Hypoxia-Inducible Factor-1α Causes Renal Cyst Expansion through Calcium-Activated Chloride Secretion

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ABSTRACT
Polycystic kidney diseases are characterized by numerous bilateral renal cysts that continuously enlarge and, through compression of intact nephrons, lead to a decline in kidney function over time. We previously showed that cyst enlargement is accompanied by regional hypoxia, which results in the stabilization of hypoxia-inducible transcription factor-1α (HIF-1α) in the cyst epithelium. Here we demonstrate a correlation between cyst size and the expression of the HIF-1α-target gene, glucose transporter 1, and report that HIF-1α promotes renal cyst growth in two in vitro cyst models—principal-like MDCK cells (pIMDCKs) within a collagen matrix and cultured embryonic mouse kidneys stimulated with forskolin. In both models, augmenting HIF-1α levels with the prolyl hydroxylase inhibitor 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetate enhanced cyst growth. In addition, inhibition of HIF-1α degradation through tubule-specific knockdown of the von Hippel-Lindau tumor suppressor increased cyst size in the embryonic kidney cyst model. In contrast, inhibition of HIF-1α by chetomin and knockdown of HIF-1α both decreased cyst growth in these models. Consistent with previous reports, pIMDCK cyst enlargement was driven largely by transepithelial chloride secretion, which consists, in part, of a calcium-activated chloride conductance. pIMDCKs deficient for HIF-1α almost completely lacked calcium-activated chloride secretion. We conclude that regional hypoxia in renal cysts contributes to cyst growth, primarily due to HIF-1α-dependent calcium-activated chloride secretion. These findings identify the HIF system as a novel target for inhibition of cyst growth.

Polycystic kidney diseases (PKDs) comprise a number of inherited disorders that lead to bilateral renal cyst development.1 The most common form, autosomal dominant polycystic kidney disease (ADPKD), affects 1 in 400–1000, and 5% of patients requiring renal replacement therapy suffer from ADPKD.1

Cyst growth is characterized by proliferation of the cyst-lining cells and fluid secretion across the epithelium into the cyst lumen.2 Apical cAMP-dependent chloride secretion is one of the major driving forces for cyst secretion.2 Data obtained with inhibitors of the cystic fibrosis transmembrane conductance regulator (CFTR) in various cyst models suggest that the CFTR chloride channel plays a major role in this process.3–5 However, there is significant heterogeneity in CFTR expression in cyst epithelia,6 raising the question of whether other chloride channels are involved in cyst secretion. In addition, activation of apical calcium-activated chloride...
channels after stimulation of Gq-coupled purinergic receptors (P2YR) has also been described in the Madin-Darby canine kidney (MDCK) cyst model and ex vivo in ADPKD cyst epithelia.

Polycystic kidneys are characterized by chronic stabilization of the hypoxia-inducible transcription factor (HIF) in cyst-lining epithelial and peritubular interstitial cells. HIF activation in cystic kidneys appears not to be due to the genetic defects underlying cyst formation, but apparently results from regional hypoxia as a consequence of compromised blood flow and a mismatch between expanding cysts and the vascularization of cyst walls. HIF transcription factors are the central mediators of the hypoxic response and induce the expression of genes that mediate adaptation to low oxygen tensions. HIF consists of a heterodimer with a constitutive β-subunit (HIF-1β) and an oxygen-regulated α-subunit (HIF-1α or HIF-2α). In normoxia, oxygen-dependent hydroxylation of HIF-α at specific prolyl residues by HIF-prolyl hydroxylases (PHDs) targets the α-subunits for proteasomal degradation. Whereas both HIF-1α and HIF-2α are barely detectable in normal adult kidneys, systemic and regional hypoxia result in stabilization of HIF-1α in tubular epithelial cells and of HIF-2α in peritubular and glomerular cells. HIF-2α in peritubular fibroblasts regulates hypoxia-dependent erythropoietin production. Therefore, HIF-2α stabilization in peritubular cells of polycystic kidneys may explain why ADPKD is characterized by less severe anemia than other types of kidney disease.

However, the functional consequences of HIF-1α stabilization in the cyst epithelium are unclear. In malignant tumors, hypoxia-induced activation of HIF can promote tumor growth by mediating adaptation of tumor cells to reduced oxygen availability. In the rather rare case of precancerous cyst formation in patients with von Hippel-Lindau syndrome (VHL), atypical stabilization of HIF-2α in tubular cells is suggested to promote cyst growth. Renal cyst growth in PKD, albeit a benign growth process, shares similarities with tumor growth, raising the possibility that HIF-1α may promote cyst expansion. However, this hypothesis has so far not been proven. Belibi et al. used 2-methoxyestradiol (2ME2) to inhibit HIF-1α and found no effect on cyst growth in murine PKD models, whereas others found a tendency toward reduced cyst growth using this compound. Because 2ME2 undergoes rapid metabolism, its efficacy for HIF inhibition in vivo remains unclear.

In this study, we assessed the functional relevance of HIF-1α for cyst expansion in two different in vitro models using pharmacologic and genetic modulation of the HIF pathway and identified a novel mechanism of HIF-1α-dependent cyst secretion.

RESULTS

Cyst Growth Is Mediated by HIF-1α in Two Different In Vitro Cyst Models

We used the MDCK cyst model to analyze the effects of HIF-1α on cyst growth. We recently showed that only MDCK cells resembling principal cells of the collecting duct form cysts in a collagen matrix and grow upon stimulation with forskolin or 8-Br-cAMP due to an increase of apical fluid secretion. Similar results have been obtained in vivo confirming a major role for the collecting duct as the origin of cysts in ADPKD. Therefore, we have modified the MDCK cyst model by the use of a cell clone of principal-like MDCK cells (pMDCKs) to study the role of HIF in in vitro cyst growth. Treatment of forskolin-stimulated cysts with the PHD inhibitor ICA [2-(1-chloro-4-hydroxysoquinoline-3-carboxamido) acetate] led to an increase of HIF-1α-positive cyst cells (Figure 1A) and increased expression of the HIF-1α target gene, glucose transporter 1 (GLUT-1) (Figure 1B). In contrast, the HIF-1α inhibitor chetomin led to a decrease of HIF-1α-positive cells (Figure 1A) and decreased expression of GLUT-1 (Figure 1B). Of note, control cysts already showed a reproducible expression of HIF-1α and GLUT-1 (Figure 1A and B), presumably due to diffusion-limited oxygen availability as shown by ratiometric luminescence imaging (RLI) of oxygen and pimonidazole staining (Supplemental Figure 1).

Treatment of forskolin-stimulated cysts with ICA significantly increased cyst growth (Figure 1C). Treatment of non-forskolin-stimulated cysts with ICA also led to a significant increase of cyst size from 68.8±3.4 pl (nonstimulated) to 105.4±7.2 pl (ICA-stimulated) (mean±SEM of six independent experiments comprising approximately 220–250 cysts per condition; P=0.001). In contrast, chetomin inhibited in vitro cyst growth (Figure 1C). In addition, we generated pMDCK clones stably expressing two distinct small hairpin RNAs (shRNAs) directed against HIF-1α. HIF-1α levels and activity were significantly reduced by both shRNAs compared with pMDCKs stably expressing control shRNA (Figure 1, D and E). Knockdown of HIF-1α inhibited cyst growth and rendered the cysts resistant to stimulation with forskolin (Figure 1F).

To test for effects of HIF-1α on cyst growth in another model, we used embryonic mouse kidneys from embryonic (E) day E12.5–E13.5 that were cultured for 5 days under various treatment regimens, each including forskolin stimulation starting at day 2 ex vivo. Stimulation with forskolin induces tubular dilation, followed by cyst formation and cyst growth with secretion mechanisms resembling those in vivo. In line with the data from the pMDCK cyst model, ICA increased the number of HIF-1α–positive cells (Figure 2A) and increased the expression of GLUT-1 (Figure 2B), whereas inhibition of HIF-1α by chetomin led to a decrease of HIF-1α–positive cells (Figure 2A) and to decreased expression of GLUT-1 (Figure 2B). Again, ICA significantly promoted cyst growth (Figure 2C), whereas chetomin treatment resulted in much smaller cyst sizes (Figure 2D).

To further evaluate HIF-dependent effects on cyst growth in the embryonic kidney cyst model, we used TPH-Cre;VHLlox/lox mice that express the Cre-recombinase gene under control of the murine Tamm-Horsfall protein (THP; uromodulin) promoter, resulting in a knockdown of the VHL protein in the
Cyst growth of pMDCKs depends on HIF-1α. (A–C) pMDCKs are cultured within a collagen I matrix in the presence of 10 μM forskolin (Ctrl), 10 μM forskolin + 10 μM of the PHD inhibitor ICA, or 10 μM forskolin+0, 5, or 10 nM of the HIF-1α inhibitor chetomin (CTM). (A) Sixty random cysts per condition out of three individual experiments are subdivided into four groups defined by the number of HIF-1α–positive cells per cyst±SD ranging from <25% to >75% positive cells per cyst. Representative photos show cysts at day 5 stained for HIF-1α. (B) Sixty random cysts per condition out of three individual experiments are subdivided into four groups defined by the extent of GLUT-1–positive staining±SD reaching from slight basal staining (+) to strong ubiquitous staining (++++). Representative photos show cysts at day 5 stained for the HIF-1α target gene GLUT-1. (C) Mean cyst sizes±SEM from three to five individual experiments comprising the analysis of approximately 230–540 cysts per condition. Photos show representative cysts at day 5. (D) pMDCKs stably expressing scrambled control shRNA (scr) or shRNA directed against HIF-1α (cell clone 5, shRNA sequence 1 [cl5.1] and cell clone 3, shRNA sequence 2 [cl3.2]) are treated with the PHD inhibitor DP (100 μM). Luciferase activities relative to untreated controls (set to 1) obtained from three individual assays of HIF-1α–dependent luciferase gene expression are summarized. (E) Control-transfected pMDCKs and pMDCK clone 5.1 described in C are treated with the PHD inhibitors DP (100 μM), dimethyloxalylglycine (1 mM), or ICA (10 μM). Intensities relative to untreated controls (set to 1) obtained from Western blots of four individual experiments are summarized. Depicted Western blot shows the representative result obtained with ICA. (F) pMDCKs described in D are cultured within a collagen I matrix±10 μM forskolin (Fsk). At day 5, cyst sizes relative to untreated controls (set to 100%) from three to four individual experiments comprising approximately 950–1440 cysts per condition are summarized. CTM, chetomin; DP, 2,2′-dipyridyl; PHD-I, PHD inhibitor. Scale bars, 50 μm in A and B; 100 μm in C. *P<0.05.

Figure 1. Cyst growth of pMDCKs depends on HIF-1α. (A–C) pMDCKs are cultured within a collagen I matrix in the presence of 10 μM forskolin (Ctrl), 10 μM forskolin + 10 μM of the PHD inhibitor ICA, or 10 μM forskolin+0, 5, or 10 nM of the HIF-1α inhibitor chetomin (CTM). (A) Sixty random cysts per condition out of three individual experiments are subdivided into four groups defined by the number of HIF-1α–positive cells per cyst±SD ranging from <25% to >75% positive cells per cyst. Representative photos show cysts at day 5 stained for HIF-1α. (B) Sixty random cysts per condition out of three individual experiments are subdivided into four groups defined by the extent of GLUT-1–positive staining±SD reaching from slight basal staining (+) to strong ubiquitous staining (++++). Representative photos show cysts at day 5 stained for the HIF-1α target gene GLUT-1. (C) Mean cyst sizes±SEM from three to five individual experiments comprising the analysis of approximately 230–540 cysts per condition. Photos show representative cysts at day 5. (D) pMDCKs stably expressing scrambled control shRNA (scr) or shRNA directed against HIF-1α (cell clone 5, shRNA sequence 1 [cl5.1] and cell clone 3, shRNA sequence 2 [cl3.2]) are treated with the PHD inhibitor DP (100 μM). Luciferase activities relative to untreated controls (set to 1) obtained from three individual assays of HIF-1α–dependent luciferase gene expression are summarized. (E) Control-transfected pMDCKs and pMDCK clone 5.1 described in C are treated with the PHD inhibitors DP (100 μM), dimethyloxalylglycine (1 mM), or ICA (10 μM). Intensities relative to untreated controls (set to 1) obtained from Western blots of four individual experiments are summarized. Depicted Western blot shows the representative result obtained with ICA. (F) pMDCKs described in D are cultured within a collagen I matrix±10 μM forskolin (Fsk). At day 5, cyst sizes relative to untreated controls (set to 100%) from three to four individual experiments comprising approximately 950–1440 cysts per condition are summarized. CTM, chetomin; DP, 2,2′-dipyridyl; PHD-I, PHD inhibitor. Scale bars, 50 μm in A and B; 100 μm in C. *P<0.05.

HIF-1α Target Gene Expression Correlates with Cyst Formation In Vivo

We previously demonstrated in the Han:SPRD rat model of polycystic kidneys that HIF accumulation correlates with the severity of cyst formation. To confirm the functional relevance of these findings three heterozygous Han:SPRD (Cy+/+) rat kidneys were stained for the HIF-1α target gene GLUT-1. The kidneys then were subdivided into squares (Figure 3A) and analyzed regarding GLUT-1 expression (Figure 3B) and cystic area (Figure 3C). The analyses provided a significant correlation for GLUT-1 and the cystic index (Figure 3D), indicating a functional role of HIF-1α–activated genes in cyst growth in vivo.
HIF-1α–Dependent Cyst Growth Is Mediated by Calcium-Activated Chloride Secretion

To analyze the mechanisms of HIF-1α–dependent cyst expansion, we further analyzed pLMDCk cyst growth. Given its physiologic expression in principal cells of the collecting duct, we studied the role of the epithelial sodium channel (ENaC) in the pLMDCk cyst model. The ENaC inhibitor amiloride had no effect on in vitro cyst growth (Figure 4A). Furthermore, amiloride was still ineffective when pLMDCks had been pretreated with dexamethasone and insulin/transferrin/selenium for 2 weeks to induce ENaC expression (Figure 4A), indicating a lack of functional significance of ENaC for secretion in this model.

We then used the CFTR inhibitor CFTRinh-172 to analyze the role of CFTR for in vitro cyst growth. CFTRinh-172 significantly inhibited forskolin-stimulated cyst growth (Figure 4B), which is in concordance with previous reports. However, ICA-stimulated cyst growth was only slightly affected by CFTRinh-172 (Figure 4B). We therefore hypothesized that ICA may affect calcium-activated chloride secretion. In line with this assumption, stimulation with extracellular ATP, which leads to an increase of intracellular calcium in MDCK cells via stimulation of purinergic receptors, significantly increased cyst growth (Figure 4B). CFTRinh-172 caused only partial inhibition of the ATP-dependent increase of cyst sizes, whereas chetomin markedly decreased ATP-dependent cyst growth (Figure 4B). Consistently, HIF-1α–deficient pLMDCk cysts did not grow upon stimulation with ICA or ATP compared with control-transfected cysts (Figure 4C). These data suggest that HIF-1α induces calcium-activated chloride secretion, thereby leading to cyst expansion.

To further analyze the mechanisms of HIF-1α–dependent chloride secretion, we performed Ussing chamber experiments. Control-transfected pLMDCks showed a luminal chloride secretion, as indicated by a robust negative transepithelial voltage deflection upon treatment with UTP (Figure 5, A and C). In contrast, HIF-1α knockdown cells almost entirely lost the UTP-dependent luminal chloride secretion (Figure 5, B and C). Using ionomycin, an ionophore that induces a sustained increase in intracellular calcium, control-transfected cells again showed a luminal chloride secretion, whereas HIF-1α knockdown cells did not (Figure 5, D and E). Consistent with the pLMDCk cyst data, nontransfected and scrambled-transfected cells showed a robust
expression of HIF-1α under baseline conditions, whereas stable expression of shHIF-1α significantly reduced HIF-1α protein level (Figure 5F). In line with the basal expression of HIF-1α, transepithelial resistance of the pMDCK monolayer from HIF-1α knockdown cells was significantly increased (control-transfected cells: 11,823.6 ± 1397.5 Ω cm²; HIF-1α shRNA-transfected cells: 21,586.1 ± 1880.8 Ω cm²). In conclusion, these data indicate that HIF-1α is essential for calcium-activated chloride secretion in pMDCKs.

DISCUSSION

Cyst expansion provoking obstruction of primarily unaffected renal tissue is considered as the major reason for the decline of renal function in ADPKD.33 Therefore, understanding the mechanisms of cyst growth has attracted significant interest. In fact, the first clinical trials aiming at the inhibition of cyst growth to preserve renal function in PKD were recently conducted.34,35

This work proposes a novel target mechanism for the treatment of PKD by demonstrating an important role of hypoxia-dependent gene expression in cyst secretion. We obtained highly consistent results using a combination of genetic approaches, a strong inhibitor of HIF degradation, and

Figure 3. Expression of the HIF-1α target gene GLUT-1 correlates with the extent of cyst formation in vivo. (A–D) Representative analysis of a polycystic kidney section obtained from a Cy/+ rat. (A) A kidney section is stained for GLUT-1 and a high-resolution image is generated as described in Concise Methods, which is then subdivided into squares. (B) GLUT-1–positive area per total square area meeting the following criteria is analyzed in each square: covering cortical area, excluding edges and tissue artifacts (green squares). (C) Cystic index defined as cyst area (black areas exceeding the threshold area defined for “healthy” tubular lumen) per total square area was determined for each square. (D) GLUT-1 expression and cystic index significantly correlate. Analysis is performed with three individual kidneys, each providing comparable results. Scale bars, 1 mm.

Figure 4. The epithelial sodium channel ENaC and the chloride channel CFTR are of minor importance for HIF-dependent in vitro cyst growth. (A) pMDCK cysts are treated with 10 μM forskolin:±5 μM amiloride in the absence or presence of 0.1 μM dexamethasone, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (D/ITS), including a pretreatment of cultured pMDCKs with D/ITS for 2 weeks allowing a robust expression of ENaC. At day 5, mean cyst sizes±SEM from three individual experiments comprising approximately 120–170 cysts per condition are summarized. (B) pMDCK cysts are stimulated with 10 μM forskolin:±10 μM CFTRinh172:±10 μM ICA:±100 μM ATP:±10 nM chetomin as indicated. At day 5, cyst sizes relative to untreated forskolin-stimulated cysts (set to 100%) from three individual experiments comprising approximately 120–230 cysts per condition are summarized. *P<0.05.

HIF-1α Transcriptionally Regulates Expression of Genes Involved in Calcium-Activated Chloride Secretion

On the basis of these findings, we were interested in genes that are involved in calcium-activated chloride secretion and tested them for HIF-dependent regulation using RT-PCR in control pMDCKs incubated with or without ICA and in scrambled-transfected pMDCKs compared with HIF-1α–deficient cells (Supplemental Figure 5, A and B). ICA led to an increased expression of the purinergic Gq-coupled receptor P2Y2R, the calcium-activated K+ channel KCa3.1, and the calcium-activated chloride channel anoctamin 6 (ANO6) (Supplemental Figure 5A). In line with these data, knockdown of HIF-1α significantly reduced the expression of P2Y2R, KCa3.1, and ANO6 in pMDCKs (Supplemental Figure 5B). ICA-dependent upregulation of P2Y2R and ANO6 has been confirmed in vivo by administration of ICA to mice and analysis of mRNA expression of the kidneys compared with vehicle-treated kidneys (Supplemental Figure 5C). In contrast to the pMDCK cyst model, we did not observe an upregulation of KCa3.1 in vivo but a significant increase of anoctamin 1 (ANO1), another calcium-activated chloride channel. These data point toward a transcriptional regulation of genes that are involved in calcium-activated chloride secretion by HIF-1α.
Figure 5. Knockdown of HIF-1α leads to a significant decrease of purinergic, calcium-activated chloride conductance. (A and B) Original recordings of open-circuit Ussing chamber experiments. pIMDCKs grown on permeable supports and stably expressing either scrambled shRNA (A) (control) or shRNA directed against HIF-1α (B) (shHIF1α, clone 5.1) are stimulated with the P2 receptor agonist UTP (100 μM) from the luminal or basolateral side of the monolayer. (C) Summary of the calculated equivalent short-circuit currents (Isc) induced by UTP in control and shHIF-1α cells. (D) Original recording of an open-circuit Ussing chamber experiment. pIMDCKs described in A and B are treated with 1 μM of ionomycin (iono) applied from the luminal side to increase intracellular calcium. (E) Summary of the Isc induced by ionomycin in control and shHIF1α cells (number of experiments). (F) Nontransfected pIMDCKs (n.t.), pIMDCKs stably expressing scrambled shRNA (scr), or shRNA directed against HIF-1α (shHIF1α, clone 5.1) are maintained under cell culture conditions described in A and B. Left panel shows analysis of HIF-1α protein levels as intensities relative to nontransfected cells (set to 100%) obtained from Western blots of three individual experiments. The right panel is the representative Western blot. *P<0.05.

in vitro

HIF-1α inhibitor in vitro to suppress or stimulate the HIF system in two independent in vitro models. In both models, HIF-1α is expressed in the kidney epithelium, which is in line with our previous results in human ADPKD and rat PKD kidneys that revealed significant tissue hypoxia.10 Besides hypoxia, additional factors such as activation of mammalian target of rapamycin36 or inflammation37 could also contribute to HIF-1α stabilization in PKD. Inhibition or knockdown of HIF-1α significantly reduced cyst enlargement in both models. In contrast, increased stabilization of HIF-1α was associated with augmented cyst expansion. Cyst growth in both models is highly dependent on secretory mechanisms4,7 and Ussing chamber analyses revealed that HIF-1α is an important stimulator of calcium-activated chloride secretion. Calcium-dependent activation of apical chloride channels mediated by Gq-coupled purinergic receptors was previously identified as an important mechanism of fluid secretion into the cyst lumen.8,9 Notably, most of these analyses were performed using in vitro assays, including the cyst models presented here. To what extent these mechanisms play a role in the complex in vivo situation and in the context of a genetic disorder due to mutation of PKD1 or PKD2 needs to be further addressed.

These findings are not contradictory to previous reports that demonstrate a significant role of cAMP-CFTR-mediated chloride secretion2 because calcium-activated secretion and cAMP-dependent chloride secretion are not entirely separate mechanisms and there is significant interplay. First, cAMP-dependent pIMDCK cyst growth depends on synergistic costimulation of Gq-coupled purinergic receptors.7 In addition, ANO1, one of the main calcium-activated chloride channels in kidney epithelial cells, is functionally suppressed by CFTR.38 CFTR, however, is repressed by HIF-1α in intestinal and airway epithelial cells,39,40 whereas chronic hypoxia enhances calcium-activated chloride secretion.41 Therefore, hypoxia and stabilization of HIF-1α with consecutive suppression of CFTR might lead to a shift from cAMP-dependent chloride secretion toward calcium-activated chloride secretion.

Apart from direct effects on secretory mechanisms, HIF-1α stabilization could also facilitate glucose utilization via anaerobic energy generation42 or uptake via upregulation of GLUT-1, which would help to maintain energy-consuming ion secretion despite the lack of oxygen. Furthermore, HIF-1α may also render cyst epithelial cells more resistant to hypoxic stress.13

Previous reports suggested that HIF-1α plays a role not only in cyst expansion but also in cyst formation. Loss of function of the VHL protein, a critical component of HIF degradation, has been associated with polycystic kidneys.30 However, the role of HIF-1α in mediating this effect remains less clear. HIF-1α was found to be of no importance for cyst formation in mice with VHL-deficient proximal tubules.18 On the other hand, loss of VHL function leads to loss of the primary cilia,43 which is a frequent cause of PKDs,44 and knockdown of HIF-1α in VHL-deficient cells could rescue the primary cilium.45 This potential link between HIF-1α and cystogenesis has been further strengthened by mice lacking fumarate hydratase 1 (FH1). Lack of FH1 leads to stabilization of HIF under normoxic conditions accompanied by the development of renal cysts that strongly express HIF-1α and THP.46 A subsequent report revealed that the combined knockdown of FH1 and HIF-1α increased cyst initiation and likely also cyst...
growth. This might be explained by the fact that cysts caused by FH1 deficiency are neoplasias and primarily grow due to hyperplasia and MYC-dependent proliferation that is antagonized by HIF-1α. In the embryonic cyst model, however, we did not find changes in cyst number in response to stabilization of HIF-1α, whereas secretion-dependent cyst growth was strongly increased. In addition, recent studies suggested that accumulation of fumarate by FH1 deficiency enzymatically inhibits various α-ketoglutarate–dependent dioxygenases resulting in altered histone and DNA methylation, thereby promoting tumorigenesis.

Because HIF-1α is a transcription factor, the mechanistic link between HIF-1α and the increase of calcium-dependent chloride secretion might be due to transcriptional regulation of genes that are involved in calcium-activated chloride secretion. This is supported by our data showing HIF-dependent regulation of P2Y2R, ANO6, and KA3.1 in the plMDCK cyst model and increased expression by ICA in vivo. However, HIF-dependent downregulation of CFTR, with inhibition of the suppression of ANO1, might also play a role. In addition, hypoxia may promote ATP release from the cyst epithelium, which then could further stimulate purinergic Gq-coupled receptors.

Irrespective of the spectrum of mechanisms by which HIF-1α might promote cyst growth, our data suggest that inhibition of HIF-1α to retard cyst growth deserves exploitation in vivo. The development of pharmacologic HIF-1α inhibitors as anticancer agents is actively pursued and once they are available, ADPKD could qualify for a noncancer indication of such drugs. In addition, based on the secretory effects of HIF-1α, we propose that targeting secretion-dependent cyst growth in PKD should comprise cAMP- and calcium-dependent chloride secretion, with the latter potentially becoming more important in later stages of PKD when hypoxia is likely more severe.

CONCISE METHODS

Animals
All animal experiments were approved by local government authorities and conformed to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Generation and characterization of mice carrying the loxP-flanked conditional alleles of HIF-1α and Vhlh were previously described. Targeted gene inactivation in thick ascending limbs was achieved by cross-breeding those mice to THP-Cre transgenic mice in a C57BL/6 background. Cre-negative homozygous littermates for the conditional alleles were used as controls. Heterozygous (Cy/+) Han:SPRD rats were maintained as previously described. Male C57BL/6N mice, weighing 20–25 g, were purchased from Charles River Laboratories (Sulzfeld, Germany). ICA (40 mg/kg) was dissolved in 5% DMSO, 0.5 M Tris buffer. Seven hours after injection and the kidneys were harvested for RNA preparation.

RNA Preparation and Real-Time PCR
Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed with M-MuLV-RT (Fermentas, St. Leon-Rot, Germany). SYBR-Green–based real-time PCR was performed using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). mRNA expression levels were normalized to 18S rRNA when analyzing mouse kidney tissue and hypoxanthine-guanine phosphoribosyltransferase when analyzing plMDCKs using the ∆∆Ct method. All primer sequences are listed in Supplemental Table 1.

Embryonic Kidney Culture
Metanephric kidneys were dissected from E12.5–13.5 embryonic mice and cultured on transparent Millicell organotypic cell culture inserts (Merck Millipore, Billerica, MA). DMEM/Ham’s F12 culture medium supplemented with 2 mM l-glutamine, 10 mM HEPES, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 25 ng/ml PGE, 32 pg/ml T3, 110 μ/ml penicillin, and 0.1 mg/ml streptomycin was added under the culture inserts, and organ cultures were maintained in a 37°C humidified CO2 incubator for 5 days. After 24 hours, the medium was changed and supplemented with forskolin, ICA, or chetomin as indicated in Supplemental Figure 1, H and I. Medium was changed every 24 hours. After 5 days, whole kidneys were photographed by means of a BZ-9000 microscope (Keyence, Osaka, Japan) and the cystic index defined as total cyst area divided by total kidney area was measured using ImageJ software (version 1.45).

plMDCK Cyst Model
plMDCKs were a kind gift of Hans Oberleithner (University of Muenter, Muenter, Germany). In vitro cyst assays were performed as previously described. In brief, plMDCKs were resuspended as a single-cell suspension in type I collagen and filled into 24-well plates (3 to 6 wells per condition). Chetomin, ATP, forskolin, amlodipine, dexamethasone, insulin, transferrin, selenium, CPT, and 172 (all Sigma-Aldrich, Steinheim, Germany), or ICA was added to the medium at day 0 in the concentration mentioned in the Figures 1 and 4, and the medium was changed every 2 days. After 4 days, two random visual fields per well were photographed with an Olympus CK40 microscope (X40 magnification; Olympus Life Science Research GmbH, Munich, Germany) and a Leica DC200 camera (Leica Microsystems, Wetzlar, Germany). Cyst diameters of all captured cysts that were nearly spherical (approximately 80–360 cysts per condition and single experimental procedure) were measured with ImageJ software (version 1.45). Cyst volume was then estimated using the formula for the volume of a sphere, 4/3πr³.

Oxygen Measurements
RLI of oxygen was performed by the use of the VisiSens imaging technology consisting of a portable RLI-device (VisiSens USB microscope; PreSens GmbH, Regensburg, Germany) and Analytical software to control the camera settings and to analyze the data. The sensor foils contained an oxygen-sensitive probe and a reference dye that were placed in an oxygen permeable polymer matrix layer. Sensor foils were placed on top of, below, and within the collagen matrix at different heights. RLI-sensor foils were two-point calibrated using Na2S2O5 and air-saturated distilled water before use.
Pimonidazole analyses were performed by administration of pimonidazole (1 μM hypoxyprobe-1; Chemicon International, Temecula, CA) to the plMDCK cysts within the matrix overnight before fixation with paraformaldehyde (4%). Whole collagen blobs then were incubated with hypoxyprobe Mab1 (1:50) conjugated with FITC.

**shRNA and Generation of shHIF-1α–Deficient Cells**
Primers complementary to two distinct regions of *Canis familiaris* HIF-1α (accession number XP_537471.2) were cloned BglII and Xhol into pSUPERIOR vector (Oligoengine, Seattle, WA). Correct cloning was verified by sequencing. As a negative control, pSUPERIOR containing a scrambled sequence was purchased from Oligoengine, plMDCKs were transfected with Fugene (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Colonies were picked after 2 weeks of treatment with G-418 (500 μg/ml; PAA Laboratories, Coelbe, Germany).

** Primer Sequences Used for shRNA Directed against HIF-1α**
The following primers were used for shHIF-1α: 2105 5’-GAT CCC CGC ACA ATT ACA GTA TTC CAT TCA AGA GAT GGA ATA CTG TAA TTG GCC TTC TTG-3’ and 5’-TCG AGA AAA AGC ACA ATT ACA GTA TTC CAT CTC TTG AAT GGA ATA CTG TAA TTG TGC GGG-3’ (sequence 1); and 2236 5’-GAT CCC CGC TCC ATC TCC TAC CCAATG TCA AGA GAC ATG GGT AGG AGA TGG AGC GGG-3’ (sequence 2). To note, in the shHIF-1α 2236 sequence, an additional thymidine was inserted in the hairpin of the shRNA that did not impair silencing.

**Luciferase Reporter Gene Assays**
plMDCKs were cultured in 24-well plates to 50% confluence and transfected with 400 ng/well of the luciferase reporter plasmid 6xHRE/ tk/luc and 100 ng of a pCMV-β-galactosidase expression vector using the Fugene transfection reagent. Transfected cells were stimulated for 18 hours with 100 μM dipyridyl (Sigma-Aldrich, Munich, Germany). Luciferase activities were normalized to the respective β-galactosidase expression.

**Immunoblotting**
Proteins were dissolved in lysis buffer containing 6.65 M urea, 10% glycerol, 10 mM Tris-HCl (pH 6.8), 1% SDS, 5 mM dithiothreitol, and Complete protease inhibitors (Roche) and sonicated. Thirty micrograms of proteins was used for immunodetection of HIF-1α. Proteins were probed with polyclonal rabbit anti-human HIF-1α (1:1000; Cayman Chemicals).

**Immunohistochemistry**
plMDCK cysts at day 4 and embryonic kidneys at day 5 were rinsed in PBS supplemented with 0.9 mM calcium chloride and 0.49 mM magnesium chloride (PBS”). Paraformaldehyde (4%) was added to fix the cysts or kidneys for 1 hour at room temperature. Glycine (200 mM) in PBS” was added for another hour to quench the excess aldehyde. Blobs of collagen gel were put into biopsy bags and paraffinized. Three-micrometer paraffin sections of the plMDCK cysts and kidneys were stained for HIF-1α and GLUT-1. Polyclonal rabbit anti–HIF-1α (Cayman Chemicals, Ann Arbor, MI) and polyclonal rabbit anti–GLUT-1 (Alpha Diagnostics, San Antonio, TX) antibody were both used at a dilution of 1:10,000. A catalyzed signal amplification system and a biotinylated secondary anti-rabbit antibody (Dako, Hamburg, Germany) were used according to the manufacturer’s instructions. Goat anti-THP antibody (ICN Pharmaceuticals, West Laval, Quebec, Canada) was used at a dilution of 1:1000 and biotinylated polyclonal rabbit anti-goat IgG antibody (Dako) was detected using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Signals were captured with a BZ-9000 microscope (Keyence, Osaka, Japan) and the background correction algorithm in ImageJ (version 1.45) was applied.

** GLUT-1–Cyst Formation Correlation Analyses**
Kidney sections from three C57l/6J rats stained for GLUT-1 were photographed at ×20 magnification and photos were stitched using BZ-9000 analyzer software (Keyence). Obtained images were further processed with ImageJ software (version 1.45) and subdivided into squares (Figure 3A). To analyze GLUT-1 expression, a color deconvolution algorithm was applied to extract the GLUT-1 (DAB [3,3'-diaminobenzidine]) signal followed by binarization and particle analysis to obtain the ratio of GLUT-1–positive area per total square area in each square meeting the following criteria: covering cortical area, excluding edges and tissue artifacts (green squares in Figure 3B). In addition, cystic index was obtained for each square by dividing cyst area (black areas in binarized images exceeding the threshold area defined for healthy tubular lumen) by total square area. R2 and statistical significance was determined by the use of Prism 5.04 (GraphPad Software, Inc., La Jolla, CA).

**Ussing Chamber Experiments**
plMDCKs stably transfected with shHIF-1α or scrambled sequence (control cells) were grown as polarized monolayers on permeable supports (Millipore) for 5 days and were mounted into a perfused micro-Ussing chamber. The luminal and basolateral surfaces of the epithelium were perfused continuously with Ringer’s solution consisting of the following in mM: NaCl, 145; KH2PO4, 0.4; K2HPO4, 1.6; glucose, 5; MgCl2, 1; and Ca gluconate, 1.3) at a rate of 6 ml/min (2 ml chamber volume). All experiments were carried out at 37°C under open-circuit conditions. Transepithelial resistance (Rte) was calculated according to Ohm’s equation (Rte=ΔVte/ΔIte) and the corresponding changes in transepithelial voltage (Vte) were recorded continuously. Values for Vte were referred to the serosal side of the epithelium. Rte was calculated according to Ohm’s law (Rte=ΔVte/ΔIte). The equivalent short-circuit current (Isc) was calculated according to Ohm’s law from Vte and Rte (Isc=Vte/Rte).

**Statistical Analyses**
The data were expressed as mean±SEM. The differences among groups were analyzed using one-way ANOVA, followed by a Bonferroni test for multiple comparisons. An unpaired t test was applied to
compare the differences between two groups. Wilcoxon signed-rank test for column statistics was used for relative values. \( P < 0.05 \) was considered statistically significant and is represented by asterisks in Figures 1, 2, 4, and 5, and Supplemental Figures 4 and 5.

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

None.

**REFERENCES**


**Table 1: PCR primer sequences**

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<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<td>P2Y2R (canine)</td>
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<td>P2Y2R (mouse)</td>
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<td>CGATCCGAGGCGCCTTCACT</td>
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**Figure legends**

**S1. PLC cysts within a collagen matrix are exposed to hypoxia. A** Typical setting of the PLC cyst assays showing PLC cysts within a collagen matrix at the bottom of a well and medium on top of the collagen/cell suspension. **B** Prior to the ratiometric luminescence imaging (RLI) of oxygen by the use of the VisiSens™ imaging technology, RLI-sensor foils (depicted by dotted red lines) were two-point calibrated. To this end, left sensor foil was incubated with Na₂S₂O₅ (which binds molecular oxygen) and the right sensor foil was
exposed to room air. C Calibration curve obtained from B. D-G Sensor foils were placed at different heights in the well as depicted by the red arrow and oxygen was measured on top of the collagen matrix (D), within the collagen matrix (E,F) and at the bottom of the well (G). H PLC cysts were incubated with pimonidazole before fixation and stained with FITC-conjugated hypoxyprobe antibody. I PLC cysts incubated with FITC-conjugated hypoxyprobe antibody without pimonidazole serving as control.

S2. THP is expressed early in embryonic kidney development. S2 shows a cross section of a metanephric kidney E13.0 stained for THP revealing expression of THP apically in branching tubules (*) and to a lesser extent also partly in comma shaped bodies (#). Scale bar whole kidney: 100µm; magnifications: 50µm.

S3. THP is expressed in the cyst epithelium of embryonic kidneys stimulated with forskolin. S3 shows a cross section of an embryonic kidney from a THP-Cre;VHLlox/lox mouse, that was dissected at E13.5 and cultured ex vivo for 5 days including forskolin stimulation for the last 4 days. Most of the cysts are stained positive for THP (*), a few cysts show a subtle or no staining (#) whereas proximal tubule segments stain negative for THP (arrow).

S4. THP-dependent deletion of VHL leads to a significant increase of tubular expression of HIF-1α in embryonic kidneys. Embryonic kidneys E13.5 with THP-dependent deletion of VHL (Cre+; n=4) were stained for HIF-1α and compared with VHL-competent kidneys (Cre-; n=4). The number of HIF-1α positive tubular cells was normalized to the total number of tubular cells. Right shows representative embryonic kidney sections. Scale bar: 200µm.

S5. HIF-1α leads to transcriptional activation of genes involved in calcium-activated chloride secretion. A,B Relative mRNA expression of the purinergic receptor P2Y2R, the
Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels anoctamin 6 (ANO6), and anoctamin 1 (ANO1), the calcium-activated K\textsuperscript{+} channel KCa3.1, the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter 1 (NKCC1), and the Cl\textsuperscript{-} channel CFTR in PLC in the presence or absence of ICA (\textbf{A}; \(n=3\); control set to 1) or in PLC stably expressing scrambled shRNA (Scr) or shRNA directed against HIF-1\textalpha{} (shHIF-1\textalpha{}, clone 5.1) (\textbf{B}; \(n=3\)). \textbf{C} Relative mRNA expression of ANO6, P2Y2R, ANO1, KCa3.1, NKCC1, and CFTR in kidneys from 7 mice treated with ICA (40mg/kg) relative to 3 control kidneys (set to 1).
Fig. S1

A. A diagram showing a culture setup with air, medium, and matrix + cysts.

B. An image showing the oxygen concentration (% O₂) in the matrix.

C. A color scale for the oxygen concentration (% O₂).

D-G. Images showing different regions with oxygen concentrations of 12.4%, 6.1%, 5.4%, and 1.1% respectively.

H. An image of Pimo + Hypoxyprobe revealing green fluorescence in a culture.

I. An image of Hypoxyprobe showing dark regions, possibly indicating hypoxic areas.

Scale bars: 100 µm.
**Fig. S4**

Supplement

THP-Cre;VHL^{lox/lox}

HIF-1α pos. tubular cells [%]

Cre- Cre+

![Image of tissue samples showing Cre- and Cre+ conditions with bar chart and asterisk indicating statistical significance.](image-url)
Fig. S5

A  PLC
Ctrl  ICA
P2Y2R  ANO6  KCa3.1  ANO1  NKCC1  CFTR

B  PLC
Scr  shHIF-1α
P2Y2R  ANO6  KCa3.1  ANO1  NKCC1  CFTR

C  kidneys
Ctrl  ICA
ANO6  P2Y2R  ANO1  KCa3.1  NKCC1  CFTR