP content as therapeutic approach for PKD could prove to be an interesting endeavor. Based on our findings with the zebrafish PKD model, pannexin-1 emerges as novel target to prevent cyst growth in PKD. Unlike probenecid, which is a well-known pannexin-1 inhibitor,濮⁸⁰ the inhibitor used here, BB-FCF, has been demonstrated to be specific for pannexin-1 and does not target P2X7.⁸¹ Thus, our findings unequivocally indicate the contribution of pannexin-1 to cyst growth and suggest this channel as a druggable target for PKD therapy. Interestingly, pannexin-1 expression levels appear to be dramatically increased in pathological conditions compared to the healthy kidney making it an especially interesting therapeutic target.⁷³,⁸¹ BB-FCF, is considered safe to use. To be effective in humans, a higher daily dose of BB-FCF should be administered than the acceptable daily intake of 6mg/kg body weight/day set by the European Union.

**FIGURE 8** Polycystin-1 regulates pannexin-1 mediated purinergic signaling. (A) Variable pro-urinary flow is sensed and translated into a molecular signaling cascade involving the inhibition of mTORC1, increased function of PC1 and in turn activation or upregulation of pannexin-1 channels to release more ATP into the extracellular lumen. The extracellular ATP, and its hydrolysis products ADP, AMP, and adenosine, will evoke an activation of purinergic signaling. Long-term exposure to increased FSS results in the increased expression of various purinergic signaling components; that is, P2Y/P2X receptors and ectonucleotidases (ENTPDs). (B) In the case of PC1/PC2 loss-of-function, ATP release via pannexin-1 is increased followed by an augmented cyst growth. Inhibition of pannexin-1 channels (e.g., using BB-FCF) decreases the elevated ATP release and reduces subsequent cyst growth when PC1/PC2 function is impaired.
Food and Safety Authority. A potential side effect using BB-FCF may be blue skin coloration. Therefore, identifying structurally related compounds or other pannexin-1 inhibitors is warranted.

In summary, we present a novel mechanism for renal FSS-modulated ATP release through pannexin-1 channels regulated via PC1 (Figure 8). Our study strengthens the notion that extracellular ATP plays a key role in disease progression of PKD. Furthermore, our data suggest that inhibition of ATP release in PKD could reduce cyst growth. Thus, pannexin-1 is presented as novel therapeutic target in PKD.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interests.

AUTHOR CONTRIBUTIONS
EV, JR, RB, DP, FA, and JH designed research; EV, JR, CM, MR, and FA performed research; EV, CM, and MR contributed new reagents or analytic tools; EV, JR, CM, MR, RB, DP, FA, and JH analyzed data; and EV, JR, RB, DP, FA, and JH wrote the paper. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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