Formation of Renal Cysts and Tumors in Vhl/Trp53-Deficient Mice Requires HIF1α and HIF2α

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Abstract

The von Hippel–Lindau (VHL) tumor suppressor gene is inactivated in the majority of clear cell renal cell carcinomas (ccRCC), but genetic ablation of Vhl alone in mouse models is insufficient to recapitulate human tumorigenesis. One function of pVHL is to regulate the stability of the hypoxia-inducible factors (HIF), which become constitutively activated in the absence of pVHL. In established ccRCC, HIF1α has been implicated as a renal tumor suppressor, whereas HIF2α is considered an oncprotein. In this study, we investigated the contributions of HIF1α and HIF2α to ccRCC initiation in the context of Vhl deficiency. We found that deleting Vhl plus Hif1α or Hif2α specifically in the renal epithelium did not induce tumor formation. However, HIF1α and HIF2α differentially regulated cell proliferation, mitochondrial abundance and oxidative capacity, glycogen accumulation, and acquisition of a clear cell phenotype in Vhl-deficient renal epithelial cells. HIF1α, but not HIF2α, induced Warburg-like metabolism characterized by increased glycolysis, decreased oxygen consumption, and decreased ATP production in mouse embryonic fibroblasts, providing insights into the cellular changes potentially occurring in Vhl mutant renal cells before ccRCC formation. Importantly, deletion of either Hif1α or Hif2α completely prevented the formation of renal cysts and tumors in Vhl/Trp53 mutant mice. These findings argue that both HIF1α and HIF2α exert protumorigenic functions during the earliest stages of cyst and tumor formation in the kidney. Cancer Res; 76(7); 2025–36. ©2016 AACR.

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most frequent renal malignancy, and up to 92% of ccRCC tumors harbor biallelic inactivation of the von Hippel–Lindau (VHL) tumor suppressor gene (1). VHL mutations occur at the earliest stage of tumor formation (2). The absence of pVHL function is clearly necessary for the growth of fully transformed ccRCC cell lines as xenografts (3). However, the fact that the frequency of VHL mutant single epithelial cells vastly outweighs the number of ccRCC tumors in kidneys of familial VHL disease patients (4) and the absence of tumors in a variety of renal epithelial cell–specific Vhl knockout mice (reviewed in ref. 5) argues that ccRCC formation requires mutations in addition to VHL. There are currently no autochthonous mouse models that fully reproduce all of the characteristic morphologic and invasive properties of human ccRCC; however, consistent with the hypothesis that multiple cooperating mutations are required for ccRCC development, kidney epithelial cell–specific codelletion of Vhl with Pten (6), or with Kif3a to genetically ablate primary cilia (7), caused the formation of simple and atypical cystic lesions that are similar to the ccRCC precursor cystic lesions found in the kidneys of patients with inherited VHL disease. Deletion of Vhl together with homozygous loss of Trp53 (8) or with heterozygous loss of Bap1 (9) gave rise to very similar phenotypes, including simple and atypical cystic lesions as well as tumors containing cells that display cytoplasmic clearing and elevated mTORC1 activity, recapitulating many of the cellular and molecular changes that are characteristic of human ccRCC.

pVHL controls many biological activities, including regulation of the stability of the hypoxia-inducible transcription factors α (HIF1α, HIF2α, and HIF3α, collectively HIFα; ref. 10), regulation of NF-kB activity (11), maintenance of the primary cilium (12), activation of p53 (13), secretion of extracellular matrix components (14), promotion of DNA double-strand repair (15), regulation of the plane of cellular division (16, 17), and suppression of aneuploidy (16, 18). The combined loss of several or all of pVHL’s functions could contribute to tumor initiation and progression. Loss of function mutations of VHL occur in ccRCC mutually exclusively to rarer mutations in TCEB1, encoding Elongin C, and collectively these cause constitutive stabilization of HIFα in up to 95% of ccRCC tumors (1), implying that HIFα activation is a major oncogenic driving force in ccRCC. HIF1α and HIF2α appear...
to play opposite roles in determining the aggressiveness of established ccRCCs; HIF1a inhibits, whereas HIF2a promotes tumor formation in ccRCC xenografts (19–21), copy loss of the chromosomal locus harboring the HIF1A gene predicts poor patient outcome (22), and sporadic ccRCCs expressing HIF1a and HIF2a exhibit lower proliferation rates than ccRCCs expressing only HIF2a (23). ccRCC cell lines frequently express only HIF2a and do not express functional HIF1a due to biallelic alterations of the HIF1A locus (24). Knockdown of HIF1A in ccRCC cell lines that express both HIF1a and HIF2a promotes xenograft tumor formation (24). These studies argue that whereas HIF2a activity is tumor promoting, there is a selection against HIF1a expression or activity during the progression of some cases of ccRCC. On the other hand, because HIF1a is strongly expressed in single and multicellular clusters of VHL-null cells and in cystic lesions in VHL patients (4), and because transgenic overexpression of HIF1a in mouse proximal tubular epithelial cells causes a clear cell appearance, increased proliferation, and a disorganized tubular morphology (25), and because many human ccRCCs express HIF1a and HIF1a target genes (26), it may also be argued that HIF1a plays an important role in promoting ccRCC development. These studies have largely focused on fully transformed, genetically complex ccRCC cell lines derived from advanced tumors, and it remains unclear if and how HIF1a and HIF2a contribute to the earliest stages of initiation of ccRCC.

An early event following VHL mutation is likely to be a profound alteration of cellular metabolic pathways. Numerous HIF1a and/or HIF2a-dependent metabolic changes occur in ccRCC cell lines, including elevated glucose uptake and conversion to lactate with a concomitant reduction in mitochondrial oxidation of pyruvate, reduced mitochondrial biogenesis, reduced mitochondrial complex I activity, altered cytochrome c oxidase activity, increased pentose phosphate pathway flux, decreased oxidative glutaminolysis, and increased lipogenesis through reductive glutamine metabolism (27–38). Because ccRCC cell lines have complex genetic backgrounds that arose during tumor evolution, it is possible that some of these metabolic changes might be driven by late-occurring mutations that further modify metabolic pathways and allow ccRCC cells to proliferate efficiently in the metabolic environment imposed by constitutive HIF1a activity. For example, ccRCCs can harbor activating mutations in the PI3K–mTORC1 pathway, inactivating TPS3 mutations or can exhibit high levels of MYC expression or decreased fructose-1,6-bisphosphatase 1 (FBP1) expression. These studies argue that whereas HIF2a promotes tumor formation in ccRCC xenografts (19–21), copy loss of the chromosomal locus harboring the HIF1A gene predicts poor patient outcome (22), and sporadic ccRCCs expressing HIF1a and HIF2a exhibit lower proliferation rates than ccRCCs expressing only HIF2a (23). ccRCC cell lines frequently express only HIF2a and do not express functional HIF1a due to biallelic alterations of the 

**Materials and Methods**

**Mouse strains**

Hif1afl/fl (40) and Hif2afl/fl (41) mice were crossed with Ksp1.3-Cre;Vhlfl/fl (6), Ksp1.3-Cre;Vhlfl/fl;Trp53fl/fl (8), or Ksp1.3-Cre/+ (42) mice. Littermate mice that lacked the Cre transgene served as wild-type controls.

**Immunohistochemistry**

Immunohistochemistry and immunofluorescence of formalin-fixed paraffin-embedded tissues were performed as previously described (6, 8) using antibodies listed in Supplementary Materials and Methods.

**Respirometry**

Biopsies (1 mm³) of renal cortex and medulla were assayed for oxygen consumption following the addition of different respiratory substrates and inhibitors using high-resolution respirometry (Oxygraph-2k, OROBOROS INSTRUMENTS Corp) according to the protocol described in Supplementary Materials and Methods.

**Microarrays**

Primary adult mouse renal epithelial cells were cultured for 3 days and infected with Adeno-GFP (Vector Biolabs, 1060) or Adeno-Cre (Vector Biolabs, 1700) as described (8). mRNA was isolated 4 days after adenovirus infection, and Cy3- and Cy5-labeled cDNA from GFP and Cre samples was competitively hybridized to Mouse GE 4 × 44 K v2 Microarrays (Agilent). Dye swap experiments were used to control potential dye-specific hybridization effects. Analysis using R/Bioconductor package limma was based on the average fold expression level changes (Cre/GFP) of three independent biologic experiments. Microarray data are accessible through GEO number GSE75745.

**Histological stains**

Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stains were conducted using standard protocols. For the visualization of lipids, frozen kidney sections were fixed with 10% formalin for 15 minutes, washed in tap water, equilibrated in 60% isopropanol, and stained with Oil Red O (3 mg/mL in 60% isopropanol) overnight at 4°C. After rinsing in 60% isopropanol, sections were washed with water, counterstained with hematoxylin, and mounted in Mowiol.

**Real-time PCR**

RNA isolation, cDNA preparation, and real-time PCR were performed as described (6) using primers listed in Supplementary Materials and Methods.

**Mouse embryo fibroblasts**

Mouse embryo fibroblasts (MEF) were isolated from E13.5 embryos of relevant non-Cre-expressing floxed mouse strains or C57BL/6 embryos (for wild-type MEFs) and cultured as previously described (8) at 5% O₂ and 5% CO₂. MEFs were infected with adenovirus expressing Cre recombinase and GFP (Ad-Cre-GFP, Vector Biolabs; #1700) or GFP only (Ad-CMV-GFP, Vector Biolabs; #1060). Detailed protocols for measuring ATP, extracellular acidification rate, oxygen consumption rate (OCR), lactate production, glucose utilization, and mitochondrial content are described in Supplementary Materials and Methods.

**Statistical analyses**

Unless otherwise stated, data are presented as mean ± SD, and statistical differences were assessed using the Student
viously reported hydronephrosis phenotype caused by Hif2a double deletion, fully rescued (data not shown) the previous morphology of kidneys. This phenotypic rescue will be described in detail elsewhere.

Results
Vhl deletion together with deletion of Hif1a or Hif2a does not cause renal tumors
Most cases of ccRCC appear to arise from the proximal tubule, although there is also evidence that some cases can arise from cells in other nephron segments (5). We employed the Ksp1.3-Cre transgene that induces epithelial cell–specific gene deletion widely throughout different segments of the nephron, including in a significant number of proximal tubular cells, as well as in epithelia of several genital tract tissues (42). We generated Ksp1.3-Cre;Vhlflfl (Vhlfl/fl, ref. 6), Ksp1.3-Cre;Hif1aflfl (Hif1afl/fl), Ksp1.3-Cre;Hif2aflfl (Hif2afl/fl), Ksp1.3-Cre;Vhlflfl;Hif1aflfl (Vhlfl/fl;Hif1afl/fl), and Ksp1.3-Cre;Vhlflfl;Hif2aflfl (Vhlfl/fl;Hif2afl/fl) mice to achieve renal epithelial-specific loss or constitutive stabilization of HIF1α and/or HIF2α (Fig. 1A and B). Immunohistochemical staining confirmed the expected stabilization of either or both HIF1α and HIF2α in the relevant Vhl mutant genotypes (Fig. 1C and D). All of the above-described mutant mice were analyzed at 6, 12, and 18 months of age. Neither Hif1a nor Hif2a deletion alone had any effect on the morphology of kidneys. Vhl/Hif1a double deletion, but not Vhl/Hif2a double deletion, fully rescued (data not shown) the previously reported hydronephrosis phenotype caused by Vhl deletion (6). This phenotypic rescue will be described in detail elsewhere.

Vhlfl/fl mice do not develop ccRCC precursor lesions or tumors (6). We reasoned that HIF1α might act as an antiproliferative factor that prevents tumor development following Vhl mutation. However, none of the genotypes exhibited cysts, dysplastic lesions, or tumors in the cortex, medulla, or papilla even when aged for 18 months. Tubular epithelial cells in Vhlfl/fl;Hif2afl/fl mice frequently displayed a highly unusual “optically-clear” nucleus, characterized by a thin ring of chromatin surrounding a nonstained region in the center (Supplementary Fig. S1). Papillary thyroid carcinomas display optically clear nuclei, but this histologic feature does not arise in ccRCCs. We conclude that the combination of loss of the many putative tumor suppressor functions of pVHL plus constitutive expression of the putative oncoprotein HIF2α plus the absence of the putative tumor suppressor activity of HIF1α does not cause cyst or tumor initiation.

Hif1a and Hif2a are both necessary for renal cyst and tumor formation caused by Vhl/Trp53 deletion
We next investigated potential requirements for Hif1a or Hif2a in the initiation of renal cysts and tumors in the Vhl/Trp53 double mutant background (8). We generated Ksp1.3-Cre;Vhlflfl;Trp53flfl, Hif1aflfl (Vhlfl/fl;Trp53fl/fl;Hif1afl/fl) and Ksp1.3-Cre;Vhlflfl;Trp53flfl; Hif2aflfl (Vhlfl/fl;Trp53fl/fl;Hif2afl/fl) mice (Fig. 2A and B) and confirmed HIF1α and HIF2α stabilization in the relevant genotypes (Fig. 2C and D). PCRs specific for the recombined Vhl and Trp53 alleles demonstrated that these genes were deleted efficiently in these mice (Supplementary Fig. S2). We analyzed cohorts at 6 and 12 months of age. Table 1 shows a summary of the incidence
Figure 2.
Deletion of $\text{Vhl}^-/\text{Trp53}^-\Delta/\Delta$, and $\text{Vhl}^-/\text{Trp53}^-/\text{Hif1a}^-\Delta/\Delta$, and $\text{Vhl}^-/\text{Trp53}^-/\text{Hif2a}^-\Delta/\Delta$ in renal epithelial cells. A and B, H&E stainings of renal cortex from mice aged 6 months (A) and 12 months (B). Arrowheads, disorganized tubules with clear cell cytoplasm; arrows, optically clear nuclei; Cy, cysts; Neo, neoplasm. C–E, immunohistochemical stainings for HIF1\text{α} (C), HIF2\text{α} (D), and GLUT1 (E) in the renal cortex. F, H&E stainings of vesicular glands from mice aged 6 months. G and H, H&E stainings of epididymides from mice aged 12 months.
of different phenotypes in each genotype, and Fig. 2 shows examples of these phenotypes. We previously demonstrated (8) that Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup> mice display hydropnephrosis, seminal vesicle developmental abnormalities (Fig. 2F), subterfity, and subvability, and by 12 months of age, these mice develop simple and atypical kidney cysts (Fig. 2B), kidney tumors (Fig. 2B), disorganized and multilayered epididymal epithelial cell growth (Fig. 2G), epididymal squamous metaplasia (Fig. 2H), and benign epithymal tumors at high penetrance. Among one third of these mice also develop a variety of genital-urinary tract carcinomas (8). All of these phenotypes were completely absent in Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>/Hif1a<sup>−/−</sup> mice. Six-month-old Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup> mice frequently displayed disorganized renal tubular epithelia with a clear cell appearance, but this phenotype was not present in Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>/Hif1a<sup>−/−</sup> mice (Fig. 2A). These mice developed only a few micro-cysts at a frequency that was not higher than littermate control mice (data not shown), and importantly no large renal cysts or tumors arose in mice aged 12 months (Fig. 2B, Table 1). Epididymides of Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>/Hif1a<sup>−/−</sup> mice displayed nuclear atypia and mild epithelial disorganization (Fig. 2G and H), similar to the phenotypes observed in younger Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup> mice (8), but did not develop epithelial dysplasia or squamous metaplasia. No malignant tumors were observed in any other genital-urinary tract organs in these mice (Table 1).

Thus, HIF1α activity is indispensable for the initiation of kidney cyst and tumor formation and for the formation of genital-urinary tract malignancies.

In contrast, Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>/Hif2a<sup>−/−</sup> mice displayed several identical phenotypes to Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup> mice, including hydropnephrosis (not shown), seminal vesicle developmental abnormalities (Fig. 2F), disorganized epididymal epithelial growth (Fig. 2G), and epididymal squamous metaplasia (Fig. 2H), although these epididymal phenotypes were present in only half of the mice (Table 1) and were much smaller lesions than those seen in Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup> mice. Similarly to Vhl<sup>−/−</sup> Trp53<sup>−/−</sup> mice, Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>/Hif2a<sup>−/−</sup> mice were also subfertile and subviable, with many mice dying at various ages, possibly due to kidney failure caused by excessive hydro nephrosis. While 6-month-old Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>/Hif2a<sup>−/−</sup> mice displayed disorganized renal tubular epithelium with optically clear nuclei (Fig. 2A), no large renal cysts or tumors (Fig. 2B), nor genital-urinary tract carcinomas were observed in 12-month-old mice. Thus, many of the preneoplastic and all of the neoplastic phenotypes resulting from combined deletion of Vhl and Trp53 are also dependent on HIF2α activity.

### Table 1. Summary of phenotypes in Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>, Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>/Hif1a<sup>−/−</sup> and Vhl<sup>−/−</sup>/Trp53<sup>−/−</sup>/Hif2a<sup>−/−</sup> mice aged 11 to 15 months

<table>
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<tr>
<th>Phenotype</th>
<th>Vhl&lt;sup&gt;−/−&lt;/sup&gt;/Trp53&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Vhl&lt;sup&gt;−/−&lt;/sup&gt;/Trp53&lt;sup&gt;−/−&lt;/sup&gt;/Hif1a&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Vhl&lt;sup&gt;−/−&lt;/sup&gt;/Trp53&lt;sup&gt;−/−&lt;/sup&gt;/Hif2a&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Number of renal cysts</td>
<td>399 (n = 30 kidneys)</td>
<td>15 (n = 30 kidneys)</td>
<td>10 (n = 22 kidneys)</td>
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<tr>
<td>Number of renal tumors</td>
<td>16 (n = 30 kidneys)</td>
<td>0 (n = 30 kidneys)</td>
<td>0 (n = 22 kidneys)</td>
</tr>
<tr>
<td>Number of genitourinary tract carcinomas</td>
<td>6 (n = 17 mice)</td>
<td>0 (n = 15 mice)</td>
<td>0 (n = 18 mice)</td>
</tr>
<tr>
<td>Incidence of epididymal dysplasia/squamous metaplasia</td>
<td>100% (n = 9 mice)</td>
<td>0% (n = 7 mice)</td>
<td>50% (n = 8 mice)</td>
</tr>
<tr>
<td>Incidence of seminal vesicle abnormality</td>
<td>100% (n = 9 mice)</td>
<td>0% (n = 7 mice)</td>
<td>0% (n = 8 mice)</td>
</tr>
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NOTE: Numbers of renal cysts and renal tumors are derived from analysis of midline longitudinal sections of n kidneys of each genotype. Numbers of genitourinary tract carcinomas represent the cumulative number of tumors arising in the epididymis, seminal vesicle, uterus, and urothelium of n mice of each genotype. The incidence of epididymal and seminal vesicle phenotypes represents the percentage of n mice of each genotype that displays the listed phenotypes.

HIF1α and HIF2α are Renal Oncogenes

To gain further molecular insights into HIF1α- and HIF2α-dependent processes in Vhl-null cells that might be necessary for tumor initiation, we developed a system to culture primary renal epithelial cells derived from kidneys of adult Vhl<sup>−/−</sup>, Vhl<sup>−/−</sup>/Hif2a<sup>−/−</sup>, Vhl<sup>−/−</sup>/Hif1a<sup>−/−</sup>, and Vhl<sup>−/−</sup>/Hif2a<sup>−/−</sup>Hif1a<sup>−/−</sup> mice. Epithelial morphology (Supplementary Fig. S3A), Cdhl1 mRNA expression (Supplementary Fig. S3B), and E-Cadherin protein expression (Supplementary Fig. S3C) were maintained for at least 12 days of culture, implying that the epithelial phenotype of these cells is largely preserved in this culture system. Cultures were infected with adenovirus expressing GFP as control or with adenovirus expressing Cre (Fig. 4A), and real-time PCR confirmed the deletion of the floxed genes (Supplementary Fig. S3D). Microarray comparisons of global mRNA expression 4 days after viral infection revealed a transcriptional signature induced by loss of Vhl that included 753 probes that were significantly (P < 0.05) upregulated more than 1.5-fold and 451 probes downregulated more than 1.5-fold. This transcriptional signature was used to probe mRNA expression data derived from human normal kidney and ccRCC samples (GSE17895). Unsupervised clustering using the Vhl-deletion gene signature accurately segregated human ccRCCs from normal kidney tissue (Supplementary Fig. S4), demonstrating that our culture system can identify...
ccRCC-relevant gene expression changes. We identified genes that were upregulated by loss of Vhl and that were dependent solely on Hif1α or solely on Hif2α or that remained upregulated when either Hif1α or Hif2α were deleted but not when both were deleted, indicating that they are targets of both HIF1α and HIF2α (Fig. 4B, Supplementary Table S1). While the Hif2α-dependent and Hif1α/Hif2α-dependent gene sets were not strongly enriched for sets of genes that participate in particular biological processes, the Hif1α-dependent target genes (Fig. 4C) encompassed numerous previously identified hypoxia and HIFα targets (Bnip3, Bnip3l, Egfb3, Loxl2, Akr1, Car5, Vegfa, P4ha2, Miff), including genes that regulate cellular metabolism (Supplementary Fig. S5) by promoting glycolytic flux (Sld2a1, Cpi1, Pfk1b, Pfkfb3, Aldoa, Aldc, Tp1, Cadph, Pdk1, Pgam1, Eno1), promoting glycolgenolysis (Pgul1, Pgm2), diverting glucose-derived carbon from mitochondrial oxidation to lactate production and excretion (Pdk1, Ldhα, Sclvha3), or regulating mitochondrial electron transport by inhibiting complex I (Ndufs1f2; ref. 36). Real-time PCR analyses of mRNA isolated from Vhl+/−, Vhl−/−/Hif1α−/−, and Vhl−/−/Hif2α−/− kidneys confirmed that several of these gene expression changes (Pkg1, Pdk1, Pfkfb3, Ldhα, Ndufs41f2), as well as upregulation of Cox2−2, which alters the efficiency of cytochrome c oxidase (37), also occur upon Vhl deletion in vivo and demonstrated that, with the exception of Ndufs41f2, all were strictly dependent on Hif1α and independent of Hif2α (Fig. 4D). A similar Hif1α-dependent, Hif2α-independent metabolic gene expression signature was observed in cultured primary renal epithelial cells (Supplementary Fig. S6A and S6B) and kidneys (Supplementary Fig. S6C) in the Vhl/Trp53 double mutant background, indicating that the induction of expression of these genes is independent of p53 function. Moreover, elevated GLIT1 protein expression was detected in renal tubules in Vhl+/−, Vhl+/−/Trp53+/−, Vhl+/−/Hif2α+/−, and Vhl+/−/Trp53+/−/Hif2α+/− mice but not in Vhl−/−/Hif1α−/− or Vhl−/−/Trp53−/−/Hif1α−/− mice (Figs. 1E and 2E).

Because this Hif1α-activated transcriptional signature predicted decreased flux of pyruvate into mitochondria and decreased mitochondrial electron transport (Supplementary Fig. S5), we developed a method to analyze mitochondrial respiration in biopsies of the renal cortex and medulla. Oxygen consumption resulting from various steps of mitochondrial oxidation was assessed by sequential addition of the following metabolic intermediates or inhibitors: (i) malate, octanoyl-carnitine, and ADP to determine medium chain fatty acid oxidation, (ii) pyruvate and glutamate to determine complex I-specific activity, (iii) succinate to determine total ATP synthase capacity, (iv) rotenone to inhibit complex I and determine complex II-specific activity, (v) antimycin A to block complex III, allowing the correction of residual O2 consumption, and (vi) N,N,N’,N’-tetramethyl-p-phenylene-diamine (TMPD) and ascorbate to determine maximal cytochrome c oxidase activity (complex IV). Vhl deletion caused a decrease in all of the assays of respiratory capacity in the medulla but not cortex of these mice (Fig. 4E), consistent with the fact that the majority of tubules in the medulla are null for Vhl and the fact that proximal tubular cells, where gene deletion occurs in a minority of cells, are the most abundant cell type in the cortex. These effects were completely rescued in Vhl−/−/Hif1α−/− but not in Vhl−/−/Hif2α−/− kidneys (Fig. 4E), consistent with the Hif1α dependency of the gene expression changes. Because Hif1α has been shown to decrease mitochondrial abundance via multiple mechanisms (32, 43), we analyzed mitochondrial abundance in AQP2 expressing duct principal cells using immunofluorescence for the mitochondrial outer membrane protein...
TOM20. Reduced TOM20 staining intensity was observed in these cells in Vhl/+/ and in Vhl/Hif1a+/ kidneys, whereas Vhl/Hif1a+/ kidneys exhibited increased TOM20 staining (Fig. A–I and U), consistent with the measures of cellular respiration. Similar results were obtained in the Vhl/Trp53 mutant background (Supplementary Fig. S7E–S7M). We conclude that HIF1α but not HIF2α stabilization decreases mitochondrial abundance and oxidative capacity independently of p53 function in Vhl mutant renal tubular cells in vivo.

We next investigated whether the characteristic clear cell phenotype of ccRCC, believed to be caused by the cytoplasmic accumulation of lipids and glycogen, was also dependent on HIF1α. Indeed, tubular epithelial cells in Vhl/+, Vhl/Hif1a+/-, Vhl/Hif2a+/-, and Vhl/Trp53/+/Hif1a+/- and Vhl/Trp53/+/Hif2a+/- mice, but not in Vhl/Hif1a+/ and Vhl/Trp53/+/Hif1a+/ kidneys, frequently demonstrated clear cell morphology (Figs. 5I–L and 2A–D). Tubules in Hif1a+/- and Hif2a+/- mice did not display morphologic alterations (Supplementary Fig. S1). Tubules in Vhl/+, Vhl/Hif1a+/-, Vhl/Hif2a+/-, Vhl/Trp53/+/Hif1a+/-, Vhl/Trp53/+/Hif2a+/-, and Vhl/Trp53/+/Hif2a+/- kidneys all displayed strong accumulation of cytoplasmic glycogen droplets as assessed by PAS staining (Fig. 5M), suggesting that this is not the cause of glycogen accumulation. Surprisingly, no accumulation of lipids was observed in tubular cells in any genotype, as assessed by Oil Red O (Fig. 5Q–T) and Nile red (data not shown).

Figure 4.
HIF1α stabilization inhibits mitochondrial oxidation in Vhl-deficient cells. A, workflow for microarray analysis of mRNA expression 4 days after deletion of Vhl alone or together with Hif1a and/or Hif2a in primary renal epithelial cells. B, gene expression clustering of differentially expressed genes between Adeno-Cre and Adeno-GFP-treated cells. Rows represent mean log2 ratios of three independent samples of each genotype. HIF1α-specific or HIF2α-specific target genes or genes that are regulated by both HIF1α and HIF2α are indicated. C, heat map of gene expression of HIF1α-specific genes. D, mRNA abundance of the indicated genes in kidneys deficient for Vhl, Vhl/Hif1a, and Vhl/Hif2a. Mean ± SD (n = 6 mice of each genotype) gene expression ratios between Ksp1.3-Cre and Ksp1.3-Cre/+ mice, normalized to expression of Rplp0. E, respirometry of biopsies from cortex and medulla of wild-type (+/+), Hif1a+/-, Hif2a+/-, and Vhl/Trp53+/- mice (right column) kidneys. Graphs depict oxygen consumption attributable to total ATP synthase capacity, complex I, complex II, complex IV, and medium chain fatty acid oxidation.

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Figure 5.
Cellular metabolic phenotypes in Vhl, Vhl/Hif1a, and Vhl/Hif2a-deficient mice. A–H, immunofluorescence stainings for AQP2, TOM20, and DAPI. A–D show overlaid channels, E–H show TOM20 fluorescence only. Arrowheads, examples of collecting duct principal cells. I–L, H&E staining of renal cortex. Arrowheads, epithelial cells with clear cell cytoplasm; arrows, optically clear nuclei. M–P, PAS staining of the renal cortex. Arrows, optically clear nuclei that display strong accumulation of glycogen. Q–T, Oil Red O staining in the renal cortex. U, average TOM20 intensity in AQP2-positive cells. A total of 43 to 64 tubules were analyzed in 7 to 11 separate regions per genotype. V, Oil Red O staining of perirenal fat. W, mRNA abundance of the indicated genes in kidneys deficient for Vhl, Vhl/Hif1a, and Vhl/Hif2a (n = 6 mice of each genotype) of the ratios of gene expression between Ksp1.3-Cre and wild-type (WT) mice, normalized to expression of Rps12.
We derived MEFs from cells rapidly dedifferentiate after trypsinization, they were not more insight at the cellular level into the metabolic alterations independently of p53

neither glycogen nor lipid accumulation appears to be the cause of capacity correlate with the presence of a clear cell cytoplasm, dependent reductions in mitochondrial abundance and oxidative positive control for these stainings (Fig. 5V). Thus, while HIF1

We next turned to a primary cell culture – based system to gain more insight at the cellular level into the metabolic alterations that follow Vhl deletion. Because primary renal tubular epithelial cells rapidly dedifferentiate after trypsinization, they were not suitable for these experiments. We instead utilized primary MEFs as a genetically tractable cell culture system and focused on alterations in glucose metabolism and oxygen consumption. We derived MEFs from Vhl\(^{+/+}\), Vhl\(^{−/−}\)Hif1a\(^{+/+}\), Vhl\(^{−/−}\)Hif2a\(^{+/+}\), and Vhl\(^{−/−}\)Hif1a\(^{−/−}\)Hif2a\(^{−/−}\) embryos and infected them with adenoviruses expressing GFP (Adeno-GFP) or Cre (Adeno-Cre). The efficient deletion of all genes in the relevant floxed genotypes was confirmed by real-time PCR (Supplementary Fig. S8). We investigated the dependence on Hif1a and Hif2a of the expression of a series of the key HIF1a-dependent metabolic genes that we identified in renal epithelial cells. The mRNA levels of Slc2a1, Pck1, Aldh1a1, Paqr1, Ndufa12, and Cox 4-2 were elevated in Vhl mutant cells in a Hif1a-dependent but Hif2a-independent manner (Fig. 6A), identically to the results seen in primary renal epithelial cells and in knockout kidneys. Deletion of Vhl induces premature senescence that can be rescued by codelletion of Trp53 (8). Vhl\(^{+/+}\)Trp53\(^{−/−}\) double null MEFs also exhibited higher mRNA abundance of these genes (Fig. 6A), indicating that the gene expression pattern is not a secondary consequence of senescence, nor is it dependent on p53. Because this gene expression pattern is expected to promote the conversion of glucose-derived pyruvate into lactate at the expense of entry of pyruvate into the mitochondria for oxidative phosphorylation, we analyzed cellular readouts of glycolytic flux in cells cultured at 20% O\(_{2}\). Modest increases in glucose utilization (Fig. 6B) and extracellular acidification rate (Fig. 6C), as well as increases in the amount of lactate secreted by the cells (Fig. 6D), were observed in Vhl and Vhl/Trp53-null MEFs. In contrast to these relatively small increases, there were large decreases in oxygen consumption (Fig. 6E) in Vhl and Vhl/Trp53-null MEFs that were not attributable to reduced mitochondrial mass, as assessed by NAO staining (Fig. 6F). These results show that Vhl deletion causes a modest increase in glucose uptake and conversion to lactate but a large decrease in the activity of the mitochondrial tricarboxylic acid cycle, reflected by a lowered OCR. The reduced levels of oxidative phosphorylation that are not compensated by a large elevation in glucose flux to lactate suggested that cells may not be able to produce normal levels of ATP. Indeed, Vhl and Vhl/Trp53 deletion caused a decrease in cellular ATP level (Fig. 6C) that is dependent on Hif1a but independent of Hif2a (Fig. 6H), consistent with the transcriptional effects of these gene deletions on metabolic regulatory genes.

Discussion

The molecular and cellular causes of ccRCC are incompletely understood. Biallelic inactivation of VHL is an early event in most ccRCCs, but deletion of Vhl in renal epithelial cells in mice does not cause tumor formation (5). Correlative and functional genetic studies of human ccRCC have demonstrated that HIF1a stabilization exerts a tumor suppressor–like activity, whereas HIF2a behaves oncogenically in the context of tumor cell proliferation (5). Here, we tested the hypothesis that ablating HIF1a stabilization might allow hyperproliferation of Vhl mutant kidney epithelial cells in vivo, potentially allowing cysts or ccRCC tumors to form. In fact, Hif1a codelletion rescued the increased epithelial cell turnover caused by Vhl deletion, and Vhl\(^{−/−}\)Hif1a\(^{−/−}\) mice developed no renal proliferative abnormalities. Similar mouse models in which Vhl and Hif1a were deleted in renal epithelial cells under the control of different nephron segment-specific Cre transgenes also did not lead to tumor formation (44, 45). Our Vhl\(^{−/−}\)Hif2a\(^{−/−}\) mice similarly did not develop renal cysts or tumors. Thus, despite the numerous putative tumor suppressor functions of Vhl, its deletion together with constitutive stabilization of HIF1a and HIF2a alone or together is insufficient for renal tumor formation. Previous studies employing various strategies to achieve HIF1a and/or HIF2a activation in Vhl wild-type renal epithelia also failed to induce tumor formation beyond the stage of simple cysts or small dysplastic lesions (25, 46–48). Vhl/Trp53 deletion leads to the formation of simple and atypical renal cysts, renal tumors as well as carcinomas in other organs of the genital tract (8). We anticipated that the tumor phenotype in these mice might be enhanced by Hif1a codelletion. In fact, all phenotypes in these mice were completely rescued. Similarly, codelletion of Hif2a in this genetic background also completely prevented the formation of renal cysts, renal tumors, and malignancies of the genital-urinary tract. Insofar as the Vhl/Trp53 deletion model mimics the initial stages of evolution of ccRCCs, these findings indicate that HIF1a and HIF2a stabilization both play essential oncogenic roles in cyst and tumor formation. It will be interesting in further mouse genetic studies to determine whether HIF1a and HIF2a are also both necessary for the formation of tumors induced by other mutations that cooperate with Vhl, such as Bap1 (9).

It seems likely that HIF1a and HIF2a might each alter multiple cellular processes that are separately or cooperatively necessary for cyst and tumor evolution. While our studies uncoupled increased epithelial turnover from cyst and tumor formation, we identified several metabolic alterations that precede tumor formation. In Vhl mutant renal epithelial cells, HIF1a induces a transcriptional program that promotes the glycolytic conversion of glucose to lactate and decreases mitochondrial oxidative phosphorylation, reducing glucose– and fatty acid–derived carbons. HIF1a also decreased the abundance of mitochondria in collecting duct epithelial cells, consistent with previous findings in cultured human cells that HIF1a decreases mitochondrial biogenesis via MXI1-MYC-PGC1β and increases mitophagy via BNIP3 (32, 43). Our microarray observation that Bnip3 expression is upregulated in Vhl-deficient renal epithelial cells (Fig. 4C) provides the basis for future studies to uncover how Vhl loss reduces mitochondrial abundance. In established ccRCC cell lines, increasing mitochondrial abundance by constitutive PGC-1β expression causes decreased xenograft tumor growth (49), consistent with our correlative data that Vhl\(^{−/−}\)Hif2a\(^{−/−}\) mice do not show decreases in mitochondrial abundance and activity and do not develop cysts or tumors. These findings argue that a reduction in mitochondrial abundance promotes tumorigenesis. On the other hand, we show in MEFs that HIF1a induces Warburg-like metabolism associated with increased conversion of glucose to lactate, decreased mitochondrial oxygen consumption, and a lowered cellular level of ATP, implying that reduced cellular ATP levels may be an early
Figure 6.
Impact of Vhl, Hif1a, Hif2a, and Trp53 mutations on glycolysis, oxygen consumption, and ATP production. A, mRNA abundance of the indicated genes between Adeno-Cre and Adeno-GFP–treated MEFs (72 hours after infection), normalized to expression of Rps12 (n = 3). B–E, relative glucose utilization (B), extracellular acidification rate (ECAR; C), relative lactate production (D), OCR of Adeno-GFP or Adeno-Cre–treated cultures (E) of MEFs 72 hours after infection. B and D show pooled data from three independent experiments, each assayed in quadruplicate, and C and E depict single representative experiments of three independent experiments, each involving five replicate assays per genotype. F, flow cytometry using 10-N-Nonyl acridine orange (NAO) staining. Filled gray curves show Adeno-GFP–infected cells, nonfilled curves show Adeno-Cre–infected cells. G and H, relative cellular ATP levels of Adeno-GFP and Adeno-Cre–treated cells of the indicated genotypes. n = 11 to 30 independent experiments, each with 3 to 5 technical replicates.
cellular consequence of VHL gene deletion. The role that this mode of metabolism might play in promoting or inhibiting ccRCC formation remains to be determined, but it is noteworthy in human ccRCCs that high mRNA expression levels of several of the HIF1α-dependent metabolic genes that we identify in this study (including PFKM, ALDOB, PKC1, PGM2, ENO1, LDHA, PDK1), as well as higher levels of phosphorylated AMPK, a predicted consequence of HIF-1α-mediated reduction in cellular ATP levels, correlate with better patient outcome (39). This finding argues that the HIF1α transcriptional signature acts to restrain aggressive tumor behavior. Thus, it appears that different metabolic activities of HIF1α may contribute differently to different aspects of tumor formation and progression. In addition, it appears likely that mutations or alterations in the expression of other metabolism-regulating genes, such as decreased expression of FBPI (38), may act together with HIF1α to further alter cellular metabolic pathways to achieve a balance of high rates of glycolytic and pentose phosphate pathway flux and sufficient oxidative phosphorylation to provide the necessary metabolic intermediates and ATP to fuel efficient biosynthesis, cellular proliferation, and tumor growth.

VHL mutant 'normal' tubular cells in VHL patient kidneys exhibit clear cell morphology (4), highlighting that this phenotype precedes tumor formation. We demonstrate that HIF1α stabilization, but not HIF2α stabilization, induces the clear cell phenotype, consistent with previous observations based on over-expression of stabilized mutants of HIF1α and HIF2α (25, 48). Somewhat surprisingly, while decreased mitochondrial activity and abundance correlated with the presence of clear cell cytoplas-m, the accumulation of glycogen deposits did not correlate and renal tubules did not accumulate lipids. These findings contradict the widely held idea that the clear cell phenotype results from cytoplasmic lipid and glycogen accumulation.

Our studies encourage a modification of the idea that HIF2α is the ccRCC oncogene and that HIF1α restrains tumor progression. We argue that while activation of HIF1α or HIF2α alone or together is insufficient for tumor formation following biallelic inactivation of VHL, their activities are each indispensable for the formation of cysts and tumors that arise as a consequence of cooperating secondary mutations. The frequent selection in human ccRCC for functional losses of the HIF1α gene (24) or for other posttranscriptional alterations that impair HIF1α stability and promote HIF2α activity (50) raises the intriguing idea that HIF1α may switch during tumor evolution from being a factor that is initially necessary for tumor formation (oncogene) to one that later restrains tumor progression (tumor suppressor).

Our findings also raise the idea that pharmacological inhibition of either HIF1α or HIF2α may be sufficient to prevent ccRCC formation in patients with inherited VHL mutations.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Formation of Renal Cysts and Tumors in Vhl/Trp53-Deficient Mice Requires HIF1α and HIF2α

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