von Hippel-Lindau Tumor Suppressor Protein and Hypoxia-Inducible Factor in Kidney Cancer

Mindy A. Maynard Michael Ohh

Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Ont., Canada

Key Words

von Hippel-Lindau disease · Renal clear-cell carcinoma · von Hippel-Lindau tumor suppressor protein · E3 ubiquitin ligase · Hypoxia-inducible factor · Hypoxia · Angiogenesis

Abstract

The development of hereditary von Hippel-Lindau (VHL) disease and the majority of sporadic kidney cancers are due to the functional inactivation of the *VHL* gene. The product of the *VHL* gene, pVHL, in association with elongins B and C, cullin 2, and Rbx1 form an E3 ubiquitin-ligase complex VEC that targets the alpha subunits of hypoxia-inducible factor (HIF) for ubiquitination. Ubiquitin-tagged HIF- α proteins are subsequently degraded by the common 26S proteasome. pVHL functions as the substrate-docking interface that specifically recognizes prolyl-hydroxylated HIF- α . This hydroxylation occurs only in the presence of oxygen or normoxia. Thus, under hypoxia, HIF- α subunits are no longer subjected to degradation and are thereby able to dimerize with the common and constitutively stable β subunits. The heterodimeric HIFs upregulate a myriad of hypoxia-inducible genes, triggering our physiologic response to hypoxia.

Inappropriate accumulations of HIF- α in VHL disease are believed to contribute to the pathogenesis via the upregulation of several of these HIF target genes. Our current molecular understanding of the roles of HIF and pVHL in the development of VHL-associated clear-cell renal cell carcinoma (CC-RCC) is the focus of this review.

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Hereditary Kidney Cancers

Kidney cancer occurs at a frequency of 1 in 30,000 individuals [1, 2]. Of the 4% of the cases that are hereditary, the most common form is CC-RCC, a highly vascular tumor derived from the epithelial cell of the proximal tubule [3, 4]. A prevalent genetic alteration observed in hereditary and most sporadic (approximately 80%) CC-RCC is the inactivation of the tumor suppressor *VHL* gene on chromosome 3p25 [5, 6]. Translocation of chromosome 3p to chromosomes 2, 6, 8, or 11 is also observed in families predisposed to CC-RCC [7–9]. The breakpoint of chromosome 3 for the translocations observed in CC-RCC frequently does not interrupt *VHL* gene, but may disrupt other putative tumor suppressor genes [10].

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Michael Ohh
Department of Laboratory Medicine and Pathobiology, Faculty of Medicine
University of Toronto, 1 King's College Circle
Toronto, Ont. M5S 1A8 (Canada)
Fax +1 416 978 5959, E-Mail michael.ohh@utoronto.ca

Other hereditary syndromes that predispose patients to kidney cancer include tuberous sclerosis (TS), papillary type 1 RCC, hereditary leiomyoma RCC, Birt-Hogg-Dubé syndrome, familial renal oncocytoma, and medullary carcinoma. TS patients have a germline mutation in either the *TSC1* or the *TSC2* tumor suppressor genes [11]. The products of these loci are hamartin and tuberlin which interact cooperatively to regulate cell growth [12]. Papillary type 1 RCC has been attributed to activating mutations in the proto-oncogene *a-met*, a receptor for hepatocyte growth factor (HGF) [13]. In hereditary leiomyoma patients, germline mutations of the fumarase gene followed by a second somatic inactivation of the remaining wild-type allele have been implicated in the pathogenesis of this papillary type 2 RCC [14]. Fumarase is a mitochondrial protein that functions in the Krebs cycle. The absence of functional fumarase may cause reduced oxidative phosphorylation and increased free radical formation [15]. Oxygen free radicals may activate the human HIF-1 (hHIF-1) transcription factor and subsequent downstream proliferative targets such as transforming growth factor α (TGF- α) [15]. Birt-Hogg-Dubé syndrome is caused by a mutation of the *BHD* gene that encodes the novel protein folliculin and results in chromophobe renal tumors [16]. Medullary carcinoma is a rare aggressive neoplasm associated with sickle cell disease [17]. The pathogenesis may be partially attributed to hemoglobinopathy, leading to hypoxia in the renal medulla [18]. hHIF-1 and the angiogenic protein vascular endothelial growth factor (VEGF) are overexpressed in these tumors [18]. Nonpolyposis colon cancer Lynch type 2 can also predispose afflicted individuals to kidney cancer, specifically squamous cell carcinoma in the renal pelvis. Lynch type 2 patients have defects in the mismatch repair genes *MSH* and *MLH1* [19, 20]. The etiology of familial renal oncocytoma, a benign glandular tumor, is unknown [10].

von Hippel-Lindau Disease

VHL disease is a rare inherited disorder that occurs at a frequency of 1 in 36,000 in the general population [21]. It is characterized by the development of cysts and tumors in multiple organ systems [21–24]. In the central nervous system, these may include hemangioblastomas of cerebellum, brainstem, and spinal cord. Retinal hemangioblastomas, endolymphatic sac tumors of the inner ear, pheochromocytomas, pancreatic islet cell tumors, epididymal (in males) and broad-ligament (in females) cystadenomas, and CC-RCC are also commonly associated with

VHL disease. VHL tumors are highly vascular due to the overexpression of angiogenic factors such as VEGF [25]. CC-RCC is the main cause of morbidity and mortality in these patients [26].

CC-RCCs occur in VHL patients at a frequency of approximately 45%, with a mean age at the time of detection of 39 years [27]. The patients usually possess multiple bilateral cysts and tumors in their kidneys which range in size, grade, and growth rate [27]. Typically, the smaller tumors are of lower grade and less invasive. Renal cysts in VHL patients are homozygous null for *VHL* and may transition to a solid tumor type [4]. Thus, *VHL* represents a classical ‘gatekeeper’, the inactivation of which represents an early and requisite step in tumor development [28].

Genetically, VHL disease is autosomal recessive [21, 25, 29]. VHL kindreds have a single germline mutation in one of the two *VHL* alleles. The somatic loss of the remaining wild-type *VHL* allele initiates tumor development [30]. This somatic event occurs at a very high frequency, resulting in a high penetrance of VHL disease (>90%) [21, 31]. Thus, clinically, VHL disease presents as an autosomal dominant disorder. The correlation between specific germline mutations of the *VHL* gene and tumor phenotype has led to the classification of VHL disease [21]. Typically, type 1 patients have a frequent loss of the entire *VHL* gene or a gross truncation of the protein product pVHL, while type 2 patients usually possess missense mutations [32, 33]. Type 1 VHL patients have a low risk of developing pheochromocytomas, but present with other tumors, including hemangioblastomas of CNS and retina and CC-RCC. Type 2 patients have a high risk of developing pheochromocytomas and are further subdivided according to their predisposition to CC-RCC. Type 2A patients are at low risk, type 2B patients are at high risk, and type 2C patients develop pheochromocytomas exclusively. Pheochromocytoma may represent a gain of function for pVHL because the risk of a pheochromocytoma is very low in type 1 patients [34].

VHL Tumor Suppressor Gene and pVHL

The *VHL* gene on chromosome 3p25 in humans consists of only three exons [35–37]. Its promoter contains three positive regulatory regions. An Sp1-binding site at +1 to +11 significantly contributes to transcriptional activation of *VHL* [38]. The two other positive regions (–49 to –19 and –114 to –91) interact with yet unidentified transcription factors [38]. The *VHL* locus produces two tran-

scripts that are translated into three proteins [39–42]. The first VHL mRNA contains exons 1–3 and is translated into two proteins due to an internal translational initiation start site at codon 54 [39–42]. The larger product is a 213 amino acid protein of approximately 24–30 kD (pVHL³⁰), and the shorter is an 18–19 kD isoform (pVHL¹⁹) of 160 amino acids [39–42]. At present, both are believed to be wild-type functional VHL proteins and are collectively referred to as pVHL. Accordingly, no tumor-derived mutations have been detected in the first 54 amino acid residues of *VHL* [33, 43]. Both proteins when expressed in *VHL*–/– CC-RCC cells are capable of suppressing tumor formation in Nude/SCID mice [39, 41, 42, 44]. The second transcript translated into the third protein from the *VHL* gene consists of exon 1, spliced directly to exon 3, and is not capable of tumor suppression [5].

pVHL Expression and Subcellular Localization

pVHL is conserved across species and ubiquitously expressed in human fetal and adult tissues [45, 46]. Homozygous germline inactivation of *VHL* is embryonic lethal due to a deficiency in placental vasculogenesis [47]. A conditional knockout of *VHL* in the liver of mice causes steatosis and blood-filled vascular cavities, indicating its importance in regulating angiogenesis in adults [48]. The steatosis observed in the conditional knockout is due to an accumulation of fat in hepatocytes that is similar to that observed in the stroma of hemangioblastomas in VHL patients [48–51]. However, unlike in humans, mice heterozygous for *VHL* are normal and do not develop tumors over their, albeit short, life span [47].

pVHL¹⁹ and pVHL³⁰ have minor differences in their subcellular localization. While pVHL³⁰ is primarily cytosolic and less nuclear, pVHL¹⁹ is equally distributed between the two compartments [40, 42, 52–55]. pVHL³⁰ has also been found to associate with the endoplasmic reticulum (ER), while pVHL¹⁹ does not [40, 54]. The functional significance of pVHL³⁰ associating with the ER is unclear, but may be related to the ability of pVHL³⁰ to bind fibronectin and its requirement for proper extracellular fibronectin matrix assembly [54, 56]. pVHL has also been found in the mitochondria of rats and may further regulate glucose and fat metabolism by mechanisms additional to its role in the hypoxia-responsive pathway (discussed below) [57].

VEC Ubiquitin Ligase Complex

pVHL associates with elongins B and C, Cul2, and RING box protein (Rbx) 1, forming the VEC E3 ubiquitin ligase complex (see fig. 1) [55, 58–65]. VEC functions in conjunction with an E2 ubiquitin-conjugating enzyme to polyubiquitinate proteins which targets them for degradation via the 26S proteasome. Cul2 is the scaffold for the E2 enzyme, Rbx1, and elongin C components of the complex [55]. Rbx1 functions to assist in recruiting the E2 [66–68]. Elongin C bridges Cul2 and the substrate recognition component of the E3 ligase, pVHL (see fig. 1) [69, 70]. pVHL also interacts with the CCT (cytosolic chaperonin-containing TCP-1) protein-folding complex which promotes proper folding of pVHL prior to its binding elongin C [71].

Thus, pVHL consists of two functionally distinct domains: an α domain at its COOH terminus (residues 156–206) and a β domain primarily at its NH₂ terminus (residues 63–155 and 207–213; see fig. 1) [70]. The α domain is required for binding elongin C, and the β domain is the substrate-docking interface for target proteins, including the HIF- α subunits [70]. Mutations in either domain of pVHL have been found in VHL patients and often cluster to the surface residues that contact elongin C or substrate [58, 72, 73]. This strongly suggests the importance of α and β domains for the tumor suppressor function of pVHL.

Hypoxia-Inducible Factors

Expression of hHIF- α Subunits

HIF is a heterodimeric transcription factor composed of alpha and beta subunits and is the primary mediator of the cellular hypoxic response [74–77]. There are three HIF- α genes in humans: *hHIF-1 α* , *hHIF-2 α* (also known as EPAS1 and MOP2), and *hHIF-3 α* (see figure 2). The hHIF-1 α tissue expression is ubiquitous and generally weak [71, 78, 79]. Recently, hHIF-1 was shown to be required for myeloid cell infiltration and activation because of its ability to activate the enzymes of glycolysis [80]. While the hHIF-2 α expression is also widespread, there are noticeable differences between tissues [79, 81, 82]. For example, in the mouse, mHIF-2 α is highly expressed in the lung, where it has been implicated in the development of the tubular system, and in vascular endothelial cells, where it is involved in vascular remodeling [83–85]. hHIF-3 α is the least characterized of the hHIF- α subunits and is unique because the gene gives rise to mul-

Fig. 1. Multiple mechanisms regulate the hypoxia-responsive pathway. Under hypoxic conditions, hHIF- α subunits are stabilized and enter the nucleus via their C-terminal nuclear localization signal (C-NLS). hHIF- α dimerizes with ARNT and binds to the hypoxia-responsive elements (HRE) of target genes, such as VEGF, EPO, and TGF- α , to activate transcription. hHIF-1 and hHIF-2 α are also reduced by REF-1/transferrin at a conserved cysteine residue in the C-terminal transactivation domain (C-TAD) which potentiates binding of transcriptional coactivators CBP/p300. Under normoxia, hHIF- α subunits are hydroxylated at a conserved proline residue within ODDD by PHD 1–3. The modified hHIF- α subunits are then recognized and bound by pVHL of the VEC E3 ubiquitin ligase complex for polyubiquitination which targets them for degradation via the 26S proteasome. In addition, a conserved asparagine residue within the C-terminal transactivation domain (C-TAD) of hHIF-1/2 α is hydroxylated by FIH-1 under normoxia which inhibits recruitment of coactivators.

Fig. 2. Domain structure of hHIF-1 α , hHIF-2 α , hHIF-3 α 1–4, and mIPAS. Functional domains are illustrated as open boxes, and the double-headed arrow represents the inhibitory domain (ID). bHLH = Basic helix-loop-helix; PAS = Per/Arnt/Sim; PAC = PAS-associated C-terminal domain; N/C-TAD = N/C-terminal transactivation domain; ODDD = oxygen-dependent degradation domain; C-NLS = C-terminal nuclear localization signal; LXXLL = LXXLL motif; LZIP = leucine zipper. P, C, N represent conserved proline, cysteine, and asparagine residues that are modified for hHIF- α regulation.

multiple transcripts in both mice and humans (fig. 1) [86, 87]. Its expression is highest in heart, placenta, lung, and skeletal muscle and weaker in liver and kidney of humans [86].

hHIF- α /ARNT Dimerization and DNA Binding

The hHIF-1 α protein is 826 amino acids and approximately 120 kD [88]. hHIF-2 α is 874 amino acids and approximately 115 kD [81, 83]. hHIF-3 α 1 (the longest HIF-3 α isoform) is 668 amino acids and approximately 73 kD (see fig. 2) [86, 89]. hHIF-1 α , hHIF-2 α , and hHIF-3 α possess a basic helix-loop-helix (bHLH), two Per/ARNT/Sim homology domains (PAS A and PAS B), and a PAS-associated C-terminal domain (PAC) for DNA binding and dimerization with hHIF-1 β (also known as ARNT; aryl hydrocarbon receptor nuclear translocator; see fig. 2) [83, 90]. ARNT is 91–94 kD and is constitutively expressed [74, 75]. Thus, the regulation of hHIF transcription factors occurs primarily at the level of hHIF- α subunits [75, 91]. The heterodimeric HIF binds to the hypoxia-responsive element (HRE) within the enhancer of hypoxia-responsive genes [90]. Other members of the PAS family of proteins such as ARNT2 and ARNT3 also interact with hHIF- α , although the significance of this diversity is presently unknown [92, 93].

Degradation of hHIF- α by VEC under Normoxia

hHIF- α subunits are labile under normoxia and are degraded after polyubiquitination by the VEC complex [73, 94–99]. The minimum domain that confers instability to hHIF- α has been mapped and appropriately named the oxygen-dependent degradation domain (ODDD; see fig. 2) [99–101]. A proline residue in the conserved LAPYIXMD motif within the ODDD (P564, P531, and P490 in hHIF-1 α , hHIF-2 α , and hHIF-3 α 1–3, respectively) and another conserved proline residue outside of the ODDD (P402 in hHIF-1 α) are hydroxylated in the presence of oxygen [86, 102–104]. The β domain of pVHL specifically recognizes and binds to the prolyl-hydroxylated ODDD (see figure 1) [86, 102–104].

Prolyl Hydroxylase Domain Containing Proteins

The enzymes prolyl hydroxylase domain containing proteins (PHD) 1, 2, and 3 in humans are responsible for prolyl hydroxylation (see fig. 1) [105, 106]. PHDs selectively hydroxylate hHIF- α subunits under normoxia, because their activity is dependent on the availability of molecular oxygen [105, 106]. Therefore, hHIF- α subunits are stabilized under hypoxia [96]. Based on hHIF-1 α , there is evidence supporting differential substrate speci-

ficity by the three PHD enzymes. All three PHDs are capable of hydroxylating P564, but PHD2 is most active in this capacity [105, 107]. PHD1 and PHD2, but not PHD3, are capable of hydroxylating P402 [105, 107]. Interestingly, PHD2 and PHD3 are transcriptionally upregulated by hypoxia, while PHD1 is constitutively high irrespective of the oxygen level [105, 107, 108]. The hypoxic induction of PHD2 and PHD3 may prepare the cell in advance for rapid hydroxylation of hHIF- α , when the normal oxygen tension resumes.

hHIF-3 α 1 Has a Unique Domain Structure

In addition to possessing bHLH, PAS A, PAS B, PAC, and ODDD domains, hHIF-3 α 1, but not hHIF-1 α or hHIF-2 α , possesses a leucine zipper at its COOH terminus and two LXXLL motifs flanking the ODDD (see fig. 2) [86]. These may mediate novel DNA binding and protein-protein interactions, respectively. The potential target DNA sequences and proteins that interact singularly with hHIF-3 α via the leucine zipper and LXXLL motifs are currently unknown.

Transactivation Properties of HIF- α Subunits

HIF-1 α contains an NH₂ terminus transactivation domain (N-TAD) and a COOH terminus TAD (C-TAD; see fig. 1 and 2). The C-TAD of hHIF-1 α encompasses residues 786–826 and interacts with the transcriptional coactivators cAMP response element binding protein (CREB) and CREB-binding protein (CBP), p300, steroid receptor coactivator (SRC-1), and transcription intermediary factor 2 (TIF-2) for transcriptional activation [82, 109–112]. CBP/p300, SRC-1, and TIF-2 have intrinsic histone acetyltransferase activity. Redox factor 1 (REF-1), a protein with cysteine-reducing activity, also interacts with the C-TAD [82]. In conjunction with transferrin, REF-1 reduces a conserved cysteine residue in the C-TAD (YDCEV) of HIF-1/2 α [82]. Cysteine reduction enhances binding of SRC-1 and CBP/p300 and, therefore, transactivation by hHIF-1 [82].

C-TAD is also regulated by oxygen tension through inhibitory hydroxylation of a conserved asparagine residue by the asparagyl-hydroxylase-factor-inhibiting HIF-1 (FIH-1; see fig. 1) [113]. Under normoxia, asparagine hydroxylation at residue 803 in hHIF-1 α inhibits binding of CBP/p300 by steric hindrance [113]. This residue is conserved in hHIF-2 α at residue 851. Additionally, there is a less well characterized inhibitory domain (ID) between N-TAD and C-TAD that negatively regulates these domains by a mechanism independent of altering stability, but dependent on oxygen tension [114].

Table 1. Transcriptional targets of hHIF-1

Vascular growth and regulation
α 1B-adrenergic receptor
Adrenomedullin
Endothelin 1
Heme oxygenase 1 (HO-1)
Nitric oxide synthase II (NOS-2)
Plasminogen activator inhibitor 1 (PAI-1)
Vascular endothelial growth factor (VEGF)
Iron metabolism and erythropoiesis
Ceruloplasmin
Erythropoietin (EPO)
Transferrin and its receptor
Glucose uptake and metabolism
Glucose transporters 1 and 3 (GLUT-1 and -3)
Adenylate kinase 3
Glycolysis: aldolases A and C, enolase 1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hexokinases 1 and 2, lactate dehydrogenase A (LDH A), phosphofructokinase L (PFK L), phosphoglycerate kinase 1 (PGK-1), pyruvate kinase M
Collagen matrix formation
Collagen type V- α_1
Prolyl-4-hydroxylase- α_1
Regulation of HIF-1 transactivation
p35srj/CITED2
Proliferation, differentiation, and cell viability
Cyclin G2
Differentiated embryo chondrocyte 1 (DEC-1)
Insulin-like growth factor 2 (IGF-2)
Insulin-like growth factor binding proteins (IGFBP) 1, 2, and 3
NIP-1
NIX
p21
Transforming growth factor (TGF)- α , - β_3
VEGF, EPO
Catecholamine biosynthesis
Tyrosine hydroxylase (TH)
Regulation of extracellular pH
Carbonic anhydrases (CA) 9 and 12
For references, refer to Semenza [151] and Maxwell et al. [152].

Nuclear Translocation of hHIF- α

All three HIF- α subunits possess two bipartite-type nuclear localization signals (NLS): N-NLS and C-NLS that consist of 2–5 basic residues separated by an extended spacer sequence (see fig. 2) [115]. The ability of N-NLS, found in the bHLH, to mediate nuclear localization is silenced by the PAS domains [110, 116]. The C-NLS is located between N-TAD and C-TAD of hHIF-1 α

and hHIF-2 α and before the second LXXLL of hHIF-3 α 1. C-NLS is required for the nuclear localization of hHIF-1 α , hHIF-2 α , and hHIF-3 α [115]. Interestingly, while the entire C-NLS is required for hHIF-1 α translocation, either bipartite half of the C-NLS of hHIF-2 α is sufficient for nuclear localization [115]. For hHIF-3 α , the minimal NLS required for nuclear translocation has not been determined.

Transcriptional Targets of hHIF-1, hHIF-2, and hHIF-3

hHIF-1 is an activator of transcription of numerous genes, including those involved in angiogenesis and vascular regulation, erythropoiesis and iron metabolism, glucose transport and glycolysis, collagen matrix formation, proliferation, differentiation, apoptosis, catecholamine biosynthesis, and extracellular pH regulation (see table 1). hHIF-2 is capable of binding the same HRE elements recognized by hHIF-1, and hence hHIF-2 has the potential to activate hHIF-1-responsive genes [81, 83, 84, 101]. To date, hHIF-2 has been documented to activate VEGF, erythropoietin (EPO), glycolytic enzymes, tyrosine hydroxylase, Flk-1, and Tie-2 [81, 83, 84, 101, 117, 118]. Furthermore, hHIF-2 is reported to have a higher transactivation potential than HIF-1 at some promoters, including those of *EPO* and *VEGF* [81]. At the *Flk-1* (VEGF-2 receptor) promoter, the Ets transcription factor interacts exclusively with the N-TAD of hHIF-2 α to activate transcription [117]. Correspondingly, the Flk-1 expression on proliferating endothelial cells correlates with the high expression of hHIF-2 α , but not hHIF-1 α . hHIF-2 is also reported to specifically upregulate the angiopoietin receptor Tie-2, whereas hHIF-1 has no activity at this promoter [83]. hHIF-3 appears to be a relatively weak transcriptional activator of hypoxia-responsive genes [89, 90]. Whether hHIF-3 has transactivation specificity that is unique from hHIF-1 and/or hHIF-2 is currently unknown.

Dominant-Negative Inhibitor of HIF Transactivation

Recently, a dominant-negative inhibitor of murine HIF-1 transactivation was identified as an alternatively spliced variant of the mouse *HIF-3 α* locus (*mHIF-3 α*) and named inhibitory PAS domain protein (mIPAS) [87, 119]. Under normoxia, mIPAS is expressed in the corneal epithelium and the Purkinje cells of the cerebellum [119].

Hypoxia-inducible expression of mIPAS through selective splicing of the *mHIF-3 α* locus has been shown to occur in corneal epithelium, cerebrum, cerebellum, heart, skeletal muscle, and lung [119]. Interestingly, Makino et al. [87] demonstrated that the selective splicing of the *mIPAS* isoform occurs in the exclusion of full-length *mHIF-3 α* in heart and lung [87].

Inhibition of angiogenesis in the hypoxic eye is critical in preventing vision impairment. The expression of mIPAS in the corneal epithelium inhibits angiogenesis under hypoxia by limiting the transcription of *VEGF* [119]. It is now known that mIPAS can dimerize with mHIF-1 α , preventing its binding to ARNT and to the HREs of HIF-regulated genes including *VEGF* [119]. Furthermore, implanting mIPAS antisense coated pellets into the eyes of mice disinhibited angiogenesis and thereby implicated mIPAS as the key negative regulator of HIF-mediated VEGF expression in the eye [119]. Significantly, as compared with parental Hepa 1c1c7 cells, Hepa 1c1c7 cells stably transfected with mIPAS produced slower-growing tumors with lower vascular density when implanted into SCID mice [119].

mIPAS has structural homology to hHIF-3 α 4 splice variant (see fig. 2). Whether hHIF-3 α 4 is capable of acting as a dominant-negative inhibitor of human HIF-mediated transactivation remains to be determined.

Contribution of HIF to the Development of CC-RCC

The *VHL* tumor-suppressor gene is considered the 'gatekeeper' for the development of CC-RCC. The development of CC-RCC in familial VHL disease and sporadic kidney cancer begins with the biallelic inactivation of the *VHL* locus [4, 30]. Reintroduction of the wild-type pVHL into pVHL $-/-$ CC-RCC cell lines suppresses their ability to form tumors in Nude/SCID mice [42, 44]. The ability of VEC to target hHIF-1 α and hHIF-2 α for ubiquitin-mediated destruction is thought to be indispensable for the tumor suppressor function of pVHL.

hHIF-1 α and hHIF-2 α are frequently overexpressed in hypoxic regions of solid tumors. Likewise, inordinately high levels of hHIF-1 α and hHIF-2 α are found in CC-RCCs devoid of functional pVHL [120]. It follows then that many of the hypoxia-responsive genes are upregulated in most human cancers [121]. For example, CC-RCCs are highly vascular due to the overexpression of angiogenic factors such as VEGF. Recently, hHIF-1 α has also been shown to be upregulated in TS CC-RCC [122].

TS CC-RCC tumors are reminiscent of VHL-associated CC-RCC due to their hypervascular phenotype and the overproduction of VEGF [123]. The product of the *TSC2* tumor suppressor gene (tuberin) has been shown to inhibit hHIF-1 α via the mammalian target of rapamycin (mTOR) and a mTOR-independent mechanism [122]. hHIF-regulated TGF- α is an epithelial mitogen that can stimulate the proliferation of primary renal proximal tubular epithelial cells (RPTEC; the suspected origin of CC-RCC) as well as quiescent CC-RCC cells stably expressing pVHL [124, 125]. RPTEC and CC-RCC express the TGF- α receptor EGFR (endothelial growth factor receptor) [124, 126]. Thus, the TGF- α /EGFR autocrine loop may contribute to CC-RCC tumorigenesis.

The relative contribution of hHIF-2 versus hHIF-1 in CC-RCC is an emerging area of research. The introduction of hHIF-1 α mutant that escapes pVHL recognition into CC-RCC cells reconstituted with wild-type pVHL does not produce a tumorigenic phenotype in SCID mice [127]. However, the treatment of these pVHL-restored CC-RCC with hHIF-1 α ODDD peptide that can block pVHL binding to hHIF- α substrates restored the tumorigenic phenotype [127]. This finding suggests that although hHIF-1 α is dispensable, other hHIF- α subunits (or other VEC substrates) are associated with the tumor suppressor function of pVHL. In support of this notion, the importance of hHIF-2 α in CC-RCC tumorigenesis was demonstrated by Kondo et al. [128]. In their study, they used hHIF-2 α that could not be targeted for polyubiquitination by VEC. Unlike hHIF-1 α , this stable hHIF-2 α was able to restore the tumor phenotype in CC-RCC cells stably expressing pVHL [128]. This suggests that hHIF-2 α is a major oncogenic player in the progression of CC-RCC.

HIF-Independent Functions of pVHL

Potential Additional Targets of the VEC Complex

hHIF- α proteins are the most established substrates of the VEC ubiquitin ligase complex. However, others have been identified. pVHL binds several of the atypical protein kinase C (aPKC) isoforms and may target aPKC λ for ubiquitin-mediated proteolysis [129]. VEC may also target the VHL-interacting deubiquitinating enzymes (VDU) 1 and 2 for polyubiquitination [130]. pVHL can also interact and target the hyperphosphorylated large subunit of RNA polymerase II (Rpb1) for ubiquitin-mediated degradation [131]. Rpb1 and associated Rpb6 both possess an ODDD-like domain which contains a hydroxylation motif for promoting the binding to pVHL

[131]. Moreover, Na et al. [132] have shown that pVHL can interact with Rpb7 of RNA polymerase II for proteasomal degradation which suppresses Rpb7-induced *VEGF* transactivation [132]. Thus, pVHL's ability to inhibit transcription under normoxia by binding to the components of the RNA polymerase II may contribute to its ability to function as a tumor suppressor.

Regulation of the Extracellular Fibronectin Matrix by pVHL

Dysregulation of the extracellular matrix (ECM) assembly is often associated with solid tumors [133]. VHL-/- CC-RCC cells are deficient in proper fibronectin matrix assembly [54]. pVHL has been shown to associate directly with fibronectin to promote normal fibronectin matrix formation [54, 134]. In three-dimensional growth assays, VHL-/- CC-RCC cells appear undifferentiated and form tightly packed spheroids [135]. Reintroduction of pVHL into these cells induces epithelial differentiation and forms loose spheroids. This observation has been attributed to the deposition and formation of fibronectin matrix by CC-RCC cells expressing pVHL [54]. pVHL is also required for the formation of β 1-integrin fibrillar adhesions from classic focal contacts which is required for organizing extracellular fibronectin into fibrillar arrays [56].

In type 2C VHL disease, pVHL binding to fibronectin is impaired, while its E3 ubiquitin ligase function is not [134]. Type-2C-causing mutations may represent an oncogenic gain of function for pVHL, because type 1 VHL patients have a low risk of developing pheochromocytomas.

pVHL's Regulation of Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs)

Another consequence of pVHL inactivation is the upregulation of MMPs such as MMP1 [136]. pVHL's negative regulation of MMP1 occurs independent of the hypoxia-responsive pathway [136]. MMPs are important for angiogenesis, morphogenesis, and tissue remodeling and are upregulated during tumor growth and metastasis. The MMP inhibitors TIMPs are upregulated by pVHL independent of the hypoxia-responsive pathway [136]. VHL-/- CC-RCC cells with upregulated MMPs and downregulated TIMPs are more invasive on artificial ECM in response to HGF than their counterparts with reconstituted pVHL [136].

Direct Regulation of Transcription by pVHL

pVHL is capable of regulating the transcription of certain genes independent of the hypoxia-responsive pathway and its ability to regulate RNA polymerase II subunits. pVHL has been shown to sequester the transcription factor SP1, but does not target SP1 for polyubiquitination [137]. This binding represses activity at the proximal promoter of platelet-derived growth factor β [138]. As well, pVHL can interact with hHIF-1 at HRE enhancer sequences and recruit the novel transcriptional repressor, pVHL-associated KRAB-A domain containing protein (VHLaK) [139]. VHLaK binds KRAB-associated protein 1 (KAP-1) which recruits histone deacetylase complexes or heterochromatin protein 1 family members for transcriptional repression [140–144]. Interestingly, the VHLaK gene, ZnF197, is located on chromosome 3p21, a common site for translocations found in CC-RCC [145].

pVHL's Regulation of the Cell Cycle

CC-RCC cells devoid of pVHL overexpress cyclin D1, downregulate the cyclin-dependent kinase inhibitor p27, and do not exit the cell cycle following serum starvation [148–151]. When pVHL is reintroduced into these cells specifically, the cyclin D1 expression is restored such that it is repressed under normoxia and activated during hypoxia [140].

pVHL can also interact with microtubules and stabilize them against depolymerization in vivo [152]. Microtubule polymerization and depolymerization occur during the chromosome division phase of mitosis. Disease-associated pVHLs that fail to bind microtubules are primarily type 2A mutants which predispose patients to hemangioblastomas and pheochromocytomas, but not CC-RCC [152]. Whether pVHL's role in microtubule depolymerization is important for tumor suppressor activity remains to be determined.

Concluding Remarks

In summary, the development VHL disease and most CC-RCCs are due to the mutation or inactivation of the *VHL* tumor suppressor protein pVHL. pVHL is a vital component of an E3 ubiquitin ligase complex called VEC and plays a role of specifying the target to be polyubiquitinated. Polyubiquitin-tagged proteins are subsequently degraded by the common 26S proteasome. The targets of VEC are the HIF- α subunits which are part of the heterodimeric transcription factor HIF. Importantly, in the absence of functional pVHL, as in VHL-associated tu-

mors including CC-RCC, HIF becomes stabilized and inappropriately overexpresses hypoxia-inducible genes such as VEGF, GLUT-1, and EPO. This likely explains why VHL-associated tumors are hypervascular. There are now at least three HIF- α subunits (HIF-1 α , HIF-2 α , and various HIF-3 α isoforms) that are targeted for pVHL-dependent ubiquitin-mediated destruction. This adds complexity to our current understanding of oxygen-sensing mechanisms and in particular to how hypoxia-inducible genes are ultimately regulated. Continued molecular characterization of the interplay between pVHL and its targets will undoubtedly help shed new insight into the

development of CC-RCC and perhaps provide new avenue(s) of anticancer intervention.

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