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

Bjoern Buchholz,* Gunnar Schley,* Diana Faria,† Sven Kroening,* Carsten Willam,* Rainer Schreiber,† Bernd Klanke,* Nicolai Burzlaff,‡ Jonathan Jantsch,§ Karl Kunzelmann,† and Kai-Uwe Eckardt*

Departments of *Nephrology and Hypertension, and ‡Chemistry and Pharmacy, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany; †Department of Physiology, University of Regensburg, Regensburg, Germany; and §Department of Clinical Microbiology, Immunology, and Hygiene, Microbiology Institute, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany

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 model2,8 and ex vivo in ADPKD cyst epithelia.9 Polycystic kidneys are characterized by chronic stabilization of the hypoxia-inducible transcription factor (HIF) in cystlining epithelial and peritubular interstitial cells.10 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.11 HIF transcription factors are the central mediators of the hypoxic response and induce the expression of genes that mediate adaptation to low oxygen tensions.12 HIF consists of a heterodimer with a constitutive β-subunit (HIF-1β) and an oxygen-regulated α-subunit (HIF-1α or HIF-2α).13 In normoxia, oxygen-dependent hydroxylation of HIF-α at specific prolyl residues by HIF-prolyl hydroxylases (PHDs) targets the α-subunits for proteasomal degradation.13a 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.14 HIF-2α in peritubular fibroblasts regulates hypoxia-dependent erythropoietin production.15 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.16 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.17 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.18 Renal cyst growth in PKD, albeit a benign growth process, shares similarities with tumor growth,19 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,20 whereas others found a trend toward reduced cyst growth using this compound.21 Because 2ME2 undergoes rapid metabolization,22 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.7 Similar results have been obtained in vivo23 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 (plMDCKs)2 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-hydroxyisoquinoline-3-carboxamido) acetate]24 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,25 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 1, A and B), presumably due to diffusion-limited oxygen availability as shown by ratiometric luminescence imaging (RLI) of oxygen and pimozadole staining (Supplemental Figure 1).

Treatment of forskolin-stimulated cysts with ICA significantly increased cyst growth (Figure 1C). Treatment of nonforskolin-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 plMDCK 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 plMDCKs 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.4,5 In line with the data from the plMDCK 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 THP-Cre;VHLlox/lox mice that express the Cre-recombinase gene under control of the murine Tamm-Horsfall protein (THP; uromodulin) promoter,26 resulting in a knockdown of the VHL protein in the
thick ascending limb of adult animals. Little is known about THP expression in embryonic kidneys. In humans, THP was detected immunohistochemically by the 14th week of gestation as well as by the end of the 16th week of gestation in 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 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 pMDCK 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 pMDCK cyst model. The ENaC inhibitor amiloride had no effect on in vitro cyst growth (Figure 4A). Furthermore, amiloride was still ineffective when pMDCKs 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 pMDCK 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 pMDCKs 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 pMDCK 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 pLMDCk monolayer from HIF-1α knockdown cells was significantly increased (control-transfected cells: 11,823.6 ± 1424.1 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 pLMDCks.

**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 cyst growth. (A) pLMDCk cysts are treated ± 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 pLMDCks 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) pLMDCk 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%) are obtained from three to six individual experiments comprising approximately 210–840 cysts per condition. (C) pLMDCk cysts stably expressing control shRNA (scrambled; 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 cultured in the presence of 10 μM forskolin (Fsk):±10 μM ICA±100 μM ATP. At day 5, cyst sizes relative to control-transfected cysts (set to 100%) from three individual experiments comprising approximately 120–230 cysts per condition are summarized. *P<0.05.

**DISCUSSION**

Cyst expansion provoking obstruction of primarily unaffected renal tissue is considered as the major reason for the decline of renal function in ADPKD. 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. 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
chetomin, which was previously used as 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 cyst epithelium, which is in line with our previous results in human ADPKD and rat PKD kidneys that revealed significant tissue hypoxia. Besides hypoxia, additional factors such as activation of mammalian target of rapamycin or inflammation 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 mechanisms 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. 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 secretion because calcium-activated secretion and cAMP-dependent chloride secretion are not entirely separate mechanisms and there is significant interplay. First, cAMP-dependent pMDCK cyst growth depends on synergistic costimulation of Gq-coupled purinergic receptors. In addition, ANO1, one of the main calcium-activated chloride channels in kidney epithelial cells, is functionally suppressed by CFTR. CFTR, however, is repressed by HIF-1α in intestinal and airway epithelial cells, whereas chronic hypoxia enhances calcium-activated chloride secretion. 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 generation 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.

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. 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. On the other hand, loss of VHL function leads to loss of the primary cilium, which is a frequent cause of PKDs, and knockdown of HIF-1α in VHL-deficient cells could rescue the primary cilium. 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. 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 KCa3.1 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 thal arriving out layer mice (C57BL/6) background. Cre-negative homozygous littermates for the conditional alleles were used as controls. Heterozygous (Cy+/-) HanSPRD 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 animals were injected once intraperitoneally with the dissolved solution, and three animals were injected with the carrier solution serving as the control. Mice were euthanized 6 hours after injection and the kidneys were harvested for RNA preparation.

**Embryonic Kidney Culture**

Metanephric kidneys were dissected from E12.5–E13.5 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 1-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 U/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 Muenster, Muenster, 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, amiloride, dexamethasone, insulin, transferrin, sodium, CFTRinh-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 (×40 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, $\frac{4}{3}\pi r^3$.

**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 AnalytiCa1 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.

**RNA Preparation and Real-Time PCR**

Total RNA was extracted with RNazol B (Biowizol, Eching, 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 SD method. All primer sequences are listed in Supplemental Table 1.
Pimonidazole analyses were performed by administration of pimonidazole (1 μM hypoxprobe-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 hypoxprobe 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. pMDCKs 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).

**Luciferase Reporter Gene Assays**

Luciferase activities were normalized to the respective TAC-3 (-galactosidase expression. Luciferase Reporter Gene Assays was added for another hour to quench the excess al-

**GLUT-1–Cyst Formation Correlation Analyses**

Kidney sections from three C57BL/6J mice 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 binned 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).

**Immunohistochemistry**

pMDCK 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.

**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.

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**DISCLOSURES**

None.

**REFERENCES**


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