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Activation of Trpv4 reduces the hyperproliferative phenotype of cystic cholangiocytes from an animal model of ARPKD

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Abstract

Background & Aims—In polycystic liver diseases, cyst formation involves cholangiocyte hyperproliferation. In PCK rats, an animal model of autosomal recessive polycystic kidney disease (ARPKD), decreased $[Ca^{2+}]_i$ in cholangiocytes is associated with hyperproliferation. We recently showed Trpv4, a calcium-entry channel, is expressed in normal cholangiocytes and its activation leads to $[Ca^{2+}]_i$ increase. Thus, we hypothesized that pharmacological activation of Trpv4 might reverse the hyperproliferative phenotype of PCK cholangiocytes.

Methods—Trpv4 expression was examined in liver of normal and PCK rats, normal humans, and patients with autosomal dominant PKD (ADPKD) or ARPKD. Trpv4 activation effect on cell proliferation and cyst formation was assessed in cholangiocytes derived from normal and PCK rats. The *in vivo* effects of Trpv4 activation on kidney and liver cysts was analyzed in PCK rats.

Results—Trpv4 was overexpressed both at mRNA (8-fold), and protein (3-fold) levels in PCK-cholangiocytes. Confocal and immunogold electron-microscopy supported Trpv4 overexpression in the livers of PCK rats and ARPKD or ADPKD patients. Trpv4 activation in PCK cholangiocytes increased $[Ca^{2+}]_i$ by 30% inhibiting cell proliferation by ~25-50% and cyst growth in 3-D-culture (3-fold). Trpv4-siRNA-silencing blocked effects of Trpv4 activators by 70%. Trpv4 activation was associated with Akt phosphorylation and β -Raf and Erk1/2 inhibition. *In vivo*, Trpv4 activation induced a significant decrease in renal cystic area and a non significant decrease in liver cysts.

Conclusions—Taken together, our *in vitro* and *in vivo* data, suggest that increasing intracellular calcium by Trpv4 activation may represent a potential therapeutic approach in PKD.

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Keywords

calcium; PKD; cholangiocytes; Trpv4

INTRODUCTION

In the polycystic liver diseases, genetic defects initiate the formation of hepatic cysts, which arise from cholangiocytes.¹⁻⁸ Specifically, ARPKD is characterized by biliary dysgenesis (i.e., ductal plate malformation, hepatic fibrosis and cyst formation) that becomes progressively more severe with age.^{1, 6, 9-12} Genetic studies indicate that mutations in *PKHD1* gene are responsible for ARPKD pathogenesis. *PKHD1* encodes a protein, fibrocystin/polyductin, with unknown functions.^{13, 14} ADPKD is the result of mutations in either PKD1 or PKD2, encoding polycystin-1 and -2, respectively.

At least 3 processes likely contribute to cyst development and expansion: (1) cholangiocyte hyperproliferation; (2) cell-matrix interactions; and (3) accelerated fluid transport. Different factors likely control these processes and promote cyst growth;^{1-8, 12} one of them is adenosine 3',5'-cyclic monophosphate (cAMP), an intracellular second messenger that influences cholangiocyte proliferation and secretion. Moreover, cAMP induces proliferation of cystic cells by activation of the B-Raf/MEK/ERK signaling pathway.¹⁵⁻¹⁹ We recently reported that cystic cholangiocytes have increased intracellular cAMP and that pharmacological inhibition of cAMP with octreotide, a somatostatin analogue, reduces cAMP levels, inhibits cholangiocyte proliferation, and retards cyst development *in vitro* and *in vivo*.⁸

Another important factor that affects cholangiocyte proliferation is $[Ca^{2+}]_i$.^{19, 21} It has been shown that both cystic renal epithelial cells and cystic cholangiocytes have decreased $[Ca^{2+}]_i$ levels.^{16, 18-20} We and others reported that a $[Ca^{2+}]_i$ elevation by the calcium ionophore, A23187, suppresses the mitogenic effect of cAMP on cell proliferation via activation of PI3K/Akt and inhibition of the B-Raf/ErK pathway.^{18, 19} Therefore, the restoration of $[Ca^{2+}]_i$ levels in cystic cells may represent a potential therapeutic approach in PKD.

We also recently demonstrated that the calcium entry channel, Trpv4, is expressed in normal cholangiocytes and its activation increases $[Ca^{2+}]_i$.²² This channel was also reported to regulate calcium responses in kidney epithelial cells.²³ Trpv4 responds to a wide variety of physical, thermal, and chemical stimuli, including osmolarity, low pH, citrate, the synthetic phorbol-derivative, 4 α -phorbol 12,13-didecanoate (4 α PDD),²⁴ and the recent developed potent and specific Trpv4 activator, GSK1016790A.²⁵ Arachidonic acid (AA) is an endogenous activator of Trpv4 and acts via the cytochrome P450 (CYP) epoxygenase-dependent formation of epoxyeicosatrienoic acids (mainly 5',6'-EET) that directly activates the channel.²⁶ In addition, nifedipine is able to increase the expression of CYP450, enhancing AA metabolism to 5',6'-EET and subsequently activating Trpv4.²⁷

Thus, we hypothesized that pharmacological activation of Trpv4 might restore the reduced $[Ca^{2+}]_i$ levels in cystic cells and thereby decrease proliferation and cyst growth. In the present work, we found that cholangiocytes from the PCK rat (an animal model of ARPKD) and from patients with ARPKD and ADPKD overexpress Trpv4 and that its activation increases levels of $[Ca^{2+}]_i$, suppressing cell proliferation and cyst growth *in vitro*, by a mechanism involving activation of Akt and inhibition of the B-Raf/ERK1/2 signaling pathway. *In vivo*, a specific Trpv4 activator, GSK1016790A, significantly decreases renal but not hepatic cystic areas.

RESULTS

Trpv4 is overexpressed in PCK rat cholangiocytes

As shown in Figure 1A, primary cultured PCK cholangiocytes overexpressed *Trpv4* at mRNA levels by 8 times compared to normal cholangiocytes. Protein levels of Trpv4 were also upregulated ~3 times in freshly isolated PCK bile ducts, as well as in cultured PCK rat cholangiocytes, PCK-CCL (Figure 1B). Confocal microscopy confirmed the overexpression of Trpv4 in PCK rat liver (Figure 2A). While in normal ducts Trpv4 is mainly localized to cholangiocyte primary cilia (as we reported),²² in PCK cholangiocytes, Trpv4 is predominantly expressed intracellularly (Figure 2A). Consistent with this observation, more Trpv4 immunoreactivity was observed in cholangiocytes of human patients with ARPKD or ADPKD than in normal (Figure 2A). To further analyze the site of Trpv4 expression, immunogold-electron microscopy was performed. By this approach, and consistent with confocal immunofluorescence microscopy and western blot, more immunogold particles were observed in cholangiocytes of PCK rats (86 ± 12) compared to normal (18 ± 3) (Figure 2B, C). Moreover, in normal rats, the particles were predominantly localized to the apical domain, while in PCK rats; the majority of them were intracellular (Figure 2B, C). To further explore Trpv4 expression, scanning immunogold-electron microscopy was performed. By this technique we detected, as previously reported,²² substantial Trpv4 expression on primary cilium as well as on the apical membrane of normal bile ducts. In contrast, PCK bile ducts showed no Trpv4 staining on primary cilia (Figure 2D). In order to confirm the apparent Trpv4 mislocalization, Trpv4-pEGFP was expressed in NRCs and PCK-CCL. While NRCs showed a predominant ciliary localization of the Trpv4-EGFP fusion protein, PCK-CCL presented a more diffuse, intracellular localization with no ciliary expression (Figure 2E).

Trpv4 activators increase intracellular calcium levels

To test if Trpv4 activation induces an increase in $[Ca^{2+}]_i$ levels, PCK cholangiocytes were incubated for 24 hrs with the following activators: (i) 4aPDD, (ii) 5',6'-EET, or (iii) combination of nifedipine and AA. Our data show that treatment with different concentrations of 4aPDD increases $[Ca^{2+}]_i$ levels in a dose-dependent manner (Figure 3A). The additional Trpv4 activators, 5',6'-EET and combination of nifedipine with AA (Nif+AA), increased $[Ca^{2+}]_i$ as well (Figure 3B). Short term $[Ca^{2+}]_i$ response to Trpv4 activators, 30 μ M 4aPDD and 300 nM GSK1016790A, were evaluated in NRCs and PCK-CCL by ratiometric analysis of fura-2 fluorescence. Both NRCs and PCK-CCL responded with an increase in $[Ca^{2+}]_i$ after Trpv4 stimulation; however, normal cells showed a delayed response compared to PCK cells (Figure 3C).

Trpv4 activators decrease cholangiocyte proliferation

To analyze the effect of Trpv4 activation and the subsequent increase in $[Ca^{2+}]_i$ on the rate of proliferation, PCK-CCL were cultured in the presence or absence of 4aPDD, 5',6'-EET, Nif+AA or GSK1016790A. We found that in response to all activators, the rate of cholangiocyte proliferation was reduced by ~20%-50%. In contrast, Trpv4 activation did not affect the rate of cell proliferation in NRCs (Figure 4).

Trpv4 activators decrease cyst growth

To test the effect of Trpv4 activation on the growth of cystic structures, freshly isolated PCK bile ducts were grown for 3 days in 3-D culture under different conditions. In the absence of activators, PCK cysts expanded in size ~10 times at day 3 compared to day 0. Treatment with 4aPDD significantly reduced cyst growth in a dose dependent manner (Figure 5A, B). In the presence of 5',6'-EET and Nif+AA, cyst growth was significantly decreased as well. The

growth of cystic structures formed by normal bile ducts was not significantly affected by Trpv4 activation (Figure 5A, B).

4αPDD decreases cyst growth in a Trpv4-dependent manner

To study the specificity of Trpv4 activation on hepatic cystogenesis, we examined cyst expansion in 3D-culture in the presence of scrambled or Trpv4-siRNAs. The siRNA reduced Trpv4 protein by 80% (Figure 6A). Cyst growth was decreased by ~50% in response to 4αPDD when bile ducts were pre-treated with scrambled siRNA (Figure 6B). In contrast, 4αPDD did not affect cyst growth when bile ducts were preincubated with the specific Trpv4-siRNA (Figure 6B), suggesting that Trpv4 plays an important role in this process.

4αPDD and GSK1016790A activate Akt and decrease the activity of B-Raf/Erk signaling pathway

We recently showed that cAMP-induced proliferation of PCK cholangiocytes is inhibited in response to calcium elevation by the ionophore A23187. Moreover, this process was associated with PI3K and Akt activation and with decreased Erk phosphorylation.¹⁹ Thus, to test if the $[Ca^{2+}]_i$ elevation by Trpv4 activation proceeds by the same mechanisms, we analyzed the status of Akt, B-Raf and Erk in response to 4αPDD and GSK1016790A treatment. Consistent with our previous observations, we found that the pAkt/tAkt ratio was increased (Figure 7A), while the activity of B-Raf and the pErk/tErk ratio were reduced (Figure 7B, C), suggesting the B-Raf/Erk axis is inhibited by the Ca^{2+} /PI3K/Akt pathway. The pErk/tErk and pAkt/tAkt ratios were not significantly affected by Trpv4 activation in NRCs.

Effect of Trpv4 activation on cyst progression in vivo

To further explore the concept of intracellular calcium restoration by Trpv4 activation as a potential approach for cyst growth retardation, we tested the effect of GSK1016790A in the PCK rat. As previously reported, GSK1016790A is lethal at a 0.3 mg/kg b.w.;²⁸ therefore we used a very low sublethal dose (0.01mg/kg b.w.). We found a significant reduction in the renal cyst area (by 28.4%) and renal fibrosis (by 58.3%); however, the decreases in liver cyst area and fibrosis (by 11.5% and by 15%, respectively) were not statistically significant, suggesting that at the low dose only kidney cystic cells are responsive to the drug (Figure 8). The treatment did not affect the serum biochemistry (supplemental Table).

DISCUSSION

The key findings reported here relate to the role of the calcium entry channel, Trpv4, as a potential target to decrease cyst growth. The data suggest that Trpv4 is overexpressed in cholangiocytes from the PCK rat and PKD human livers, and its pharmacological activation increases intracellular calcium levels, which as we reported is reduced in cystic cholangiocytes. Moreover, the increase in intracellular calcium levels induced by Trpv4 activation decreased cell proliferation and cyst growth *in vitro* and diminished cyst growth *in vivo*, by a mechanism involving Akt and B-Raf/Erk1/2 signaling pathway.

It is now well established that in the PKDs, genetic mutations of ciliary proteins result in defective intracellular calcium homeostasis, decreasing intracellular calcium levels with a permissive effect on cAMP-induced proliferation.²¹ The first example of a therapeutic strategy assessing the role of calcium elevation in PKD was recently published;²⁹ treatment of murine model of ADPKD with triptolide, an active diterpene used in traditional Chinese medicine, arrested cell proliferation and inhibited renal cyst formation. Triptolide targets the calcium channel, polycystin-2, increasing $[Ca^{2+}]_i$ concentrations. To date, no studies have been published targeting $[Ca^{2+}]_i$ in liver cystogenesis. A second strategy to increase intracellular calcium was the use of calcimimetics, which function as agonists of the calcium-sensing

receptor (CaR), a G-protein coupled receptor activation of which induces calcium mobilization from intracellular stores. There are two recently published studies assessing the use of calcimimetics in PKD; one with negative results,³⁰ while the other established that CaR modulation may inhibit late-stage cyst growth.³¹ However, the lack of expression of these receptors in biliary epithelia³⁰ suggests that the use of calcimimetics for the treatment of liver cysts will not be effective.

In the work reported here, we proposed Trpv4 as a target to increase intracellular calcium levels of cystic cells, and subsequently reduce cyst development. Trpv4 belongs to the vanilloid subfamily of the transient receptor potential channels that function as integrators of physical and chemical stimuli. This Ca^{2+} -permeable channel functions as an osmosensor, being activated by extracellular hypo-osmolarity and inhibited by extracellular hyper-osmolarity.³²⁻³⁷ In previous work, we found that in normal cholangiocytes, Trpv4 is localized in primary cilia and functions as an osmosensor. The activation of Trpv4 by luminal hypo-osmolarity induces an increase in intracellular calcium leading to bicarbonate secretion mediated by a mechanism involving luminal ATP release and purinergic receptors.²² In the present work, we found that Trpv4 is upregulated in cholangiocytes of an animal model of ARPKD and widely distributed in the cell, being present on the apical and basolateral membrane as well as heavily expressed intracellularly. Moreover, our limited data in patients with ARPKD and ADPKD suggest similar Trpv4 overexpression in cystic cholangiocytes. Why Trpv4 is overexpressed remains to be elucidated, but the overexpression and mislocalization of key receptors and transport proteins is a common feature in polycystic diseases.³⁸

At first glance, the overexpression of Trpv4 should be associated with increased intracellular calcium level.³⁹ However, the predominant intracellular localization of the channel in PCK cholangiocytes may reduce the contribution of Trpv4 to basal intracellular calcium levels. Also, the plasma, bile and cystic fluid hyper-osmolarity and basal pH in PCK rats compared to normal (data not shown), may function as inhibitory stimuli for Trpv4 channels. Interestingly, increased pH values were observed in liver cyst fluid from ADPKD patients.⁴⁰ Finally, in the absence of Trpv4 activation, we observed no differences in areas of cystic structures formed by PCK cholangiocytes in 3-D culture treated with scrambled or specific Trpv4 siRNAs, consistent with the notion that Trpv4 is not participating in the regulation of basal $[\text{Ca}^{2+}]_i$ levels.

Why the levels of intracellular calcium are decreased in cystic cells is unknown. Different factors could be responsible for this phenomenon. In the animal model of ARPKD, the PCK rat, cystic cells have abnormal and dysfunctional cilia deprived of fibrocystin.⁴¹ Fibrocystin interacts with the calcium-modulating cyclophilin ligand (CAML),⁴² suggesting that ciliary fibrocystin may contribute to intracellular calcium regulation. Also, fibrocystin was recently shown to be involved in the calcium signaling induced by mechanosensation. Furthermore, its inhibition by an extracellular antibody inhibited intracellular calcium responses.⁴³ In ADPKD, genetic mutations in either polycystin-1 and -2 lead to defects in the intracellular trafficking of calcium 21. Even though polycystin-2 expression seems to be unaltered in a human-derived ARPKD cell line,⁴⁴ recent reports showed that this calcium channel is regulated by the direct interaction with fibrocystin.^{45, 46} Finally, both polycystin-2 and Trpv4 interact with the inositol 1,3,5-trisphosphate receptor, a key regulator of cholangiocyte intracellular calcium levels.⁴⁷ Therefore, malfunction of these cilia-associated proteins (i.e. polycystin-1, polycystin-2 or fibrocystin) may cause disruption of calcium homeostasis.

In the present study, the specific Trpv4 activators, 4αPDD and GSK1016790, as well as other activators (i.e., combination of AA and nifedipine or the AA metabolite, 5',6'-EET) increased intracellular calcium in cystic cholangiocytes. These observations, together with the fact that Trpv4 silencing inhibits the effect of 4αPDD on cyst growth, strongly suggest that the

intracellular calcium elevation and the associated decreased cholangiocyte proliferation and cyst growth are the result of specific Trpv4 activation. Interestingly, Trpv4 and polycystin-2 form a functional mechanosensory complex in the kidney, where Trpv4 is essential for the calcium signaling induced by flow.²³ Thus, Trpv4 activators may also be useful in ADPKD, mainly resulting from PKD2 mutations, where triptolide would be not effective.²⁹

We found that the restoration of intracellular calcium in cystic cholangiocytes is associated with Akt activation and inhibition of B-Raf and Erk1/2. These observations are consistent with previous reports showing that elevation in intracellular calcium activates Akt in a PI3K-dependent manner subsequently inhibiting B-Raf.^{15, 16, 18, 19, 48, 49}

The overexpression of Trpv4 makes this channel an attractive candidate for *in vivo* activation using systemic Trpv4 agonist administration. Nevertheless, *in vivo* Trpv4 stimulation should be done with caution since hyper-activation leads to negative secondary effects, like induction of Trpv4-dependent hyperalgesia^{50, 51} or cardiovascular disorders.²⁸ In the present work we tested the *in vivo* effect of the novel Trpv4 activator, GSK1016790A. This activator induces an acute circulatory collapse when administered at 0.3 mg/kg.²⁸ Therefore, our study was limited to a sublethal dose of 0.01 mg/kg. At this dose, we found a significant decrease in both kidney cystic areas and fibrosis, but no significant effects on liver cystogenesis. These may be due to insufficient cholangiocyte Trpv4 activation at the tested dose. However, the deleterious effect of Trpv4 activation on the circulatory system hampered the possibility of using higher doses of GSK1016790A. Other potential factors such as more effective calcium ATPases that actively pump calcium out from the cytosol to the extracellular space or back into the calcium stores may explain the differences in kidney and liver responses and are currently under study in our group. Therefore, we believe that different and/or less toxic Trpv4 activators or the combinatory targeting approach of both intracellular calcium and cAMP may be of most benefit to decrease cyst growth.

In summary, we showed for the first time that Trpv4 is overexpressed in PKD cholangiocytes. The pharmacologic activation by four different Trpv4 agonists restores intracellular calcium levels subsequently decreasing proliferation and cyst growth via a mechanism involving Akt activation and inhibition of B-Raf/Erk pathway. Taken together, our *in vitro* and *in vivo* results have identified a novel target for decreasing cystic cell growth and support the notion that the restoration of intracellular calcium is a potential tool in reducing cyst progression. Our work also provides the rationale for the development of combined therapeutic strategies (i.e., reduction of cAMP and increase of intracellular calcium) for the treatment of PKD including Trpv4 activation.

Materials and Methods

Animals and models

Wild-type Sprague–Dawley and PCK rats (225–250 g) were maintained on a standard diet. All experimental procedures were approved by the Animal Use and Care Committee of the Mayo Clinic. Animals were anesthetized with pentobarbital (50 mg/kg bw i.p.). Livers were harvested, fixed in 10% formaldehyde, and embedded in paraffin for histology. We used cell lines derived from normal and PCK rats: NRCs and PCK-CCL, respectively,⁵² as well as cholangiocytes in primary culture isolated from normal and PCK rats.¹⁹ Freshly isolated bile ducts were used for 3D-culture experiments and protein expression analysis. Cells and bile ducts were incubated on forskolin-containing media (NRC media, supplemental information). For *in vivo* experiments, three-week-old PCK rats were injected daily intraperitoneally during 8 weeks with 0.01 mg/kg bw GSK1016790A (Sigma-Aldrich) or vehicle (5% DMSO, 10% cremophor, in saline). Cystic area in liver and kidney and hepato-renal fibrosis were assessed as previously described⁸ (supplemental information).

Human samples

Paraffin blocks from five normal, three ARPKD and three ADPKD patients were obtained from Mayo Clinic Tissue Registry Archives. All experimental procedures were approved by the Mayo Clinic Institutional Review Boards (IRB: 08-005681).

RT-PCR

Total RNA was obtained from primary cultured normal and PCK rat cholangiocytes with TRIZOL reagent (Invitrogen, Carlsbad, CA). Quantification of mRNAs were carried out by real-time qPCR using specific primers for TRPV4²² and 18S rRNA as normalizing control.

3-D culture system

Freshly isolated normal and PCK bile ducts ranging in diameter between 40 and 100 μ m were grown in 3-D culture as described¹⁹ (supplemental information). For siRNA studies, bile ducts were pre-incubated for 24 for hours with scrambled or Trpv4 siRNA²² prior adding the activators.

Measurement of intracellular Ca²⁺

Intracellular calcium levels were compared as previously described.^{19, 22} Briefly, PCK cholangiocytes cultured on 96-well microplates were incubated for 24 hours in the presence of different Trpv4 activators and then were loaded with 3 μ M fluo-4-AM. After washing, fluorescence was read in a microplate reader at 480 and 540 nm wavelengths for excitation and emission, respectively. Fluo-4 fluorescence is expressed as increase percentage compared to control conditions. We also measured intracellular calcium by ratio imaging (Supplemental information).

Proliferation assay

proliferation assays were carried out using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega).

B-Raf activity

The activity of B-Raf kinase was measured employing the commercial available kit IP Kinase Activity Assay Kit (BioSource Intenational).

Western Blots

Protein fractions were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, blots were incubated overnight at 4°C with one of the following antibodies: Trpv4 (1:5000, Alomone Labs Ltd.), Erk (1:2000, Abcam Inc.), p-Erk (1:1000, BD Biosciences), Akt (1:1000, Sigma), p-Akt (1:1000, Santa Cruz Biotechnology); washed and incubated 1 h at room temperature with HRP conjugated corresponding secondary antibody (1:5000 dilution). For protein detection, ECL system was employed.

Immunofluorescence confocal microscopy

Liver sections were incubated with antibodies against acetylated α -tubulin (1:500, Sigma-Aldrich) and Trpv4 (1:100) o/n at 4°C followed by incubation for 1 h with fluorescent secondary antibodies (1:100). Nuclei were stained with 4',6-diamino-2-phenylindole. For human liver staining a sheep polyclonal antibody to TRPV4 was used (1:100, Abcam Inc.). For Trpv4-EGFP expression analysis, NRC and PCK-CCL were transfected with the Trpv4-EGFP plasmid⁵³ using Fugene (Roche). After five days of incubation in NRC media without

serum, cells were fixed and staining for the ciliary marker acetylated α -tubulin and ciliated cells were analyzed under the confocal microscopy.

Immunogold electronmicroscopy

Normal and PCK livers were perfused with 4% paraformaldehyde in 0.1 mo/L PBS and processed as previously described.⁵⁴ Thin cryo sections (60 nm) were cut with a Leica cryomicrotome (Leica Microsystem Inc., Bannockburn, IL), and Trpv4 immunolabeling was performed and analyzed as previously described²² (supplemental information).

Immunogold scanning electron microscopy

Isolated bile duct units were fixed and processed as previously described,⁵⁵ (supplemental information)

Statistics

Data are expressed as mean $\pm$ SE. Statistical analyses were performed by One-way ANOVA with Bonferroni posthoc test to compare more than two groups and by the Student t test to compare two groups. Results were considered statistically different at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ARPKD	autosomal recessive polycystic kidney disease
ADPKD	autosomal dominant polycystic kidney disease
IBDs	intrahepatic bile ducts
TRPV	transient receptor potential vanilloid
NRCs	normal rat cholangiocytes
4 α PDD	4 α -phorbol 12,13-didecanoate
AA	arachidonic acid
EET	epoxyecosatrienoic acid

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Figure 1. Trpv4 is overexpressed in PCK cholangiocytes: qPCR and western blot

A, Quantitative RT-PCR for Trpv4 on primary cultured cholangiocytes from normal and PCK rats (n=5). **B**, Representative western blot showing overexpression of Trpv4 in freshly isolated bile ducts from normal and PCK rats (n=5) and in cultured NRC and PCK-CCL (n=3). Data are shown as percentage of actin-normalized Trpv4 band compared to normal. *p<0.05.

Figure 2. Trpv4 is overexpressed in cystic cholangiocytes: confocal immunofluorescence and immunogold electron microscopy

A, Confocal immunofluorescence images showing expression of Trpv4 in normal and PCK rats and in normal and ARPKD and ADPKD human liver samples (L, lumen; Trpv4 in green; acetylated α -tubulin in red; DAPI nuclear staining in blue). Original magnification 1000X (bars, 10 μ m). **B** and **C**, Immunogold electron microscopy confirmed Trpv4 overexpression and showed its localization on apical and basolateral domains. Intracellular Trpv4 is significantly increased in PCK rat livers, while in normal liver gold particles were mainly on the apical domain. Bars, 500 nm; * $p < 0.05$. **D**, immunogold scanning electron microscopy of the apical surface of normal bile ducts and PCK cysts showed loss of ciliary Trpv4 in cystic

cells. SEM, scanning electron microscopy; IEM immunogold electron microscopy. **E**, NRC and PCK-CCL cholangiocytes transfected with Trpv4-EGFP were stained with the ciliary marker acetylated α -tubulin (ac- α -tubulin, in red). Ciliary Trpv4-EGFP fluorescence was only found in NRC. Original magnification 1000X.

Figure 3. Trpv4 activation increases intracellular calcium levels in PCK cholangiocytes
A, 4αPDD induced intracellular calcium increases in a dose dependent manner. **B**, the alternative Trpv4 activators 5',6'-EET and nifedipine (Nif) + arachidonic acid (AA) also increased intracellular calcium levels. (* $p < 0.05$, $n = 10$). **C**, Fura-2 ratiometric analysis of NRCs and PCK-CCL treated with 4αPDD and GSK1016790A (GSK). The figures show the average traces of fura-2 340/380 ratio over the time.

Figure 4. Effect of Trpv4 activation on cholangiocyte proliferation

A, Effect of Trpv4 activation by 4αPDD, the Nif+AA combination, 5',6'-EET, and GSK1016790A on cell proliferation (n=10 for each activator) were analyzed by MTS assay over 3 days of culture. Data show the decrease in proliferation induced by the Trpv4 activators compared to control vehicle-treated cells. *p<0.05.

Figure 5. Effect of Trpv4 activation on cystogenesis

A, Representative images of cystic structures formed by PCK and normal bile ducts in the absence (Control) or presence of different Trpv4 activators: 5 μ M 4 α PDD, 0.5 μ M 5',6'-EET (EET) and 1 μ M Nifedipine + 1 μ M AA (Nif+AA). Original magnification 40X **B**, Quantitative assessment of circumferential areas of cystic structures after 3 days of incubation showed that Trpv4 activation impaired PCK cyst expansion. Data are expressed as fold-increase compared to day 0. * p <0.05.

Figure 6. 4αPDD inhibits cyst growth in 3D-culture in a Trpv4-dependent manner

A, western blot of PCK cholangiocytes treated with scrambled (scr) or Trpv4-siRNA showed 80% decrease of Trpv4 protein induced by specific siRNA treatment. **B**, Cystic structures formed by PCK bile ducts where treated with a specific Trpv4-siRNA and then incubated in the absence or presence of 5 μM 4αPDD. Scrambled siRNA (scr) was used as a control. Quantitative assessment of circumferential areas of cystic structures after 3 days of incubation showed that Trpv4 is essential for the inhibition of cystogenesis induced by 4αPDD. Data are expressed as fold-increase compared to day 0. *p<0.05.

β -Raf Activity (%)

Figure 7. Trpv4 activation is associated with Akt activation and inhibition of active Erk and B-Raf
NRC and PCK-CCL cells were treated with 4αPDD or GSK1016790A (GSK) and the activities of Akt, B-Raf and Erk were assessed. **A**, representative western blots of phospho- and total Akt isoforms and densitometric analysis showed that Trpv4 activation induced Akt activity in the PCK cells. **B**, PCK-CCL B-Raf activity, expressed as percentage of decrease compare to control. **C**, representative blots for phospho- and total-Erk isoforms and densitometric analysis suggested that Trpv4 activation induced a decrease in Erk activity in the PCK cells. Data represent percentage of ratio compared to each control (n=4). *p<0.05.

Figure 8. Effect of Trpv4 activation on cyst progression *in vivo*

A, representative liver and kidney sections stained with picrosirius red from PCK rats treated with vehicle (n=6) or GSK1016790A (n=4). Bar, 2500 μ m **B, C** quantification analysis of cystic and fibrotic area expressed as percentage of total parenchyma area. *p<0.05.