Renal Cyst Development in Mice with Conditional Inactivation of the von Hippel-Lindau Tumor Suppressor

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Abstract
Inactivation of the von Hippel-Lindau tumor suppressor, pVHL, is associated with both hereditary and sporadic renal cysts and renal cell carcinoma, which are commonly thought to arise from the renal proximal tubule. pVHL regulates the protein stability of hypoxia-inducible factor (HIF)-α subunits and loss of pVHL function leads to HIF stabilization. The role of HIF in the development of VHL-associated renal lesions remains to be determined. To investigate the functional consequences of pVHL inactivation, two transgenic mouse lines were generated in which pVHL function was disrupted. pVHL deficient renal cyst development was associated with increased erythropoietin levels and polycythemia. Increased expression of the HIF target gene erythropoietin was limited to the liver, whereas expression of carbonic anhydrase 9 and multidrug resistance gene 1 was up-regulated in the renal cortex of mutant mice. Inactivation of the HIF-α binding partner, arylhydrocarbon receptor nuclear translocator (Arnt), but not HIF-1α, suppressed the development of renal cysts. Here, we present the first mouse model of VHL-associated renal disease that will provide a basis for further genetic studies to define the molecular events that are required for the progression of VHL-associated renal cysts to clear cell renal cell carcinoma. (Cancer Res 2006; 66(5): 2576-83)

Introduction
The von Hippel-Lindau (VHL) gene is a classic tumor suppressor gene that is a critical regulator of cyst development and tumor growth in the kidney. Inactivation of VHL is found in both hereditary and sporadic clear cell renal cell carcinomas (CCRCC; ref. 1). Patients with the familial VHL tumor syndrome inherit one mutated VHL allele. Inactivation of the remaining allele in somatic cells results in the development of CCRCC that is often associated with retinal and central nervous system hemangioblastomas (type 1 VHL disease) or with hemangioblastomas and pheochromocytomas (type 2B VHL disease; ref. 2). Somatic inactivation of both VHL alleles leads to the development of sporadic CCRCC (3). VHL tumor suppressor activity has been shown in mouse xenograft assays, whereby re-expression of VHL in CCRCC cell lines inhibits tumor formation (4, 5).

Loss of VHL results in activation of hypoxia-inducible transcription factors (HIF) and increased expression of HIF target genes (5–7). pVHL is the substrate recognition component of an E3 ubiquitin ligase complex that targets hydroxylated HIF-α subunits for ubiquitination and degradation by the 26S proteasome at normoxic oxygen concentrations (8–11). Under conditions of hypoxia or loss of pVHL function, HIF-α subunits are stabilized and form transcriptionally active heterodimers with the constitutively nuclear HIF-β subunit arylhydrocarbon receptor nuclear translocator (Arnt). HIF transcription factors are members of the PAS (Per-Arnt-Sim) family of basic helix-loop-helix transcription factors and bind to hypoxia response elements within regulatory elements of hypoxia-regulated genes (12). HIF-mediated gene transcription is part of an adaptive cellular response to hypoxia and facilitates glucose uptake, glycolysis, and oxygen delivery by increasing angiogenesis and erythropoiesis (13, 14).

Two HIF-β and HIF-α subunits are expressed in the adult kidney. HIF-1β, also known as Arnt, is expressed ubiquitously throughout the kidney, whereas a second isoform, ARNT2, is expressed in the thick ascending limb of Henle (15). HIF-1α is expressed throughout the kidney, whereas HIF-2α expression is restricted to distinct cell populations. Under conditions of renal hypoxia or ischemia, HIF-1α is detectable in epithelial cells within the proximal and distal nephron segments, connecting tubules, and collecting ducts, whereas HIF-2α is only detectable in endothelial cells and renal interstitial cells (16, 17). In kidneys from VHL patients, HIF-1α protein is expressed in morphologically normal VHL-deficient renal epithelial cells and maintains expression in advanced tubular lesions, cysts, and CCRCC. In contrast, HIF-2α is undetectable in normal-appearing tubule cells but is highly expressed in tubular cysts and CCRCC (18–20). HIF-2α is not only associated with VHL-deficient renal lesions but is also required and sufficient for the induction and maintenance of CCRCC tumor growth in mouse xenograft assays (21–23).

The cellular origin of VHL-associated CCRCC remains controversial. Although it is commonly thought that VHL-associated renal cell carcinomas arise from the renal proximal tubule, renal cysts and carcinomas have been found to express molecular markers of both proximal and distal tubule cell origin (18, 24–26). To investigate the functional consequences of pVHL inactivation and the role of HIF signaling in renal epithelial cells, we used the phosphoenolpyruvate carboxykinase (PEPCK) promoter to generate transgenic mice in which Cre-recombinase is expressed in the renal proximal tubule and in hepatocytes. We found that conditional inactivation of VHL in PEPCK-Cre mutants resulted in renal cyst development that was associated with increased erythropoietin levels and polycythemia. Increased expression of the HIF target gene erythropoietin was limited to the liver, whereas expression of carbonic anhydrase 9 and multidrug resistance gene 1 was up-regulated in the renal cortex of mutant mice. Inactivation of the HIF-α binding partner, arylhydrocarbon receptor nuclear translocator (Arnt), but not HIF-1α, suppressed the development of renal cysts. Here, we present the first mouse model of VHL-associated renal disease that will provide a basis for further genetic studies to define the molecular events that are required for the progression of VHL-associated renal cysts to clear cell renal cell carcinoma. (Cancer Res 2006; 66(5): 2576-83)

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Materials and Methods

Generation and genotyping of mice. The generation of mice carrying the Vhlh, Hif-1x, and Arnt conditional alleles has been previously described (27–29). PEPCK-Cre transgenic mice were generated by targeted single-copy transgenesis in embryonic stem cells. Hoxpoxanthine phosphoribosyltransferase (HPRT)-deficient embryonic stem cells (E14Tg2a sc foi 30) were electroporated with the PEPCK-Cre-targeting vector that was generated by inserting Cre recombienase under the control of the rat PEPCK promoter (−500 to +73) containing a mutation in the P3(I) region (−248 to −230) into a modified PMc8 targeting vector (31). Embryonic stem cells were selected in hypoxanthine-aminopterin-thymidine medium and homologous recombinants were identified by Southern blot analysis as previously described (27). Internal and external probes for transgene detection included a 400 bp EcoRV/KpnI fragment derived from the 3′ end of the Cre-recombienase gene and a 150 bp BsrFl fragment derived from intron 3 of the HPRT gene. Targeted embryonic stem cells were injected into C57/BL6 blastocysts. Mutant mice were generated in a mixed genetic background (Balb/C, 129svJ, and C57BL/6). All procedures involving mice were done in accordance with the NIH guidelines for care and use of live animals and were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania Institutional Animal Care and Use Committee.

The primer sequences used to detect 2-lox, 1-lox, and the wild-type alleles for Vhlh, Hif-1x, and Arnt have been previously described (32).

DNA and RNA isolation. Mouse tail DNA was isolated according to Laird et al. (33) and used for genomic PCR. DNA and RNA from mouse tissues was isolated using Trizol reagent according to the guidelines of the manufacturer (Invitrogen, Carlsbad, CA).

Immunohistologic analysis. Kidneys were fixed overnight in 10% phosphate-buffered formalin and processed for routine stains in paraffin. Paraffin sections were incubated with the following primary antibodies: antihuman Tamm-Horsfall glycoprotein (THP) 1:1,000 (Biomedical Technologies, Inc., Stoughton, MA), vimentin 1:150 (Novus Biologicals, Littleton, CO), vascular endothelial growth factor (VEGF) 1:100 (Lab Vision, Fremont, CA), and Ki67 1:250 (Abcam, Cambridge, MA). Tissue sections were incubated for 1 hour at room temperature with primary antibody followed by incubation with the secondary anti-rabbit antibody 1:200 (Vector Laboratories, Burlingame, CA) for 30 minutes at 37°C. Negative controls for all samples were done using the secondary antibody alone. Antigen-antibody complexes were visualized using the VECTASTAIN ABC system (Vector Laboratories) and DAB Substrate Kit for Peroxidase (Vector Laboratories) following the protocols of the manufacturer. Staining with fluorescein-conjugated lectins Lotus tetragonolobus 1:100 (LTA, Vector Laboratories) and Griffonia bardeiniae simplicifolia II 1:50 (BSAII, Vector Laboratories) was done on rehydrated slides for 4 hours at room temperature followed by a 4′,6-diamidino-2-phenylindole counterstain and treatment with Sudan black. HE staining was done using standard procedures. LacZ staining was done on tissues sections frozen in OCT and stained with X-gal 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (β-gal) at 37°C (34).

Laser capture microdissection and PCR amplification. Mouse kidney sections 6 μm in thickness were cut onto nuclease-free Membrane Slides for Laser microdissection (Molecular Machines and Industries, Fremont, CA), and Ki67 1:250 (Abcam, Cambridge, MA). Tissue sections were incubated for 1 hour at room temperature with primary antibody followed by incubation with the secondary anti-rabbit antibody 1:200 (Vector Laboratories, Burlingame, CA) for 30 minutes at 37°C. Negative controls for all samples were done using the secondary antibody alone. Antigen-antibody complexes were visualized using the VECTASTAIN ABC system (Vector Laboratories) and DAB Substrate Kit for Peroxidase (Vector Laboratories) following the protocols of the manufacturer. Staining with fluorescein-conjugated lectins Lotus tetragonolobus 1:100 (LTA, Vector Laboratories) and Griffonia bardeiniae simplicifolia II 1:50 (BSAII, Vector Laboratories) was done on rehydrated slides for 4 hours at room temperature followed by a 4′,6-diamidino-2-phenylindole counterstain and treatment with Sudan black. HE staining was done using standard procedures. LacZ staining was done on tissues sections frozen in OCT and stained with X-gal 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (β-gal) at 37°C (34).

Real-time PCR analysis. cDNA was synthesized from 1 μg DNA (Invitrogen) treated RNA isolated from mouse renal cortex and liver using SuperScript first-strand synthesis system for reverse-transcription PCR (Invitrogen). One microliter of cDNA was subjected to PCR amplification using either SYBR Green PCR Master Mix or Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The following primer and probe sets were used to amplify specific target genes. SYBR Green primers; carbonic anhydrase 9 (Car9), forward 5′-CGTATTTCCTGGCTAACGTGA-3′; reverse 5′-GGAAGGAAGGCTCATTGTCAT-3′, multidrug resistance gene 1 (Mdr1); forward 5′-TCCACAGAGAGAGCAGACAG-3′; reverse 5′-CCAGGAGGGACATCTCTTACCA-3′. Primers for Vegfa, Epo, Bnip3, and 18S were previously published (32). PCR amplification was done on the ABI Prism 7700 Sequence Detection System (Applied Biosysytems) as previously described (32). 18S was used to normalize mRNA. Relative quantitation of mRNA expression levels was determined using the relative standard curve method according to the instructions of the manufacturer (Applied Biosysytems).

Statistical analysis. For statistical analysis and comparison of number of renal cysts in PEPCK-Vhlh and PEPCK-Vhlh/Hif-1x mutant mice, the χ2 test was done. For statistical analysis of gene expression data, ANOVA followed by the unpaired Student’s t test was done. P < 0.05 were considered statistically significant.

Results

Generation of mice with Cre-recombienase activity in renal proximal tubule cells. To express Cre-recombienase in the renal proximal tubule, we generated mice with Cre-recombienase expression under control of the rat cytosolic PEPCK promoter. PEPCK catalyzes the first step of gluconeogenesis converting oxaloacetate to phosphoenolpyruvate and is predominantly expressed in peritubular hepatocytes and in renal proximal tubules (for review, see refs. 35, 36). The PEPCK-Cre transgene was generated using a mutated version of the PEPCK promoter, which reduces PEPCK expression in the liver by 60% and increases PEPCK expression in the kidney by 10-fold in transgenic mice (37). The PEPCK-Cre transgene was inserted as a single-copy transgene on the X-chromosome upstream of the HPRT promoter by homologous recombination in embryonic stem cells (Fig. 1A). Embryonic stem cells that underwent homologous recombination and carried the PEPCK-Cre transgene were identified by Southern blot analysis and were injected into C57/BL6-derived blastocysts (Fig. 1B). Analysis of PEPCK-Cre expression in adult male mice with ROSA26-driven LacZ Cre reporter mice showed that PEPCK-Cre is expressed in the renal cortex, outer renal medulla, and in a subset of peritubular hepatocytes (Fig. 1C; ref. 32). Colocalization of LacZ expression with expression of renal proximal tubule markers LTA and BSAII lectins, which localize to the brush border of the S1, S2, S3, or S3 segments of the proximal tubule, respectively (38), revealed that PEPCK-Cre is expressed in most S1 and S2 segments and all S3 segments of the renal proximal tubule (Fig. 1D). Localization of LacZ expression with THP expression in both 2- and 24-month-old PEPCK-Cre/ROSA LacZ mice (n = 4) revealed that PEPCK-Cre expression does not overlap with THP expression, indicating that PEPCK-Cre is not expressed in the early renal distal tubule or the medulla thicck ascending limb of Henle (Fig. 1E; ref. 39).

Efficient deletion of Vhlh, Vhlh/Hif-1x, and Vhlh/Arnt in the renal cortex of PEPCK-Cre mutant mice. VHL-deficient CCRCC is associated with HIF activation and requires HIF for maintenance of tumor growth in nude mouse xenograft assays (18, 19, 21–23). In situ, CCRCC arise from cells lining premalignant renal cysts whose cellular origin remains controversial (40, 41). Morphologic and immunohistochemical analyses of renal cysts and tumors have shown that they express molecular markers from cortical distal and proximal tubule cells (18, 24–26). To investigate the functional consequences of pVHL and pVHL/HIF deletion in the proximal tubule, we inactivated Vhlh, Vhlh, and Hif-1x, or Vhlh and Arnt using PEPCK-Cre-mediated recombination. Mouse harboring conditional alleles for Vhlh, Vhlh and Hif-1x, or Vhlh and Arnt were bred to mice containing the PEPCK-Cre transgene generating mice deficient for

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Vhlh (PEPCK-Vhlh), Vhlh and Hif-1α (PEPCK-Vhlh/Hif-1α), or Vhlh and Arnt (PEPCK-Vhlh/Arnt) in PEPCK-Cre-expressing cells. The Vhlh conditional allele (2-lox) contains loxP sites flanking the promoter and exon 1 of Vhlh, resulting in complete ablation of Vhlh gene transcription upon Cre-mediated recombination (Fig. 2A; ref. 27). The conditional alleles for Hif-1α (2-lox) and Arnt (3-lox) contain loxP sites flanking the exon containing the basic helix-loop-helix domain (Fig. 2A). Cre-mediated recombination of the Hif-1α and Arnt conditional alleles results in an out-of-frame deletion and absence of HIF-1α or ARNT protein (28, 29).

The PEPCK-Cre transgene is expressed in a tissue-specific pattern similar to endogenous PEPCK and mediates efficient recombination of the Vhlh, Hif-1α, and Arnt conditional alleles. Recombination of the Vhlh conditional allele was readily detectable by genomic PCR in the liver and kidney and weakly detectable in the small intestine of PEPCK-Vhlh mutant mice (Fig. 2B). Similarly, recombined (1-lox) alleles for Vhlh, Hif-1α, and Arnt were detected in the renal cortex of PEPCK-Vhlh/Hif-1α and PEPCK-Vhlh/Arnt mutant mice (Fig. 2C).

Differential expression of HIF target genes in the renal cortex of PEPCK-Vhlh mutant mice. Inactivation of pVHL results in constitutive HIF stabilization and increased expression of HIF target genes (6). To determine HIF target gene expression levels in the renal cortex of PEPCK-Cre mutant mice, we performed real-time PCR analysis on RNA isolated from the renal cortex of PEPCK-Vhlh, PEPCK-Vhlh/Hif-1α, and PEPCK-Vhlh/Arnt mutant mice. Because PEPCK-Cre is integrated on the X-chromosome and is subject to X-chromosome inactivation in heterozygous female mice, we analyzed gene expression levels in 2-month-old male PEPCK-Cre mutant mice with normal-appearing renal parenchyma. Real-time PCR analysis revealed that mRNA transcripts for a subset of HIF target genes that are preferentially regulated by HIF-1α, including Car9, Mdr1, and proapoptotic Bnip3 (20, 42, 43), were significantly increased in the renal cortex of PEPCK-Vhlh mutant mice, whereas mRNA transcript levels in PEPCK-Vhlh/Hif-1α and PEPCK-Vhlh/Arnt mutant mice were comparable with control (Cre−) mice (Fig. 3A). A subset of HIF target genes that are preferential HIF-2 targets, including transforming growth factor α, cyclin D1, and Vegf...
Patients with VHL disease can develop polycythemia that is associated with EPO production from renal cell carcinomas, pheochromocytomas, or hemangioblastomas (41). We recently reported that 100% of PEPCK-Vhlh mutant mice develop polycythemia by 2 months of age. The development of polycythemia in PEPCK-Vhlh mutant mice occurs in an ARNT-dependent and HIF-1α-independent manner and is associated with increased EPO expression in the liver (32). To investigate whether the development of polycythemia in PEPCK-Vhlh mutant mice also correlates with increased EPO expression in the kidney, we determined Epo mRNA transcript levels in the renal cortex of 2-month-old PEPCK-Cre mutant mice by real-time PCR. As expected, control and PEPCK-Vhlh/Arnt mice expressed Epo at low basal levels in the kidney and at undetectable levels in the liver, whereas Epo levels in the renal cortex seemed suppressed although they did not statistically significantly change compared with control mice (Fig. 3B). Inactivation of Vhlh and Vhlh/Hif-1α in PEPCK-Cre mutant mice resulted in increased Epo mRNA transcript levels in the liver, whereas Epo levels in the renal cortex seemed suppressed although they did not statistically significantly change compared with control mice (Fig. 3B). Our data suggest that the development of polycythemia in PEPCK-Vhlh mutant mice is a result of HIF-2-mediated Epo expression in the liver and that inactivation of Vhlh in the renal proximal tubule is not sufficient to activate Epo transcription. Furthermore, our data support the notion that fibroblast-like renal interstitial cells are the cells that physiologically produce EPO in the adult kidney and that EPO expression in CCRCC, which are derived from renal epithelial cells, most likely requires additional genetic changes (44–46).

Renal cyst development in PEPCK-Vhlh mutant mice was ARNT dependent. To investigate the effects of PEPCK-Cre-mediated inactivation of pVHL and HIF in the renal proximal tubule, we examined the kidneys of PEPCK-Vhlh, PEPCK-Vhlh/Hif-1α, and PEPCK-Vhlh/Arnt mutant mice macroscopically and histologically. For analysis, mice were divided into two age groups, 2 to 11 months of age (group 1) and 12 to 25 months of age (group 2).
We observed that both PEPCK-Vhlh and PEPCK-Vhlh/Hif-1α mutant mice developed unilateral and bilateral macroscopic renal cysts that varied in size and number between mice (Fig. 4A). The largest cyst observed encapsulated the entire kidney (Fig. 4A). Macroscopic cysts were observed in 18% (4 of 22) of PEPCK-Vhlh mutant mice and in 30% (3 of 10) of PEPCK-Vhlh/Hif-1α double mutants >12 months of age, resulting in no statistically significant difference ($P = 0.453$) in the incidence of macroscopic cysts between the two mutant strains (Fig. 4B). Macroscopic cysts were not observed in age-matched PEPCK-Vhlh/Arnt or Cre-negative littermate controls.

In addition to macroscopic cysts, multiple microcysts were found in the kidneys of PEPCK-Vhlh and PEPCK-Vhlh/Hif-1α mutant mice. On morphologic examination, microcysts were found to be either of cortical tubular or glomerular in origin. Tubular and glomerular microcysts were defined as cystic structures >80 μm in diameter. In addition, glomeruli were considered cystic if the tuft occupied ≤25% of the cystic lumen. Tubular cysts were typically lined by a single layer of cuboidal eosinophilic epithelial cells, whereas glomerular cysts were lined by a layer of flat cells analogous to parietal epithelial cells of normal glomeruli (Fig. 5A). The majority of epithelial cells lining microcysts seemed morphologically normal, did not exhibit changes in nuclear morphology or nuclear to cytoplasmic ratio, and did not show evidence of CCRCC. Both types of microcysts were surrounded by otherwise normal-appearing nephron architecture and were not associated with tubulo-interstitial fibrosis. Other pathologic findings in PEPCK-Vhlh kidneys included vascular ectasia, dilated lymphatic vessels, and pockets of inflammation (data not shown).

Tubular microcysts were observed in 25% (5 of 20) of PEPCK-Vhlh and in 30% (3 of 10) of PEPCK-Vhlh/Hif-1α mutant mice. Glomerular cysts were observed in 35% (7 of 20) of PEPCK-Vhlh and in 70% (7 of 10) of PEPCK-Vhlh/Hif-1α mutant mice at >12 months of age (Fig. 5B). Statistical analysis indicated that there was no significant difference in the incidence of either tubular or glomerular cysts between the two mutant strains. The development of tubular and glomerular microcysts was not mutually exclusive, as 6 of 16 mice developed both types of microcysts. One control and one PEPCK-Vhlh mutant mouse (>18 months of age) developed end-stage renal disease and were omitted from further analysis. Renal cysts were observed in 35% (7 of 20) of PEPCK-Vhlh and in 70% (7 of 10) of PEPCK-Vhlh/Hif-1α mutant mice at >12 months of age (Fig. 5B). Statistical analysis indicated that there was no significant difference in the incidence of either tubular or glomerular cysts between the two mutant strains. The development of tubular and glomerular microcysts was not mutually exclusive, as 6 of 16 mice developed both types of microcysts. One control and one PEPCK-Vhlh mutant mouse (>18 months of age) developed end-stage renal disease and were omitted from further analysis. Renal cysts were observed in 35% (7 of 20) of PEPCK-Vhlh and in 70% (7 of 10) of PEPCK-Vhlh/Hif-1α mutant mice at >12 months of age (Fig. 5B). Statistical analysis indicated that there was no significant difference in the incidence of either tubular or glomerular cysts between the two mutant strains. The development of tubular and glomerular microcysts was not mutually exclusive, as 6 of 16 mice developed both types of microcysts. One control and one PEPCK-Vhlh mutant mouse (>18 months of age) developed end-stage renal disease and were omitted from further analysis. Renal...
Renal Cyst Development in VHL-Deficient Mice

Cysts were not observed in PEPCK-Vhlh/Arnt mutant mice or control mice. Taken together, our data suggest that PEPCK-Cre-mediated inactivation of pVHL predisposes mice to the development of glomerular and tubular renal cysts that are associated with otherwise normal-appearing tubular architecture. Furthermore, we conclude that renal cyst development is not dependent on renal HIF-1α.

Renal cysts in PEPCK-Vhlh mutant mice are pVHL deficient and express molecular markers associated with multiple nephron segments. To determine if renal tubular cysts in PEPCK-Vhlh mutant mice are Vhlh deficient, we examined whether cysts have undergone Cre-mediated recombination of the Vhlh conditional allele by laser capture microdissection. PCR analysis of DNA isolated from six captured microcysts revealed that the Vhlh recombined (1-lox) allele was present, indicating that tubular microcysts in PEPCK-Vhlh mutant kidneys have lost expression of Vhlh (Fig. 6A and B; data not shown). The epithelial lining of glomerular cysts was difficult to capture and therefore could not be analyzed.

Because PEPCK-Cre is expressed in the renal proximal tubule, we next investigated whether tubular microcysts express proximal tubule–specific molecular markers. For immunohistochemical analysis, kidney sections from three PEPCK-Vhlh mutant mice were stained and a total of 20 tubular microcysts were analyzed. Sections were first stained with the fluorescein-labeled lectins LTA and BSAlAII. Surprisingly, we only observed LTA staining in one small microcyst, and BSAlAII was absent from all cysts, suggesting that <5% of tubular cysts express markers of the proximal tubule (Fig. 6C; data not shown). We next examined the expression of vimentin, a mesenchymal marker that is normally not expressed by differentiated tubular epithelial cells but has been found in VHL-associated renal cysts and in regenerating nephron segments after injury, indicating dedifferentiation (18, 26, 47). We observed that 35% of tubular microcysts expressed vimentin, suggesting that epithelial cells that line tubular microcysts may be dedifferentiated (Fig. 6D), thus providing one potential explanation for the absence of LTA expression in renal tubular cysts. A number of renal cysts examined expressed neither LTA nor vimentin. Because VHL-associated renal cysts were found to express both proximal and distal tubule markers, we examined cysts for expression of THP, which labels the thick ascending limb of Henle and early distal tubules (39). THP was expressed in 11 of 20 cysts, of which eight were vimentin negative and three were vimentin positive (Fig. 6E). These findings were unexpected as PEPCK-Cre expression in mice with the ROSA26-driven LacZ Cre reporter did not co-localize with expression of THP (Fig. 1E). We cannot, however, rule out the possibility that rare cells derived from the distal tubule expressed PEPCK-Cre in mice that developed renal cysts. Nevertheless, our data show that inactivation of Vhlh in renal epithelial cells renders mice susceptible to the development of macroscopic and microscopic renal cysts.

Our data from PEPCK-Vhlh/HIF-1α mutant mice suggests that HIF-1α does not contribute to renal cyst development. To investigate the role of HIF-2α in renal cyst development, we examined HIF-2α protein levels in PEPCK-Vhlh mutant kidneys. Although we were able to detect HIF-2α in Vhlh-deficient livers by immunoblot (32), we were unable to detect increased levels of HIF-2α in renal cortex homogenates from PEPCK-Vhlh mutant mice (data not shown). We next examined HIF-2α expression by immunohistochemistry. In our hands, immunohistochemistry was not sensitive enough to reliably detect HIF-2α in Vhlh-deficient tissues, including the liver and kidney. Therefore, we were unable to conclusively determine whether HIF-2α is expressed in the renal tubular cysts.

Given that loss of VHL results in HIF stabilization and increased HIF target gene expression in CCRCC and associated renal cysts (18), we examined the expression of the global HIF target VEGF in tubular microcysts by immunohistochemistry. We observed that, in addition...
to normal VEGF expression in the outer medulla (48), four of nine cysts in one of the three mice examined expressed VEGF (Fig. 6F). Because renal cysts in VHL patients exhibit increased rates of proliferation compared with normal renal tubule cells (18), we assessed the expression of the cellular proliferation marker Ki67. We found that Ki67 was expressed in microcysts from all three mice (66% of microcysts) and observed an average 3-fold increase in the percentage of epithelial cells that expressed Ki67 in tubular cysts compared with normal-appearing renal tubules (Fig. 6G). From these studies, we conclude that inactivation of Vhlh in PEPCK-Cre mutant mouse results in the development of tubular microcysts that are pVHL deficient, express HIF target genes and protein markers associated with multiple segments of the nephron, and display evidence of dedifferentiation and increased proliferation.

Discussion

Besides its role in VHL-associated renal cancer, pVHL is an important gatekeeper in the pathogenesis of sporadic CCRCC, the most common form of kidney cancer (49). In the United States, ~30,000 patients are diagnosed annually with CCRCC and therapeutic options are limited (25). VHL-associated CCRCC is often preceded by multifocal and bilateral neoplastic renal cysts and are commonly thought to originate from the renal proximal tubule (24, 25).

Here, we show that mice with conditional inactivation of pVHL mediated by PEPCK-Cre are prone to the development of renal cysts, a pathologic finding that is associated with both sporadic and hereditary forms of CCRCC. PEPCK-Vhlh mutant mice develop both glomerular and tubular renal cysts. Although glomerular cysts are usually not found in VHL patients, tubular cysts in PEPCK-Vhlh mutant mice share morphologic and molecular features with renal cysts found in VHL patients. Morphologically, tubular microcysts in PEPCK-Cre mutant mice were lined by an eponophilic cuboidal epithelium that appeared benign and did not display evidence of atypia or nuclear abnormalities. Renal cysts in VHL patient kidneys are classified into three histopathologic categories: benign, atypical, and malignant (50, 51). Eleven percent of renal cysts in VHL patient kidneys show morphologic features of malignancy; the majority of renal cysts, however, are benign and lined by cells that on histologic examination have clear cell morphology or appear eosinophilic (26, 50). “Clearing” is a common feature of VHL-associated cysts and renal cell carcinoma, and it has been proposed that cysts lined by clear cells represent neoplastic lesions (50). Although loss of pVHL function is a frequent event in CCRCC, other genetic events appear to be required for progression of renal cysts into CCRCC, which could be one explanation why PEPCK-Vhlh mutant mice do not develop CCRCC (49).

In addition to morphologic features, renal cysts in our model share molecular features with VHL-associated renal cysts. We found that the tubular cysts in PEPCK-Vhlh mutant mice expressed protein markers of both proximal and distal tubules. Surprisingly, the majority of cysts expressed the distal tubule marker THP. Although localization of PEPCK-Cre with THP in PEPCK-Cre/ROSA LacZ mice suggested that the PEPCK-Cre transgene is not expressed in THP-positive cells, we cannot rule out the possibility that rare cells derived from the distal tubule expressed the PEPCK-Cre transgene in mice that developed renal cysts. It is interesting to note that renal lesions in VHL patients were found to express molecular markers from the renal proximal and distal tubule (18, 24, 26). A recent report by Mandriota et al. (18) suggested that in kidneys from VHL patients single-cell foci of HIF-target gene expression were frequently found in proximal tubular cells. On the other hand, “early” multicellular lesions appeared to be more frequently derived from the distal tubule based on expression of THP. Additionally, we found that renal tubular cells in PEPCK-Vhlh mutant mice display evidence of dedifferentiation and increased proliferation as suggested by expression of vimentin and increased staining for Ki-67, thus further recapitulating histologic findings from cystic lesions in kidneys from VHL patients (18, 24, 26).

Renal cyst development in our model occurs in an ARNT-dependent and HIF-1α-independent manner. These data suggest a role for HIF-2α in VHL-associated renal cystogenesis. Although we were able to detect HIF-2α in Vhlh-deficient livers by immunoblot (32), we were unable to detect increased HIF-2α in renal cortex homogenates from PEPCK-Vhlh mutant mice. Immunohistochemical studies with available antibodies were not sensitive enough to detect HIF-2α in Vhlh-deficient tissues, including the liver and kidney. Therefore, we were unable to determine whether HIF-2α is increased in renal cysts and thus had contributed to cystogenesis. Alternatively, if renal cysts in our model did not express HIF-2α, our data would suggest that hepatic HIF-2α could mediate activation of EPO. EPO has been shown to be a mitogen in CCRCC cell lines, raising the possibility that EPO could have provided a growth stimulus for cystogenesis in Vhlh-deficient renal tubules (53). However, the presence of elevated serum EPO or polycythemia alone does not seem to be sufficient for renal tubular cyst development in kidneys with wild-type VHL, suggesting that VHL deficiency in renal epithelia is required for cyst development.

In conclusion, we show that conditional inactivation of VHL in mice results in the development of renal cysts, thus generating the first mouse model of VHL-associated renal disease. Additionally, we provide genetic evidence that renal cyst development is HIF-1α independent. This novel model will provide a basis for further genetic studies to define the molecular events that are required for the progression of VHL-associated renal cysts to CCRCC.

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Erinn B. Rankin, John E. Tomaszewski and Volker H. Haase


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