Hypoxia Review Series *J. Cell. Mol. Med. Vol 15, No 2, 2011 pp. 187-195*

Guest Edior: M. Ivan & W.Y. Kim

Two sides to every story: the HIF-dependent and HIF-independent functions of pVHL

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Received: September 2, 2010; Accepted: October 4, 2010

Abstract

von Hippel–Lindau (VHL) disease is a hereditary cancer syndrome caused by inherited mutations that inactivate the *VHL* tumour suppressor gene. The *VHL* locus encodes pVHL, whose best studied function is to bind to and down-regulate the hypoxia-inducible factor (HIF) family of oxygen-dependent transcription factors. Early efforts have established the fundamental role of HIF in *VHL*-defective tumorigenesis and in particular renal cell carcinoma. However, recent findings have revealed an alternate side to the story, the HIF-independent tumour suppressor functions of pVHL. These include pVHL's ability to regulate apoptosis and senescence as well as its role in the maintenance of primary cilium and orchestrating the deposition of the extracellular matrix. To what extent these HIF-dependent and HIF-independent functions cooperate in *VHL*-defective tumorigenesis remains to be determined.

Keywords: von Hippel–Lindau • pVHL • HIF • hypoxia-inducible factor • tumorigenesis • renal cell carcinoma • pheochromocytoma • senescence • cilia

Introduction

VHL disease was first described in the medical literature in the late 19th century by the British surgeon and ophthalmologist, Treacher Collins, who reported on the occurrence of bilateral retinal haemangiomas in a pair of siblings [1]. Subsequent observations by Eugen von Hippel and Arvid Lindau linked the occurrence of retinal haemangiomas to central nervous system haemangioblastomas [2]. The term VHL disease was later coined by the neurosurgeon Harvey Cushing.

Patients with VHL disease are at increased risk for a variety of cancers, including renal cell carcinoma (RCC) of the clear cell histology, central nervous system haemangioblastomas (especially of the cerebellum and spinal cord), retinal haemangiomas and pheochromocytomas [2]. Other manifestations include visceral cysts of the kidney and pancreas, pancreatic islet cell tumours and epididymal or broad ligament papillary cystadenomas (in men and women, respectively) (Fig. 1). In affected families, cancer risk is transmitted in an autosomal-dominant manner.

Genetic linkage studies performed in the 1980s indicated that the VHL gene (*VHL*) resides on chromosome 3p25, which is a region of the genome that is commonly deleted in sporadic kidney cancers [3]. This information was used to successfully isolate the *VHL* gene in 1993 [4]. Although RCC and haemangioblastomas are the leading cause of death in patients with VHL disease, retinal haemangiomas have the potential to cause significant morbidity (blindness) because of their association with posterior retinal detachment. Over the past century, studies focusing on the

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doi:10.1111/j.1582-4934.2010.01238.x

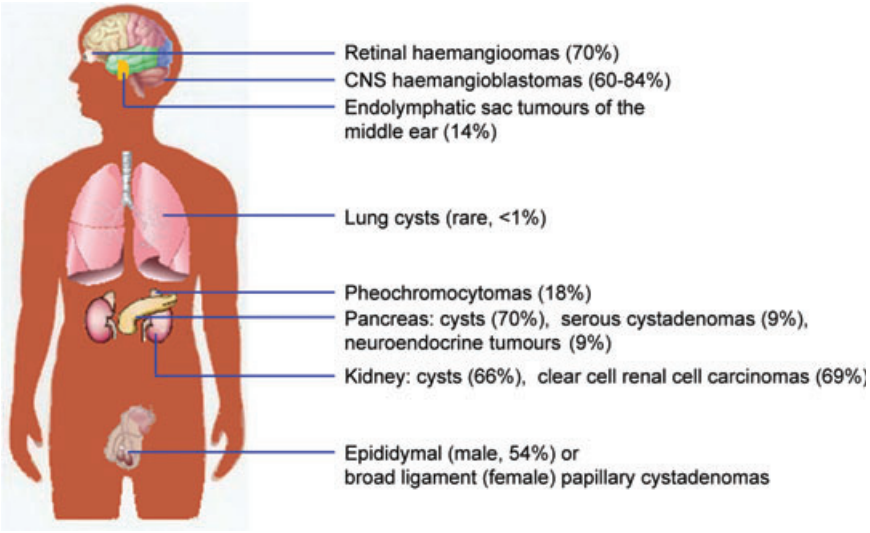

Fig. 1 Clinical manifestations of VHL disease. Summary of the spectrum of benign and malignant tumours seen in association with VHL disease. Special notes: Retinal haemangiomas are found in up to 70% of VHL patients who survive to age 60 years. Haemangioblastomas are the most common lesions associated with VHL disease and are found most frequently in the cerebellum and spinal cord. Lung cysts appear to be rare, occurring in <1% of VHL patients. Pancreatic neuroendocrine tumours are relatively rare (9%) and are typically non-functional. The incidence of broad ligament papillary cystadenomas in women is not known secondary to their asymptomatic nature.

structure and function of the *VHL* tumour suppressor gene and its protein product, pVHL, have been highly informative with respect to the pathogenesis of clear cell renal carcinoma as well as the molecular mechanisms of oxygen sensing.

The VHL protein, pVHL

The *VHL* gene consists of three exons and is ubiquitously expressed. Translation of the *VHL* mRNA gives rise to two different protein products secondary to the presence of two distinct in-frame ATG codons (codon 1 and 54), which can both serve as translational initiation sites [5–7]. In most biochemical and functional assays, the two proteins ($pVHL_{30}$ and $pVHL_{19}$) behave similarly and unless otherwise noted are referred to generically as pVHL. pVHL is primarily a cytoplasmic protein but can also be found elsewhere, including the nucleus, the mitochondria and in association with the endoplasmic reticulum [8]. In fact, pVHL shuttles back and forth between the nucleus and the cytoplasm, and pVHL cannot suppress tumour growth when artificially restrained from doing so [9, 10].

HIF-dependent pVHL functions

Many functions have been attributed to pVHL; however, the one best characterized and most clearly linked to the development of pVHL-defective tumours, is targeting of the hypoxia-inducible factor (HIF) transcription factor for proteolytic degradation (Fig. 2). HIF is a heterodimeric transcription factor consisting of an unstable α subunit and a stable β subunit. Three HIFα genes (HIF1α, HIF2α and HIF3α) have been identified in the human genome [11]. Both HIF1α and HIF2α have two transcriptional activation domains, the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD), which activate target genes upon DNA binding [12].

HIF1α and HIF2α do not appear to be fully redundant in function. Although germline knock-out of *HIF1α* and *HIF2α* results in embryonic lethality the timing and cause of death appear to differ [13–15]. Moreover, post-natal inactivation of HIF1α and HIF2α leads to differing phenotypes as well [16]. Finally, the global gene expression changes induced by HIF1 and HIF2 show that they produce overlapping yet distinct gene expression profiles in both cells and in mice [17–21]. The role of HIF3α, which possesses a NTAD but lacks a CTAD, in transcriptional regulation is less well defined, and some HIF3α splice variants appear to inhibit HIF-dependent transcriptional activation *in vitro* and *in vivo* [22–25].

Hydroxylation of HIF

When oxygen levels are high (normoxia), HIFα subunits are enzymatically hydroxylated on one or both prolyl residues that reside near the NTAD by members of the oxygen- and 2-oxoglutarate-dependent prolyl hyroxylase (PHD) family [26–29]. There are at least three PHDs identified to date: *PHD1* (*EGLN2*), *PHD2* (*EGLN1*) and *PHD3* (*EGLN3*) [30]. Although PHD2 is believed to be the primary hydroxylase for both HIF1α and HIF2α, other studies indicate that PHD3 may be mainly responsible for HIF2α hydroxylation [31, 32]. Hydroxylation of one or both proline residues within HIF1α and HIF2α creates a high affinity pVHL binding site. pVHL is part of a multi-subunit ubiquitin ligase complex composed of elongin-B, elongin-C, Cullin-2 and ring-box 1 (Rbx1) [33]. pVHL serves as a substrate recognition component that brings the ubiquitin conjugating machinery into proximity of its substrate, HIFα subunits, and leads to HIFα polyubiquitylation and destruction [26, 27, 34, 35].

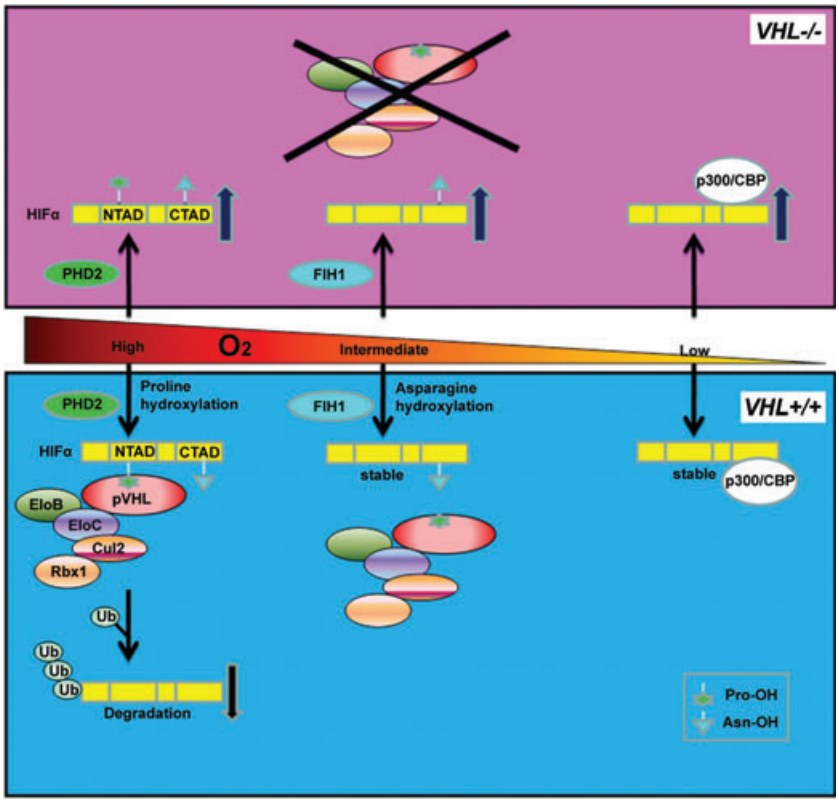

Fig. 2 pVHL controls HIF *via* oxygen-sensitive hydroxylation. HIFα subunits have both an NTAD and a CTAD transactivation domain. When O_2 levels are high, HIFα is hydroxylated on one or both conserved prolyl (Pro) residues located within the NTAD by the oxygen-dependent PHD2. This prolyl hydroxylation event generates a high affinity binding site for the pVHL E3 ubiquitin ligase complex composed of Cullin 2 (Cul2), Elongin B (EloB), Elongin C (EloC) and Rbx1. The pVHL complex polyubiquitinates HIFα, leading to its destruction by the proteasome. When O_2 levels are intermediate, HIFα is hydroxylated by factor inhibiting HIF (FIH1) at a conserved asparaginyl (Asn) residue located in the CTAD, inhibiting HIF's interaction with the transcriptional co-activators p300/CBP. When O_2 levels are low, HIFα subunits are stabilized, able to heterodimerize with the constitutively stable HIFβ, interact with p300/CBP and promote the transcription of downstream target genes. In cells lacking *VHL*, PHD2 and FIH1 remain active but HIFα subunits are not polyubquitinated and therefore allowed to accumulate.

When oxygen availability is limiting (hypoxia), the PHDs are enzymatically inactive, HIFα is therefore not hydroxylated, and does not interact with the pVHL complex. HIFα subunits therefore accumulate, translocate to the nucleus, heterodimerize with HIFβ (also called ARNT [aryl hydrocarbon nuclear translocator] and activate transcription of numerous target genes involved in cell proliferation, angiogenesis, glucose metabolism, apoptosis and other cellular processes. Similarly, in the setting of *VHL* inactivation, although HIFα subunits are prolyl hydroxylated, they are not degraded, and similar to hypoxia, are free to transactivate HIF target genes (Fig. 2).

Soon after the discovery that HIFα subunits were prolyl hydroxylated, they were noted to be post-translationally hydroxylated in an oxygen-dependent manner on a conserved asparaginyl residue located in the CTAD by the asparaginyl hydroxylase, factor-inhibiting HIF (FIH1) (Fig. 2) [36, 37]. Asparaginyl hydroxylation of HIF prevents recruitment of the transcriptional co-activators p300 and CREB (cAMP response element binding protein) binding protein (CBP), and disrupts HIF-mediated transactivation [37]. In contrast to the PHD family, FIH1 remains active even under conditions of moderate hypoxia suggesting that in this setting it may act as a secondary mechanism to inhibit HIF transcriptional activity [38]. Interestingly, the CTAD of HIF2α appears to be relatively more resistant to inhibition of FIH1 under normoxia than the HIF1α CTAD [39].

HIF is a key mediator of VHL defective tumorigenesis

Given the early age of onset of VHL-associated tumours such as retinal haemangiomas, *VHL* inactivation is likely to be sufficient for their development. Other VHL associated tumours, such as RCC however, have a longer latency and more variable penetrance, suggesting that *VHL* loss alone is insufficient for their tumorigenesis. Nonetheless, it is clear that *VHL* inactivation is necessary for their development and that HIF dysregulation plays an important role in this process. Indeed there are several lines of evidence that implicate HIFα, and in particular HIF2α, as playing an active role in $VHL^{-/-}$ renal cell carcinogenesis. First and foremost, RCC-associated pVHL mutants are invariably defective with respect to HIFα polyubiquitination and therefore all *VHL* defective RCCs produce either HIF1α and HIF2α or solely HIF2α [2, 40–42]. This would suggest that there may be selective pressure to maintain HIF2α expression, but not HIF1α. Indeed, whole exosome sequencing from sporadic RCCs detected a significant, but low frequency of truncating mutations in *HIF1α* suggesting that in RCC it may function as a tumour suppressor [43]. Second, an apparent switch from HIF1α to HIF2α expression occurs in preneoplastic lesions arising in human $VHL^{+/-}$ kidneys in association with increasing dysplasia and cellular atypia [42]. Furthermore, HIF2α activation in mice appears to induce gene expression changes similar to mice with *VHL* inactivation, whereas HIF1α activation does so to a

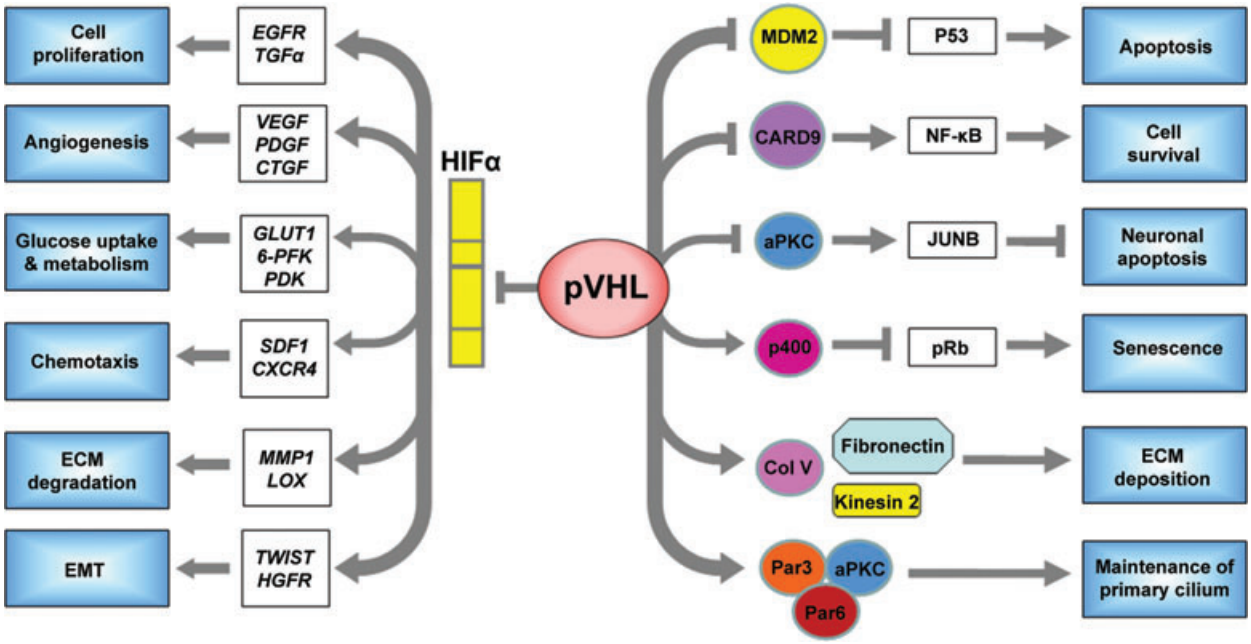

Fig. 3 pVHL inactivation mediates both HIF-dependent and HIF-independent pathways. HIF-responsive gene products play important roles in tumorigenesis. *EGFR* and *TGFα* promote cell proliferation and survival. *VEGF*, *PDGF* and connective tissue growth factor (*CTGF*) stimulate angiogenesis. Some proteins encoded by HIF-targeted gene products are responsible for regulating glucose uptake and metabolism, such as *GLUT1*, *6-PFK* and pyruvate dehydrogenase kinase (*PDK*). *CXCR4* and its ligand SDF1 stimulate chemotaxis and may also contribute to tumour cell invasion and metastases. *MMP1* and lysyl oxidase (encoded by *LOX*) are implicated in ECM breakdown and tumour cell invasion/migration. Finally, dysregulation of TWIST (*TWIST1* and *TWIST2*) and activation of HGFR (encoded by *c-MET*) are involved in EMT. pVHL has a number of HIF-independent functions as well. pVHL interacts with MDM2 and suppresses its ability to ubiqutinate p53, resulting in p53 accumulation and apoptosis. It can also act as an adaptor to bind CK2, which inactivates the NF-kB agonist CARD9, leads to inhibition of NF-kB signalling and overall inhibits cell survival. pVHL also down-regulates atypical protein kinase C (aPKC), which secondarily results in decreased levels of JUNB (an antagonist of JUN) thus permitting JUN-dependent neuronal apoptosis. Acute pVHL loss causes a senescent-like phenotype. It appears that pVHL increases p400 activity, which results in inactivation (hypophosphorylation) of the retinoblastoma protein (pRb) and prevents senescence. pVHL also interacts with collagen IV (Col IV), Kinesin 2 and fibronectin to ensure proper ECM deposition. Finally, pVHL plays an important role in primary cilium function by promoting microtubule stabilization and binding with aPKC and the polarity proteins Par3 and Par6.

much less significant degree [19]. Finally, in $VHL^{-/-}$ RCC cell lines, HIF2α, but not HIF1α, appears to be necessary and sufficient for tumour growth [44–47].

Germline inactivation of *Vhl* in mice is embryonic lethal and whereas $Vhl^{+/-}$ mice do not have a cancer prone phenotype, they do develop liver haemangiomas as a result of loss of the remaining wild-type *Vhl* allele [48]. Similarly, conditional inactivation of *Vhl* in hepatocytes results in vascular liver lesions accompanied by hepatic steatosis [48]. HIF expression is both necessary and sufficient to recapitulate the hepatic phenotypes seen in mice as *inactivation* of *Arnt* or dual *activation* of *HIF1α* and *HIF2α* were able to abrogate or induce the hepatic phenotypes seen in *Vhl* loss, respectively [19, 49]. Unfortunately at this time there are no autochthonous mouse models of $Vhl^{-/-}$ RCC that can be used to investigate HIF's role in that setting.

Several clues exist as to why HIF2α may be more oncogenic than HIF1α. First, HIF2α is less sensitive than HIF1α to the inhibition by FIH-1 and is therefore more transcriptionally active under normoxia [50]. Second, HIF1α more than HIF2α, remains susceptible to proteasomal degradation in $VHL^{-/-}$ cell lines [19]. Third, HIF2α appears to cooperate with MYC to activate MYC transcriptional targets whereas HIF1α antagonizes MYC transcriptional activation [51]. Interestingly, a recent genome-wide analysis of copy number alterations noted that a region of chromosome 8q encoding MYC is often amplified in both sporadic and VHL disease associated tumours [52]. Whether or not HIF2α activation in concert with MYC overexpression cooperate *in vivo* has yet to be determined.

HIF responsive genes

More than 100 direct HIF-responsive genes have been described with a number of these genes active in carcinogenesis (Fig. 3) [53]. These include genes that encode proteins responsible for cell proliferation (transforming growth factor [*TGFα*] and epidermal growth factor receptor [*EGFR*]); angiogenesis (vascular endothelial growth factor [*VEGF*], platelet-derived growth factor B [*PDGF-B*] and interleukin-8 [*IL-8*]); glucose uptake and metabolism (glucose transporter 1 [*GLUT1*], 6-phosphofructokinase 1 [*PFK1*]);

and chemotaxis (stromal cell-derived factor [*SDF1*] and its receptor C-X-C chemokine receptor 4 [*CXCR4*]). A number of gene products that are expected to have effects on the tumour microenvironment such as extracellular matrix (ECM) formation and turnover (membrane type 1 matrix metalloproteinase [*MMP1*] and lysyl oxidase [*LOX*]) are HIF responsive. Moreover, epithelial to mesenchymal transition (EMT) related genes (Twist [*TWIST1* and *TWIST2*] and hepatocyte growth factor receptor [*HGFR*]) are known HIF target genes as well [53].

HIF-independent pVHL functions

Recent evidence has accrued to indicate that pVHL has functions other than regulation of HIF-related pathways. The majority of these alternate functions have been discovered through biochemical interactions. However, gene expression studies also support the notion that there are HIF-independent gene expression changes induced by *VHL* loss [54, 55]. To what extent the HIF-independent functions of pVHL cooperate with HIF dysregulation in *VHL* defective tumorigenesis remains to be delineated.

Regulation of apoptosis

RCCs are notable for their insensitivity to conventional cytotoxic chemotherapies. The efficacy of chemotherapy is tightly linked to p53-mediated apoptosis [56]. However, most RCCs do not appear to harbour p53 mutations or loss suggesting either functional modulation of p53 activity or activation of alternative anti-apoptotic pathways [57, 58]. Both HIF and pVHL appear to be able to influence p53 function. Previous reports have shown that HIF can directly bind to and modulate p53 activity [59–61]. In addition, pVHL is able to regulate p53 function in a HIF-independent manner through suppression of MDM2-mediated ubiquitination and nuclear export resulting in an increase in its transcriptional activity [62]. Therefore pVHL loss appears to result in p53 inactivation by both HIF-dependent and HIF-independent effects.

The nuclear factor κB (NF-κB) pathway can mediate resistance to chemotherapy-induced apoptosis as well. pVHL deficient cells have been noted to have heightened NF-κB activity at least partially dependent upon HIF signalling [63–66]. In addition, pVHL can modulate NF-κB activity directly by binding with casein kinase 2 (CK2) and promoting the inhibitory phosphorylation of the NF-κB agonist CARD9 [67]. It seems possible that the ability of pVHL loss to both activate NF-κB and inactivate p53 may contribute to its profound chemoresistant phenotype.

Although sporadic RCC and haemangioblastomas harbour a high percentage of *VHL* mutations, *VHL* mutations are uncommon in truly sporadic pheochromocytomas [2, 68]. Indeed, 11% of apparently sporadic pheochromocytomas (defined by a lack of a family history or a spectrum of tumours suggestive of VHL disease) are actually due to occult germline, not sporadic, mutation of *VHL* [69]. This peculiarity, along with the knowledge that some *VHL* mutations that are associated with the development of pheochromocytoma (without an increased risk of RCC or haemangioblastoma) retain their ability to downregulate HIF, suggests that the development of VHL-associated pheochromocytomas is related to a HIF-independent function of pVHL [70, 71].

Insight into these apparent discrepancies has been recently elucidated by Kaelin and colleagues. It has been known for some time that during development there is an excess number of cells destined to become sympathetic neurons and that these cells' survival is dependent upon nerve growth factor (NGF). As NGF becomes limiting, these cells undergo JUN-dependent apoptosis. *VHL* mutations that are linked to pheochromocytoma development result in the HIF-independent accumulation of JUNB, which is known to antagonize the pro-apoptotic function of JUN during NGF withdrawal [72]. Thus, patients inheriting pheochromocytoma associated *VHL* mutations, presumably have an excess number of sympathetic neurons due to a relative insensitivity to NGF withdrawal induced apoptosis. However, whether the increased risk of pheochromocytoma development in patients with VHL disease is merely a reflection of an increased number of cells susceptible of forming pheochromocytomas or a distinct oncogenic mechanism associated with these mutations is unclear.

Control of cell senescence

Cellular senescence is the phenomenon of irreversible growth arrest in response to DNA damage (including shortened telomeres) but is also an important *in vivo* tumour suppressor mechanism [73, 74]. Interestingly, it has been recognized that physiological oxygenation can extend the replicative lifespan of cells in culture, which has typically been attributed to a relative decrease in the amount of oxidative stress [75]. Several reports have now confirmed that this phenomenon is at least in part due to stabilization of HIF [76, 77]. Interestingly, acute pVHL inactivation (with resultant HIF stabilization) was observed to induce senescence both *in vitro* and *in vivo* [78]. In this setting however, senescence appeared to be independent of both HIF and p53 function but primarily relied on activation of the retinoblastoma protein (pRb) and down-regulation of the SWItch/Sucrose NonFermentable (SWI2/SNF2) chromatin remodelling protein, p400. Recent work showing that induction of senescence by *VHL* loss is highly dependent upon oxygenation along with the differences in the senescence assays examined (*i.e.* replicative *versus* oncogene-induced senescence) may begin to explain the contrasting results [79].

Microtubule stabilization and maintenance of the primary cilium

pVHL associates with and is able to stabilize microtubules. This function of pVHL appears to be independent of its ability to either down-regulate HIF and its ubiquitin ligase function. Moreover, pVHL's ability to stabilize microtubules is lost in *VHL* mutations

that predispose to the development of haemangioblastomas and pheochromocytomas, but not those associated with the development of RCC [80]. The primary cilium is a specialized structure on the cell surface that serves an antenna of the cell, and regulates the transduction of both chemical and mechanical signals [81]. The ciliary axoneme is composed of microtubules arranged in nine peripheral doublets that are templated from the basal body or mother centriole. Thus microtubule dynamics and formation and maintenance of the primary cilium are intimately linked.

Preneoplastic renal cysts are a common feature of VHL disease. Immunohistochemical and laser capture microdissection studies have demonstrated that the renal tubular epithelial cells lining these cysts have lost expression of *VHL* [42, 82, 83]. Other inherited familial syndromes are characterized by the development of renal cystic diseases of the kidney (*e.g.* autosomal dominant and recessive polycystic kidney disease [ADPKD and ARPKD, respectively], and Bardet Biedl syndrome) and despite being phenotypically diverse and having distinct extrarenal manifestations, these disorders are intriguingly unified by genetic defects that converge on the regulation of ciliogenesis and function [84].

pVHL's affects on microtubule dynamics is negatively regulated by its phosphorylation by glycogen synthase kinase 3β (GSK-3β) and appears to be HIF-independent, although some studies suggest that HIF dysregulation may play at least a partial role in the loss of microtubule stability imparted by pVHL inactivation [85–87]. Interestingly, active GSK-3β itself can promote microtubule stability and cilium maintenance in a pVHL-independent manner. When GSK-3β is inactive, for example following activation of the PI3Kinase-Akt pathway, microtubule stability and cilium maintenance appear to rely on pVHL once again. In keeping with the notion that GSK-3β and pVHL redundantly maintain primary cilia, it appears that the combined loss of *VHL* and *PTEN* in a genetically engineered mouse model cooperate to promote renal and genital tract cysts [88].

It is an apparent paradox that VHL mutants predisposing to RCC maintain the ability to regulate microtubule dynamics. One possibility is that the development of renal cysts secondary to loss of primary cilia on renal tubular cells lack significant malignant potential. In this scenario, the majority of RCCs associated with VHL disease would be expected to arise *without* an antecedent cystic phase. To some degree, this is in keeping with the observation that patients with polycystic kidney disease, despite having a high renal cystic burden are not clearly at a significantly higher risk for RCC [89].

Regulation of extracellular matrix formation and cell – cell adhesion

The ECM, a physical barrier to cancer cell migration and invasion, can provide survival signals to cancer cells and aide in the maintenance of cell polarity in concert with intercellular junctions [90]. pVHL can bind directly to both fibronectin and hydorxylated collagen IV, and interestingly all pVHL mutants studied to date are defective in this capacity [71, 91]. The inability of *VHL* deficient cells to bind ECM components results in ineffective ECM organization that is not mediated by HIF [92–94]. Moreover, pVHL's ability to orchestrate proper ECM deposition does not require binding to the other components of the pVHL complex such as Cullin2 and Elongins B and C and is regulated at least partially by the post-translational modification of pVHL by the ubiquitin-like molecule, NEDD8 [95, 96]. Similarly, cell polarity and assembly of intercellular junctions (*i.e.* adherens junctions and tight junctions) are defective in cells lacking *VHL* in a HIF-independent process [97]. How an intracellular protein such as pVHL modulates the assembly of the extracellular ECM components remains to be fully elucidated.

pVHL and synthetic lethality

Synthetic lethality occurs when two non-allelic mutations, which by themselves are not lethal, result in cell death when they exist simultaneously [98]. Synthetic lethality provides a framework to discover drugs that might preferentially kill cancer cells harbouring a cancer-relevant gene, yet leave normal cells unharmed. Two screens have been performed in attempt to target *VHL* deficient cells. A cell based small molecule synthetic lethality screen identified a compound, STF-62247, that selectively induces autophagic cell death in *VHL*-deficient RCC cells but not in those expressing wild-type *VHL* [99]. In addition, an shRNA screen targeting a select group of kinases identified and validated that silencing of *CDK6*, *MET* and *MAP2K1* (MEK1) preferentially inhibited the growth of $VHL^{-/-}$ cells compared with their isogenic *VHL* wild-type counterparts [100]. Interestingly, in both screens the selective killing of cells lacking *VHL* was HIF-independent leaving open the possibility that therapies targeting these pathways might cooperate with those targeting HIF.

Conclusions

The *VHL* tumour suppressor gene is mutated or silenced in the majority of clear cell RCC. Loss of pVHL function results in the stabilization of HIFα and activation of HIF responsive genes. Many of these gene products have been shown to be oncogenic in the context of RCC. In recent years, our understanding of pVHL function has broadened to include several HIF-independent functions and it seems likely that more will be uncovered. Despite this broadened understanding of the consequences of *VHL* loss, the therapies in clinical use for RCC to date are primarily focused on dampening of HIF signalling and although effective have not achieved remarkable results. It will be interesting to determine whether targeting of HIF-independent pVHL functions either separately or in concert with HIF will lead to improved results.

Acknowledgements

W.Y.K. is a Damon Runyon Merck Clinical Investigator. This work was supported by NIH grants R01CA142794 (W.Y.K.) and T32CA128590 (M.L.) and the UNC University Cancer Research Fund (W.Y.K.).

Conflict of interest

The authors state that there is no conflict of interest.

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