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Review

The VHL tumor suppressor and HIF: insights from genetic studies in mice

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The von Hippel–Lindau tumor suppressor gene product, pVHL, functions as the substrate recognition component of an E3-ubiquitin ligase, which targets the oxygen-sensitive α-subunit of hypoxia-inducible factor (HIF) for rapid proteasomal degradation under normoxic conditions and as such plays a central role in molecular oxygen sensing. Mutations in pVHL can be found in familial and sporadic clear cell carcinomas of the kidney, hemangioblastomas of the retina and central nervous system, and pheochromocytomas, underscoring its gatekeeper function in the pathogenesis of these tumors. Tissue-specific gene targeting of *VHL* in mice has demonstrated that efficient execution of pVHL-mediated HIF proteolysis under normoxia is fundamentally important for survival, proliferation, differentiation and normal physiology of many cell types, and has provided novel insights into the biological function of individual HIF transcription factors. In this review, we discuss the role of HIF in the development of the VHL phenotype.
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Germ-line mutations in the von Hippel–Lindau tumor (VHL) suppressor can be found in patients with VHL disease, a rare familial tumor syndrome characterized by the development of highly vascularized tumors in multiple organs. Major clinical manifestations of VHL disease include hemangioblastomas of the retina and central nervous system (CNS), renal cysts and renal cell carcinoma of the clear cell type (CC-RCC), pancreatic cysts and tumors, as well as pheochromocytomas.[1] The VHL tumor suppressor is also mutated in the majority of sporadic CC-RCCs,[2] highlighting its critical and central role in the regulation of cell growth and differentiation of epithelial kidney cells. Although the molecular function of the VHL gene product, pVHL, was initially not known when it was first identified by positional cloning in 1993,[3] observations that oxygen-dependent regulation of hypoxia-inducible genes was lost in *VHL*-deficient cell lines suggested a role for pVHL in oxygen sensing.[4–6] In a seminal paper, Maxwell *et al.*[7] showed that pVHL was critical for targeting the α-subunit of hypoxia-inducible factor (HIF) for oxygen-dependent proteolysis, thus providing a direct molecular link between VHL-associated tumorigenesis and oxygen sensing via HIF. HIFs belong to the PAS (Per-arylhydrocarbon receptor nuclear translocator (ARNT)-Sim) family of basic helix-loop-helix (bHLH) transcription factors and bind DNA as heterodimers. They consist of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, also known as ARNT or HIF-β. pVHL associates with the elongins B and C, cullin2 and Rbx[8–12] and functions as the substrate recognition component of an E3-ubiquitin ligase that ubiquitylates HIF-α.[13–19] All three HIF α-subunits, HIF-1α, HIF-2α and HIF-3α interact with pVHL.[7,20] This pVHL–HIF-α interaction is highly conserved between species, requires iron- and oxygen-dependent hydroxylation of specific proline residues (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in human HIF-2α) within the oxygen-dependent degradation domain of HIF-α and is necessary for the execution of HIF proteolysis under normoxia.[21–27] Therefore, absence of pVHL results in HIF-α stabilization, increased HIF transcriptional activity and upregulation of HIF target genes, such as *vascular endothelial growth factor (VEGF)*, *glucose transporter 1* and *erythropoietin (EPO)* irrespective of oxygen levels.

To understand the role of pVHL-mediated HIF proteolysis in normal tissue physiology, tumorigenesis and during development, we have used cell-type-specific gene targeting based on Cre recombinase (Cre)/loxP-mediated recombination. In this review, we will summarize the findings from a variety of developmental and physiological studies in conditional VHL and HIF knockout mice and provide insights into the biological function of individual HIF transcription factors in a *VHL*-deficient background.

Normoxic Stabilization of HIF-α in *VHL*-Deficient Tissues

pVHL is a master regulator of HIF-α proteolysis, and genetic inactivation of the *VHL* gene results in HIF-α stabilization and increased activity of HIF transcription factors. The most

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Abbreviations: ARNT, arylhydrocarbon receptor nuclear translocator; CC-RCC, renal cell carcinoma of the clear cell type; Cre, Cre recombinase; EPO, erythropoietin; HIF, hypoxia-inducible factor; PEPCK, phosphoenolpyruvate carboxykinase; PHD protein, prolyl-hydroxylase domain protein; pVHL, von Hippel–Lindau protein; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau; THP, Tamm–Horsfall protein
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extensively studied PAS family transcription factors that are targeted by pVHL are HIF-1 and HIF-2 (here collectively referred to as HIF). HIFs regulate gene expression through interaction with hypoxia response elements, specific DNA recognition sequences that are located in hypoxia enhancer regulatory regions and bind HIF heterodimers.[28] HIF-1 and HIF-2 have distinct cell type- and tissue-specific biological functions and only overlap partially with regard to their target genes. For example, genes encoding glycolytic enzymes appear to be predominantly controlled by HIF-1,[29] whereas HIF-2 appears to be the main regulator of *VEGF* and *EPO* in tissues that express both HIF-1 and HIF-2.[30–33] Target gene preference may be the result of tissue-specific interactions with other nuclear factors, differential interactions with transcriptional cofactors or a reflection of tissue- and cell-type-dependent differences in the ratios of HIF-α protein levels (for a review on this topic see Wenger *et al.*[28]).

In addition to heterodimerization with HIF-β, which results in the formation of a bHLH transcription factor that mediates the canonical hypoxia response, stabilization of HIF-α subunits has also been shown to regulate biological processes through functional interaction with other non-PAS domain proteins. These include, among others, tumor suppressor protein p53 and the c-Myc proto-oncogene.[34–36] A more recent example is the ability of HIF-1α to associate with the intracellular domain of Notch (Notch ICD), thereby increasing the expression of Notch-target genes, such as *Hey* and *Hes*.[37] This and other HIF-α/non-PAS domain protein interactions have to be considered when interpreting experimental phenotypes that result from *VHL* gene deletion in the laboratory mouse.

A second hypoxic switch operates in the carboxy-terminal transactivation domain of HIF-α with the hydroxylation of an asparagine residue.[38] During hypoxia, asparagine hydroxylation is blocked and CBP/p300 recruitment is facilitated enabling increased levels of transcription. Factor-inhibiting HIF (FIH) hydroxylates the asparagine residue at position Asn803 in human HIF-1α, which corresponds to asparagine Asn851 in HIF-2α.[39,40] Inhibition of FIH can result in increased HIF target gene expression even under severe hypoxia or in certain *VHL*-deficient cell lines.[41]

Biological Functions not Involving HIF

Aside from mediating HIF-α proteolysis, pVHL is involved in extracellular matrix (ECM) assembly matrix turnover,[42–47] the regulation of intracellular junctions,[48] NF-κB signaling,[49] the regulation of c-Met receptor responsiveness to hepatocyte growth factor (HGF) involving β-catenin[45,50] and the regulation of p53 transcriptional activity by suppressing Mdm2-mediated ubiquitination and nuclear export.[51] Furthermore, pVHL has been shown to regulate microtubule stability and cilia maintenance[52–55] and controls the activity of plant homeodomain protein Jade-1,[56,57] and atypical protein kinase C isoforms.[58–62] Other pVHL targets include a KRAB-A domain protein, VHLak, repressing HIF transcriptional activity,[63] de-ubiquitinating enzymes,[64] the large subunit of RNA polymerase II[65] and the RNA-binding protein hnRNP A2.[66] How these HIF-independent pVHL functions and recently discovered protein interactions exactly contribute to the initiation and progression of VHL-associated tumorigenesis is unclear and requires further investigation.

pVHL during Embryonic Development

Genetic inactivation of pVHL in the mouse germ line results in death of the embryo during mid-gestation. Although placenta and embryo appear normal until embryonic day (E) E9.5, placentae from *VHL*-deficient embryos lack a properly developed syncytiotrophoblast and labyrinth and show evidence of hemorrhage by E11.5–12.5, suggesting that abnormal placental vascularization has led to the embryo's demise.[67] This phenotype appears to be largely mediated by HIF, since germ-line inactivation of HIF prolyl-hydroxylase domain (PHD) protein 2 resulted in similar, however, not identical pathology,[68] indicating HIF-independent functions of either pVHL and/or PHD2 during placental development. pVHL is not only essential for normal placental development but also plays a critical role in the development, growth and differentiation of many other tissues. For example, tissue-specific inactivation of pVHL in neuro-epithelial progenitor cells resulted in abnormal neuronal differentiation and embryonic lethality during late gestation (VH Haase, unpublished data), and chondrocyte-specific inactivation of pVHL in the growth plate caused stunted bone growth, most likely a consequence of a HIF-dependent increase in cell cycle inhibitor $p57^{kip2}$.[69] In contrast, HIF-1α inactivation in chondrocytes resulted in a lack of $p57^{kip2}$-mediated growth arrest, followed by massive apoptosis.[70]

VHL Disease Manifestations in Mice with Germ-line Mutations

Patients with VHL disease develop highly vascularized tumors in multiple organ systems, hemangioblastomas of the retina and CNS, CC-RCC and pheochromocytomas being the most prominent clinical manifestations.[1] VHL disease is grouped into two subtypes depending on the presence or absence of pheochromocytoma. Different clinical subtypes are associated with specific *VHL* mutations, which result in distinct functional and biochemical properties of the mutated *VHL* gene product (for an overview see Neumann and Bender[71], Clifford *et al.*[72] and Hoffman *et al.*[73]). Clinical manifestations of VHL disease occur relatively early in life, usually between 10 and 40 years of age, whereas sporadic tumors with *VHL* mutations, such as CC-RCC present later in life. In addition to tumor formation, mutations in the *VHL* gene can result in the development of polycythemia.[74] Congenital Chuvash polycythemia is a rare endemic disease in central European Russia and is associated with a specific mutation of *VHL* codon 200 (C598T $\Rightarrow$ Arg200Trp). This mutation is transmitted in an autosomal-recessive manner, and affected individuals, who are homozygous for the mutation, are not predisposed to the development of typical VHL-associated tumors.[75]

In mice, germ-line loss of one *VHL* allele (*VHL*+/−) results in the development of cavernous liver hemangiomas,[76] which is a rare manifestation of VHL disease in human.[77,78] This phenotype is strongly dependent on the genetic background of heterozygous mice, as the incidence of liver hemangiomas in BALB/c mice was 88%, but only 18% in a C57BL/6

background, most likely reflecting polymorphic differences in modifier genes.[79]

Although the molecular mechanism for this liver phenotype has not been systematically investigated, it is most likely that, following Knudson's two-hit hypothesis, inactivation of the remaining *VHL* wild-type allele in hepatocytes resulted in the formation of cavernous hemangiomas through an increase in HIF-dependent vascular growth factor production. Hepatocyte-specific inactivation of *VHL* using the Albumin-Cre and phosphoenolpyruvate carboxykinase (PEPCK)-Cre transgenes (Figure 1 and Table 1) phenocopies the liver pathology found in heterozygotes and suggests that liver hemangiomas in *VHL* heterozygotes are the result of pVHL loss in hepatocytes and not in other liver cell types, such as endothelial cells. Endothelial cells, which express wild-type *VHL* in this model, respond to uncontrolled and constitutive production of hepatocyte-derived vascular growth factors with proliferation. This is mechanistically similar to human VHL-associated hemangioblastomas, in which 'stromal cells' and not endothelial cells are *VHL*-deficient and represent the neoplastic component of these tumors.[94] The histogenetic origin of stromal cells is uncertain; however, correlative evidence suggests that they may be derived from angiogenic progenitor cells.[95,96]

Another major visceral manifestation of VHL disease is the development of kidney cysts, which can occur in up to 60% of patients with *VHL* germ-line mutations.[1,97] Renal cysts, which occur more frequently than CC-RCC, are considered pre-neoplastic precursor lesions of CC-RCC. In *VHL*+/– mice renal cysts were only found at extremely low frequency (<5%) by Haase *et al*.[76] and Kleymenova *et al*.[98] In both reports, CC-RCCs were not observed, nor were CNS hemangioblastomas or retinal angiomas. Treatment with streptozotocin did not result in increased susceptibility to renal carcinogenesis, but increased the incidence of hemangiomas and hemangiosarcomas in the liver and other organs such as the uterus and ovaries.[98]

Although erythrocytosis is seen in a small fraction of patients with VHL disease,[99,100] elevated hematocrits are not found in *VHL*+/– mice. Mice homozygous for a *VHL*

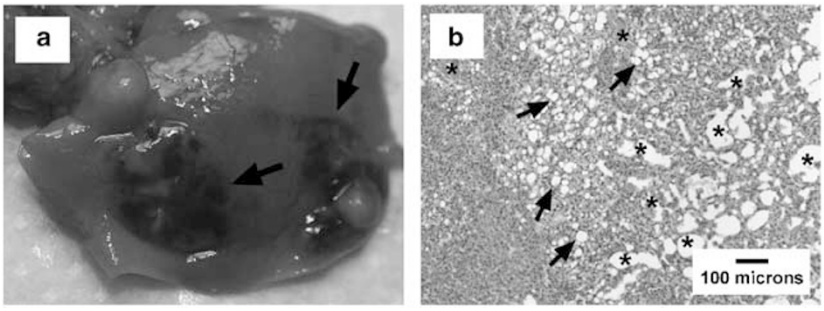

Figure 1 Hepatic vascular tumors in mice that lack pVHL in hepatocytes. (**a**) Gross photograph of a liver with multiple cavernous hemangiomas (arrows). (**b**) Histological features of cavernous liver hemangiomas. A paraffin-embedded tissue section stained with H&E is shown. Asterisks depict endothelial cell-lined, blood-filled cavities and areas of hemorrhage. VHL-associated liver hemangiomas are typically associated with macro- and microvesicular hepatocellular accumulation of neutral lipids,[76] here depicted by arrows. Lipid accumulation is also a feature of stromal cells, the neoplastic component of VHL-associated central nervous system hemangioblastomas[95,96]

Table 1 Phenotypes generated by cell-type-specific inactivation of pVHL in mice

References	Cre transgenic (promoter)	Cell type	Phenotype
			Kidney
Ding *et al*.[80]	NPHS2 (podocin)	Podocytes	Acute nephritis (4 weeks) with hematuria, proteinuria and renal insufficiency; features characteristic of pauci-immune RPGN (crescentic glomerulonephritis with prominent segmental fibrin deposition and fibrinoid necrosis, no immune deposits); absence of circulating ANCA antibodies; both HIF-1α and HIF-2α protein levels were increased; upregulation of HIF-dependent genes (including CXCR4) as well as RPGN-associated genes; *de novo* expression of CXCR4
Brukamp *et al*.[81]	NPHS2 (podocin)	Podocytes	No prominent disease pattern *in vivo*; preserved glomerular development but glomerulomegaly at a young age and occasional glomerulosclerosis; no significant proteinuria; increased podocyte apoptosis *in vitro*
Steenhard *et al*.[82]	NPHS2 (podocin)	Podocytes	Initially normal glomerular development; at 16 weeks ultrastructural changes, such as foot process broadening, widespread irregular GBM thickening with subepithelial hump formation and focal subendothelial delamination; proteinuria
Rankin *et al*.[83]	PEPCK (phosphoenolpyruvate carboxykinase)	Proximal renal tubule	Renal cysts at low frequency; inactivation of ARNT, but not HIF-1α suppressed the development of renal cysts
			Liver
Haase *et al*.[76] Rankin *et al*.[31] Rankin *et al*.[32]	Albumin	Hepatocytes	Growth deficiency, angiectasis, hemangiomas, endothelial cell proliferation, severe hepatic steatosis (neutral fat accumulation in hepatocytes), inflammatory cell infiltration, erythrocytosis from increased liver EPO production; HIF-2 is the main mediator of this phenotype; HIF-1 regulates glycolytic gene expression

Table 1 *(Continued)*

References	Cre transgenic (promoter)	Cell type	Phenotype
Peyssonnaux *et al.*[84]	Albumin	Hepatocytes	Relative iron deficiency with hypochromia, poikilocytosis, microcytosis, decreased total iron and ferritin levels; spleen also strongly iron deficient; decreased hepcidin mRNA and protein levels (ARNT dependent); hepcidin downregulation specifically due to the stabilization of HIF; significantly elevated IL-6 and IL-1 levels; IL6- or IL1-mediated stimulation of hepcidin expression is subordinate to the suppression by HIF; increased ferroportin expression in brush border enterocytes of the duodenum, Kupffer cells and hepatocytes
Rankin *et al.*[31,32]	PEPCK (phosphoenolpyruvate carboxykinase)	Hepatocytes	Liver hemangiomas, polycythemia; HIF-2α is required for the development of polycythemia and inactivation of HIF-2α is sufficient to suppress hepatic EPO expression in *VHL*-KO livers despite increased HIF-1 activity
			Immune system
Neumann *et al.*[85]	Lck (lymphocyte protein tyrosine kinase)	Thymocytes	Diminished maximal Ca_i^{2+} together with retarded initial rates of Ca_i^{2+} elevation in response to CD3/CD4 co-ligation; Ca^{2+} signaling was completely restored in *VHL−/−/HIF-1α−/−* double KO thymocytes; stabilization of HIF-1α increases flux of Ca^{2+} from the cytoplasm to the mitochondria and results in increased protein levels of SERCA2 (Ca^{2+} pump that refills ER stores); overall SERCA-mediated Ca^{2+} transport is increased; both hypoxic and non-hypoxic-mediated HIF-1α stabilization may minimize the strength and duration of Ca^{2+} signaling by means of acceleration of cytoplasmic Ca^{2+} clearance
Biju *et al.*[86]	Lck (lymphocyte protein tyrosine kinase)	Thymocytes	Small, highly vascularized thymi; increased apoptosis in CD4/CD8 double-positive cells involving a caspase8-dependent mechanism; phenotype is HIF-1 dependent.
Cramer *et al.*[87]	LysM (lysozyme M)	Myeloid cells	Increase in inflammatory response through HIF-1
			Epithelium
Karhausen *et al.*[88]	Fabp (fatty acid-binding protein)	Colonic epithelial cells	Increased expression of HIF-regulated barrier protective factors such as intestinal trefoil factor; protection from inflammatory bowel disease clinically; accumulation of glycogen in luminal epithelial cells
Seagroves *et al.*[89a]	WAP (whey acidic protein)	Mammary gland epithelium	Abnormal differentiation of gland epithelium during lactation in multiply bred mutants, collapsed alveoli, little or no milk production, enlarged blood vessels, no hyperplasia or tumors
Boutin *et al.*[90a]	K14 (keratin 14)	Epidermis	Increased dermal vasculature, higher metabolic rate, low weight, early death, erythrocytosis
			Vascular system
Tang *et al.*[91]	Tie2	Endothelial cells	Intrauterine death with hemorrhages at E12.5; loss of HIF-1α did not rescue the embryonic lethality; endocardial collapse, abnormal yolk sac vascular organization, reduced fibronectin deposition around vitelline vessels, defective extracellular fibronectin matrix assembly, defective vasculogenesis in the placental labyrinth, dilated vessels and reduced vessel complexity; increased permeability of the endothelial monolayer; impaired migration and impaired adhesion partially rescued with external fibronectin; regulation of fibronectin assembly independent of HIF-1
			Connective tissue
Wang *et al.*[92]	OC (osteocalcin)	Osteoblasts	Bone modeling is increased within the first week of life but appears to decline at latter stages of development; extremely dense, heavily vascularized long bones; progressively increased vessel numbers with age; trabecular separation reduced; no detectable changes in calvarial bone morphology; upregulation of HIF-1 and HIF-2 and VEGF, serum level of VEGF was not elevated; conversely, inactivation of HIF-1α in the osteoblast produced the reverse phenotype, that is, thinner and less vascularized bones
Pfander *et al.*[69]	ColII (collagen II)	Growth plate/ chondrocytes	Severe dwarfism, severe decrease in chondrocyte proliferation, upregulation of HIF-1-dependent $p57^{kip2}$, increased extracellular matrix deposition
			Mosaic and neurons
Hong *et al.*[93]	ROSA26-tamoxifen-inducible ER-Cre	Mosaic	Distinct changes at E14.5; embryonic lethality; small, pale embryos; extensive hemorrhage; vascular defects and liver damage in the embryos, but no major placental defects prior to death; no significant increase in apoptosis or decrease in proliferation
Ma *et al.*[79]	β-actin-tamoxifen-inducible ER-Cre	Mosaic	Hepatic vascular tumors; angiectasis in heart, liver, pancreas, lung, kidney; infertility, abnormal spermatogenesis
Haase *et al.*, unpublished observation	Nestin	Neuro-epithelial progenitor cells	Abnormal neuronal differentiation, embryonic lethality

A compilation of mouse phenotypes organized by organ system and cell types, which were made pVHL deficient, is presented. The gene regulatory elements used for the construction of Cre transgenes to induce cell-type-specific inactivation of *VHL* are listed in the second column. [a]Meeting abstract; ANCA, anti-neutrophilic cytoplasmic antibody; CXCR4, chemokine receptor 4; RPGN, rapidly progressive glomerulonephritis

mutation at codon 200, which does not result in embryonic lethality when bred to homozygosity, developed mild HIF-2-dependent erythrocytosis, thus mimicking features of human Chuvash polycythemia.[101]

pVHL and Renal Cell Cancer

A molecular hallmark of the most common form of kidney cancer, CC-RCC, is mutated pVHL. In contrast to patients with VHL disease, who transmit germ-line mutations, patients with sporadic CC-RCC have acquired somatic mutations in both copies of the *VHL* gene. VHL-associated CC-RCCs are often preceded by multifocal and bilateral renal cysts, which can be found in up to 60% of patients with VHL disease and can sometimes mimic polycystic kidney disease.[1,97] VHL-associated renal cysts can be malignant or benign, and should be viewed as preneoplastic lesions. Renal cystogenesis in general is associated with altered signaling through the primary cilium, a microtubule-based organelle, which is found in many cell types such as neurons, photoreceptors, fibroblasts and others, and predominantly functions as a luminal flow sensor on renal epithelial cells and regulates renal tubular cell proliferation.[102] More recent studies have proposed that pVHL is essential for cilium maintenance.[52–55,103] pVHL was reported to cooperate with GSK3β in an interlinked signaling pathway that maintains the primary cilium.[53] Schermer *et al.*[54] demonstrated that pVHL localizes to the monocilia of kidney cells, interacts with Par3-Par6-aPKC and controls ciliogenesis via coordinated extension of microtubules toward the cell periphery and Esteban *et al.*[103] proposed a role for HIF in cilium maintenance. The latter study showed that pVHL inactivation is associated with the loss of the primary cilium in VHL-defective cell lines and in kidney cysts found in patients with VHL disease, and that reconstitution of cell lines with wild-type pVHL restored primary cilia in a HIF-dependent manner. Although CC-RCCs are traditionally believed to arise from the proximal renal tubule,[97,104] their histogenetic origin remains controversial. Recent immunohistochemical studies suggested that CC-RCC originates from distal nephron segments, which express Tamm–Horsfall protein (THP).[105] While certain features of *VHL*-deficient tumors, for example, their highly vascular nature resulting from increased VEGF production, can be directly linked to constitutive HIF stabilization, renal carcinogenesis is more difficult to understand and cannot be explained by activated HIF signaling alone. However, loss of pVHL function and subsequent HIF-α stabilization represent the earliest detectable molecular events in renal tumorigenesis.[105] Esteban *et al.*[106] showed that pVHL inactivation is associated with downregulation of intercellular adhesion molecule E-cadherin in pre-cancerous lesions and that both HIF-1 and HIF-2 contributed to decreased E-cadherin expression with HIF-2 mediating a more potent suppression in CC-RCC cell lines. Similarly, Evans *et al.*[107] demonstrated that knockdown of pVHL resulted in E-cadherin suppression via HIF-dependent induction of E2 box-dependent transcriptional repressors Snail and SIP1, and Krishnamachary *et al.*[108] reported that HIF-1 activation in *VHL*-deficient cells downregulated E-cadherin, led to the loss of cell–cell adhesion and promoted epithelial to mesenchymal transition through the induction of transcriptional repressors TCF3, ZFHX1A and ZFHX1B/SIP1. Therefore, cellular changes, such as loss of intercellular junctions and epithelial de-differentiation involving HIF-dependent as well as HIF-independent molecular pathways[48,106–108] in addition to HIF-dependent and -independent alterations in p53 or NF-κB activity,[34,35,49,51] HGF signaling,[45,50,109] and modifications in ECM turnover and re-modeling[42–47] create the molecular environment for the development CC-RCC, which most likely requires additional genetic events. The importance of HIF activation in CC-RCC pathogenesis and growth is furthermore underscored by experimental and clinical studies, which demonstrated that inhibition of HIF-α translation by pharmacological targeting of mTOR correlated with reduced tumor growth,[110] and that increased expression of certain HIF target genes, such as CXCR4, as well as E-cadherin suppression was associated with disease progression.[111,112] With regard to the contribution of individual HIF-α homologs to renal tumor development, it is of interest that a substantial number of *VHL*-defective CC-RCC cell lines do not express HIF-1α, but express HIF-2α.[7] Furthermore a bias toward HIF-2α expression was also found in clinical CC-RCC samples with confirmed *VHL* defect.[113] This is in contrast to normal, non-transformed renal epithelial cells, in which HIF-2α is usually not detectable during hypoxia/ischemia.[114] Thus, VHL-associated tumor development may depend on a shift in the ratio of HIF-1α *versus* HIF-2α levels toward an increase in HIF-2α. In support of this hypothesis are studies in CC-RCC cell lines, which suggest that HIF-2 in contrast to HIF-1 is oncogenic and is able to override pVHL's tumor suppressor function.[115–118] HIF-2 has been proposed to preferentially regulate molecular pathways critical for renal cell growth, such as signaling through the TGF-α/epidermal growth factor receptor pathway, cyclin D1 and the c-Myc proto-oncogene.[119–124] Taken together, there is substantial evidence that HIF-1 and HIF-2 have diverse functions with regard to VHL renal tumorigenesis, which could be exploited therapeutically, and may be strongly context dependent.[125] Not much is known about the role of HIF-3α in VHL-associated tumorigenesis. Recent studies, however, have suggested that a specific splice form of HIF-3α, HIF-3α4, inhibits renal tumor growth through abrogation of HIF-2 signaling.[126]

To overcome embryonic lethality and to generate a mouse model of VHL-associated CC-RCC, we have used Cre/loxP-mediated gene targeting to inactivate pVHL in adult renal tissues. In the conditional *VHL* allele, promoter and exon 1 are flanked by loxP sites and are deleted upon Cre-mediated recombination, resulting in a mutated *VHL* allele that is transcriptionally inactive.[76] Similar to our approach, Ma *et al.*[79] generated a conditional *VHL* allele in which exons 2 and 3 were floxed. To specifically target renal epithelial cell types from which CC-RCCs are thought to originate, we generated transgenic mice, which express Cre in proximal and distal renal tubule segments under control of either a mutated version of the rat PEPCK or the THP promoter[83] (also EB Rankin *et al.*, unpublished observations). PEPCK-Cre mutant mice developed renal cysts at a frequency of $\sim$20%, but CC-RCC was not observed (Figure 2). Renal cyst development in PEPCK-Cre mutant was dependent on intact HIF signaling, but not on HIF-1α, as cysts were not observed in *VHL/ARNT* double knockout mice and HIF-1α was not

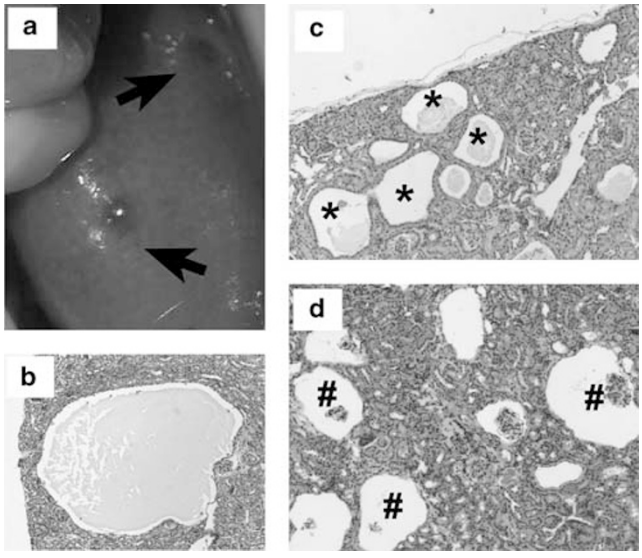

Figure 2 Mice that lack pVHL in the proximal renal tubule develop tubular and glomerular cysts. (**a**) Gross *in situ* photograph of a kidney from a male PEPCK-Cre mutant with two macroscopically visible cysts (arrows). The PEPCK-Cre transgene was used to inactivate pVHL in the proximal renal tubule. Genetic analysis with compound knockout mice demonstrated that this phenotype was HIF dependent.[83] (**b**–**d**) Histological features of cystic changes found in H&E-stained paraffin sections from *VHL*-deficient kidneys. A large cortical cyst (**b**), a cluster of renal tubular cysts lined by cuboidal epithelium (asterisks in **c**) and an area of glomerular cysts (number sign in **d**) are shown. CC-RCC or dysplastic changes in cysts were not found in *VHL*-deficient kidneys. Magnification is $\times$ 100

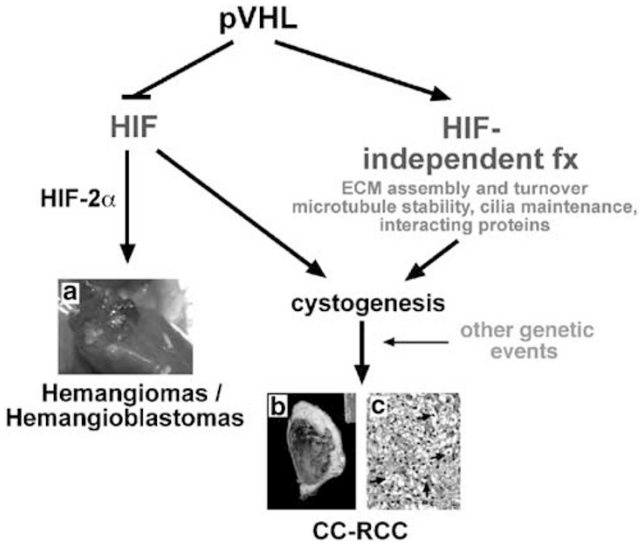

Figure 3 Schematic illustrating the role of pVHL and HIF in the development of the VHL phenotype. While activation of HIF signaling is required and sufficient for vascular tumor development, renal cystogenesis in humans may be a result of HIF activation ± loss of HIF-independent functions of pVHL. CC-RCC development most likely requires additional mutations in other tumor suppressor genes or in certain oncogenes. (**a**) Gross photography of a liver hemangioma found in a *VHL* mutant mouse. (**b**, **c**) Human renal cell carcinoma of the clear cell type (CC-RCC); (**b**) gross photography; (**c**) PAS stain of CC-RCC, clear cells are depicted by arrows, magnification $\times$ 400; images (**b**, **c**) were kindly provided by Dr. John Tomaszewski, Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

required for cyst formation.[83] Activation of HIF signaling has also been suggested to promote renal cystogenesis in a mouse model of fumarate hydratase deficiency, which leads to HIF prolyl-hydroxylase inhibition and HIF-α stabilization,[127] indicating that HIF-mediated renal cystogenesis is not dependent on *VHL* status. In contrast to pVHL inactivation with PEPCK-Cre transgenics, inactivation of pVHL using THP-Cre, which targets the medullary thick ascending loop of Henle (mTAL) and the early distal tubule, did not produce renal cysts. CC-RCCs were not observed in THP-Cre mutant animals. Renal tumors were also not found by Ma *et al*.[79] when an inducible β-actin promoter-driven Cre was used to generate mice which lacked pVHL in a mosaic pattern. The absence of renal tumorigenesis in *VHL*-deficient mice in conjunction with genetic data from humans suggests that transformation of renal cysts into CC-RCC most likely requires additional genetic events such as mutations in other tumor suppressor genes or oncogenes (Figure 3).

VHL Phenotypes in Non-Renal Tissues: is it All HIF?

To study the role of pVHL in growth, differentiation and normal tissue physiology, we have used different Cre transgenic lines to inactivate pVHL in multiple tissues. Table 1 summarizes clinical phenotypes that we and others have observed. Inactivation of pVHL results in abnormal cellular differentiation and growth causing significant organ pathology in many tissues, one common feature being increased vascularity. Although HIF-mediated stimulation of vascular growth factor production can explain increased vascularity, the effects of pVHL gene deletion on cellular growth and differentiation, metabolism and organ function are not as easily understood. In a non-RCC tumor model, Mack *et al*.[128] found significant growth retardation in teratocarcinomas derived from *VHL*−/− embryonic stem cells. These findings were surprising, but are in concordance with observations made by Carmeliet *et al*.[129] who showed that HIF-1α-deficient teratocarcinomas had a growth advantage compared to wild type. However, growth suppression in *VHL*−/− teratocarcinomas may not be entirely dependent on HIF-1, as introduction of a mutated pVHL species (Y112H) into the *VHL*−/− background remained growth suppressive despite restoration of HIF-α regulation,[130] suggesting HIF-independent functions of pVHL in this model.

To determine the role of individual HIF transcription factors in the development of the VHL phenotype in mice, we generated tissue-specific pVHL knockout mice, which lack HIF-1α and/or HIF-2α or HIF-1β (ARNT). Using this compound knockout approach, Biju *et al*.[86] was able to show that a caspase-8-dependent pro-apoptotic phenotype in *VHL*−/− thymocytes was completely HIF-1 mediated. HIF-2α, on the other hand, although expressed in thymocytes, was found to be non-functional and inactivation of HIF-1α alone was sufficient to completely suppress *VEGF* mRNA levels and the vascular phenotype in *VHL*−/− thymi.[86] This is contrast to the *VHL*-deficient liver, where inactivation of HIF-1α, expressed and functional in hepatocytes, did not affect VHL-associated vascular tumorigenesis, while inactivation of HIF-2α was sufficient to prevent the development of

Table 2 Mouse phenotypes resulting from HIF activation in tissues with wild-type *VHL*

References	Cre transgenic (promoter)	Cell type	Phenotype
(a)			
			Liver
Kim *et al.*[133]	Albumin; HIF1dPA (non-degradable HIF-1α)	Hepatocytes	Normal lifespan, normal appearing liver but with fine vacuolization of hepatocytes; moderate lipid accumulation (microvesicular pattern); normal proliferation
	Albumin; HIF2dPA (non-degradable HIF-2α)	Hepatocytes	Death at 6–8 weeks, marked hepatomegaly, polycythemia from increased liver EPO production; angiectasis; minimal vacuolization of hepatocytes; increased proliferation
	Albumin; HIF1dPA/ HIF2dA	Hepatocytes	Death at 6–8 weeks; marked hepatomegaly; angiectasis; micro- and macrovesicular hepatic steatosis (histologically identical to Albumin-Cre *VHL*$-/-$ mutants), increased proliferation; polycythemia
			Skin
Kim *et al.*[133]	K14 (keratin 14); HIF1dPA	Basal keratinocytes	No gross skin phenotype
	K14 (keratin 14); HIF2dPA	Basal keratinocytes	Formation of non-leaky blood vessels; partial alopecia; runting; epidermal proliferation; weight loss
(b)			
			Early embryo and mosaic
Takeda *et al.*[68]	EIIa (adenovirus promoter)	General deleter, early embryo prior to implantation	*PHD2–/–*: Embryonic lethality occurred between E12.5–E14.5; placental defects included significantly reduced labyrinthine branching morphogenesis, widespread penetration of the labyrinth by spongiotrophoblasts and abnormal distribution of trophoblast giant cells; in heart underdevelopment of the trabeculae, remarkably thinner myocardium, and incomplete formation of the interventricular septum; increased HIF-α in the placenta and the embryo proper but not in the heart; *VEGF* unchanged in heart and placentae; increased expression of *MASH2* and decreased expression of *TFEB* and *GCM1*
	EIIa (adenovirus promoter)	General deleter, early embryo	*PHD1–/– and PHD3–/–*: Viable; normal placenta and cardiovascular development; HIF-α and VEGF protein levels unchanged
Takeda *et al.*[134]	ROSA26-tamoxifen-inducible ER-Cre	Mosaic	*PHD2–/–*: At 6 weeks, after tamoxifen treatment, ear, trachea, liver, lung, renal cortex and brain with increased angiogenesis and angiectasia; glomerulomegaly with capillary dilatations; severe polycythemia; serum VEGF levels increased but no localized *VEGF* mRNA upregulation
	ROSA26-tamoxifen-inducible ER-Cre	Mosaic	*PHD1–/– and PHD3–/–*: No significant vascular phenotype; serum VEGF levels not increased

A summary of mouse phenotypes resulting from the conditional activation of non-degradable forms of HIF-1α (HIF1dPA) and/or HIF-2α (HIF2dPA) using Cre-loxP-mediated recombination (a), and from conditional inactivation of PHD1, 2 and 3 (b) is presented

cavernous hemangiomas (EB Rankin and VH Haase, submitted). This finding is consistent with the observation that angiogenic gene expression in hepatocytes, similar to the regulation of *EPO*, is strongly HIF-2 dependent[32] (also EB Rankin and VH Haase, submitted) and illustrates, that although HIF-1 and HIF-2 have the capability to regulate the same target genes, preferential transcriptional activation by HIF-1 or HIF-2 in the setting of VHL deficiency and/or hypoxia is tissue- or context-dependent.[120] Whether this requires tissue-specific transcriptional co-activators or repressors, certain DNA or protein modifications or other signaling events warrants further investigation.[131] Whatever the underlying mechanisms, clinical studies have also demonstrated a correlation between HIF-2α expression and the development of VHL-associated angiogenic lesions,[132] suggesting that pharmacological targeting of HIF-2 may by an effective therapy for the treatment of these tumors.

pVHL- and pVHL/ARNT-deficient liver tissues are histologically similar and direct comparison of gene expression changes has not revealed major differences[31] (also VH Haase *et al.*, unpublished observation), suggesting that the effects of pVHL gene deletion in the liver are largely HIF mediated. Indeed, forced expression of non-degradable HIF-1α and HIF-2α phenocopied the VHL phenotype in the liver and skin[133] (Table 2a). Similarly, genetic inactivation of PHD2 in the mouse germ line resulted in placental changes that resembled, but were not identical to those observed in *VHL* knockout mice.[68] PHD2, one of three major mammalian HIF prolyl-hydroxylases, is essential for HIF-α degradation under normoxia,[68,134,135] while PHD3 seems to be important for hydroxylation of HIF-α during re-oxygenation.[136] Analysis of tissue-specific *PHD* knockout mice and direct comparison with VHL phenotypes is therefore likely to provide novel insights into HIF-dependent and -independent functions of pVHL (see Table 2b).

Concluding Remarks

The pVHL E3-ubiquitin ligase complex is essential for HIF-α proteolysis under normoxia and thus plays a critical role during embryonic development and for normal tissue physiology in the adult. In this review, we have summarized findings from the analysis of tissue-specific *VHL* knockout mice and have discussed the role of individual HIF transcription factors in the development of VHL phenotypes and in the context of VHL-associated tumorigenesis. While pVHL has biological functions that do not involve the HIF pathway, most organ pathologies in *VHL* knockout mice result from constitutive activation of HIF signaling with context- and tissue-specific contributions of HIF-1 and HIF-2 transcription factors. For a more comprehensive understanding of pVHL's HIF-independent functions in tumor suppression, development and normal tissue physiology, additional genetic studies are needed that investigate and directly compare HIF signaling in wild-type and *VHL*-deficient backgrounds.

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