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Systemic *VHL* gene functions and the VHL disease

Hannah L. Bader and **Tien Hsu***
Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118, United States

Abstract

The von Hippel-Lindau tumor suppressor gene (*VHL*) is best known as an E3 ubiquitin ligase that negatively regulates the hypoxia inducible factor (HIF). *VHL* mutations are the genetic defects underlying several human diseases including polycythemia, familial VHL tumor syndrome and sporadic renal cell carcinoma. *VHL* mutations can lead to cell-autonomous phenotypes in the tumor cells. However, non-tumor cell-autonomous functions of *VHL* have also been noted. VHL tumor-derived cytokines can promote inflammation and induce mobilization of endothelial progenitor cells. Up-regulation of HIF caused by *VHL* loss-of-function mutants, including heterozygotes, has been shown to increase the activities of hematopoietic stem cells, endothelial cells and myeloid cells. As such, systemic functions of *VHL* likely play important roles in the development of VHL disease.

Keywords

Erythropoietin; Hematopoiesis; Hemangioblastoma; Haploid insufficiency; Inflammation

Introduction

The von Hippel-Lindau tumor suppressor gene (*VHL*) encodes a multifunctional protein, the mutations of which underlie the genetic defect in the familial VHL disease. Germ line mutations in *VHL* predispose the patients to several highly vascularized benign and malignant tumors, including renal cell carcinoma of the clear-cell type (ccRCC), hemangioblastoma (HB) and pheochromocytoma (tumor in the adrenal glands). Less frequent VHL tumors include those in pancreas (pancreatic cysts, serous cystadenoma and pancreatic neuroendocrine tumors), inner ears (endolymphatic sac tumor) and testes (epididymal cystadenomas). In these tumors, the remaining wild-type allele is inactivated through somatic mutation. Biallelic loss of *VHL* function has also been found in a majority of the sporadic ccRCC [1–3]. The protein encoded by the VHL gene is best known as the substrate-binding subunit of an E3 ubiquitin ligase [4–8]. The best-known degradation target of VHL-containing E3 ligase is the α-subunit of hypoxia-inducible factor (HIF-α) in normal physiological conditions [9]. At normal oxygen level, HIF-α is hydroxylated at the proline residues within an oxygen-dependent degradation domain. The prolyl-hydroxylated HIF-α is recognized by VHL, leading to poly-ubiquitination and degradation. The hydroxylation

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*Corresponding author at: Department of Medicine, Boston University School of Medicine, 650 Albany St., Rm. 440, Boston, Massachusetts 02118, United States, Tel: 1-617-638-7599, Fax: 1-617-638-7530, tienh@bu.edu.

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reaction is mediated by the prolyl-hydroxylase domain proteins (PHDs) [10]. In hypoxic conditions, the prolyl-hydroxylases are inactive and HIF-α is stabilized. HIF-α then dimerizes with the β-subunit (HIF-β) and translocates to the nucleus where the dimer functions as a transcription factor. Its best-known target genes encode proteins involved in glycolysis (e.g., phosphoglycerate kinase), glucose transport (Glut-1), angiogenesis [vascular endothelial growth factor (VEGF)] and erythropoiesis (erythropoietin); that is, proteins that mediate the cellular response and adaptation to hypoxic conditions [11]. In addition, CXC chemokine receptor 4 (CXCR4) and the ligand stromal cell-derived factor (SDF)-1 were also identified as HIF targets [12, 13], which indicates that HIF activation may contribute to the metastatic potential of cancer cells. These functions support a critical role of VHL in regulating tumor progression, especially in hyper-vascularized tumors such as ccRCC and HB. However, accumulated evidence has indicated that many HIF-independent activities of VHL also exist [14, 15]. Some of these functions are mediated through stabilizing VHL targets, contrary to its known E3 ligase activity. Therefore, VHL is a multifunctional adaptor protein that, depending on the interacting partners, can promote protein degradation or serve as a chaperon. Some of these diverse activities likely also contribute significantly to the tumor suppressor and other physiological functions.

These diverse functions also suggest that there may not be a simple, unified pathophysiological mechanism that can explain the etiology of VHL diseases. Although cell-autonomous mechanisms in *VHL* mutant tumors might be explained by up-regulation of cyclin D1 [16], increased Akt-mTOR signaling [17, 18], elevated FGF receptor signaling [19, 20], disruption of cilia formation [21–23], down-regulation of p53 [24], among others, it is also well established that *VHL* mutant cells secret a large repertoire of growth factors and cytokines, including erythropoietin (Epo), VEGF, TGF-β, PDGFβ, TNF-α, among many others [11]. Furthermore, in the last decade, it has become apparent that HIFs play a central role in the hematopoietic system [25–28]. *VHL* mutations and the resulting HIF up-regulation therefore can impact many vasculo/hematopoietic lineages. Also interestingly, some of these abnormalities can occur in partial *VHL* loss-of-function mutants and in heterozygous mice and heterozygous immune cells from VHL patients [20, 29, 30]. During development, the *Drosophila VHL* gene also shows haploid insufficiency [31]. The haploid-insufficient function of *VHL* may be profoundly important in considering the etiology of VHL disease since all VHL patients are, by definition, *VHL* heterozygotes. Such understanding prompted the idea that a non-cell autonomous and systemic consideration of the *VHL* function in the context of VHL disease may need to be evaluated. This review examines the current knowledge in this regard. The role of VEGF and PDGFβ over-expression in VHL tumor angiogenesis is well known and will not be discussed herein.

1. VHL-associated polycythemia

A homozygous missense mutation in the *VHL* gene, first identified in the Chuvash region of Russia and called the Chuvash mutation (R200W), was linked to a familial form of polycythemia — overexpansion of erythrocytes [32]. Subsequently, a knockin R200W transgenic mouse and a zebrafish *VHL* null mutant also exhibited polycythemia [33, 34]. Erythropoiesis is regulated by Epo, and polycythemia can be caused by hypersensitivity to Epo (called primary polycythemia or polycythemia vera), or by overproduction of Epo (secondary polycythemia) [33, 35, 36]. Interestingly, VHL-associated polycythemia has features of both primary and secondary polycythemia [36]. Epo is normally produced by interstitial fibroblasts in the kidney [37, 38] and by perisinusoidal (Ito) cells in the liver [39]. HIF over-expression leads to up-regulation of Epo, not only in the normal Epo-producing cells but also in the *VHL* mutant tumor cells [40–43]. However, erythrocyte progenitors harboring the R200W mutation are also more sensitive to Epo [33, 35, 36]. The mechanism of this hypersensitivity has been discovered only recently. The Epo receptor (EpoR) signals

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through the JAK-STAT pathway: upon ligand binding, the receptor-associated tyrosine kinase JAK2 is phosphorylated and in turn phosphorylates the transcription factor STAT5 [36, 44]. In a negative feedback loop, phosphorylated JAK2 (pJAK2) is targeted for proteasomal degradation by ubiquitination [44]. VHL was found to mediate pJAK2 polyubiquitination, and in polycythemia-associated *VHL* mutations, a failure to eliminate pJAK2, leading to prolonged JAK-STAT signaling, was observed [35]. This phenotype could be rescued both *in vitro* and *in vivo* by administration of the JAK2 inhibitor Tg101209, indicating that JAK2 inhibitors may prove useful in the treatment of polycythemia. Surprisingly, VHL-mediated pJAK ubiquitination did not require the formation of the well-characterized ElonginB/C-Cul2-VHL E3 ligase complex [5]. Instead, pJAK2 ubiquitination depended on the formation of a complex of pJAK2, SOCS1 and VHL. The presence of primary polycythemic phenotype also indicates that *VHL* mutant erythrocyte progenitors may be a contributing factor to the disease.

2. The role of Epo in angiogenesis and tumorigenesis

Polycythemia has been considered an isolated branch of the VHL syndrome, mainly because the Chuvash disease (R200W mutant) patients do not exhibit increased incidences of tumors [45]. This notion may need to be reconsidered, however, since Epo has been shown to have pleiotropic effects [46, 47], including a role in tumorigenesis. Epo signaling is not restricted to red blood cells. Also, the Epo/EpoR system is known to induce proliferation, chemotaxis, and angiogenesis, and inhibit apoptosis [46, 48, 49]. Epo has been found to exert a strong cyto-protective effect in animal models of brain, cardiac and renal ischemia [reviewed in (47)]. Epo signaling appears to inhibit apoptotic pathways triggered by ischemia, but may in addition reduce hypoxic injury by promoting angiogenesis. Epo administration to ischemic patients is therefore currently subject to clinical trials. Epo and EpoR are also expressed in various tumors including those of head-and-neck [50], breast, colon, lung, prostate [51], ovary [51, 52], uterine [52], kidney [40, 42, 43] and cervix [53], as well as neuroblastoma, astrocytoma, and other solid nervous system tumors [54], and numerous malignant cell lines [55, 56]. Epo added to cancer cell lines *in vitro* elicited secretion of angiogenic growth factors and promoted proliferation and chemotaxis of endothelial cells [54]. Neutralizing anti-Epo monoclonal antibody and soluble EpoR antagonist injected into *ex vivo* cultured tumor tissue blocked tumor growth [52] and soluble EpoR antagonist injected into mice carrying cancer cell xenografts reduced angiogenesis and tumor cell survival [55]. Furthermore, Epo pretreatment of some cancer cell lines rendered them less sensitive to the cytotoxic effects of the chemotherapy drug cisplatin [56]. Of note, polycythemic mice were shown to be iron deficient [57], most likely because the Epo-induced erythropoiesis led to an exhaustion of iron stores. Since iron is a necessary co-factor of proline hydroxylases, it is tempting to speculate that Epo-induced iron deficiency may be a mechanism by which tumors mediate systemic HIF up-regulation.

Interestingly, although full-blown polycythemia is infrequent in VHL disease, consistently elevated Epo is detected in a majority of VHL patients [58]. In addition, certain heterozygous *VHL* mutations can lead to polycythemia [59–61]. There is also an established association between hemangioblastoma and polycythemia [62]. It is therefore conceivable that elevated Epo level in VHL patients, whether or not it manifests into polycythemia, may contribute to progression of various VHL tumors.

3. VHL and endothelial progenitor cells

Based on the observation that mononuclear cells isolated from bone marrow or peripheral blood can give rise to endothelial colonies, the existence of endothelial stem cells – called endothelial progenitors cells (EP or EPCs) or, if detected in the peripheral blood, circulating

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EPCs (CEP/CEPC) – has been postulated (reviewed in [63, 64]). Importantly, EPCs need to be distinguished from a subset of monocytes that express endothelial markers such as VEGFR1, VEGFR2, Tie2 and CD31. These monocytes are sometimes referred to as circulating angiogenic cells (CACs). CACs contribute to angiogenesis by releasing proangiogenic cytokines, but do not differentiate into endothelial cells; instead they differentiate into a subset of tumor promoting macrophages (discussed in section 5) [63, 65]. CACs and CEPCs are CD45 positive and negative, respectively.

It has been proposed that CEPCs may contribute to tumor angiogenesis, although this is still somewhat controversial (see for instance [48, 66]). Nevertheless, there is evidence that CEPCs are relevant to VHL tumors. Patients with ccRCC were found to have elevated levels of CEPCs [67]. In addition, high levels of VEGFR2-positive cells in the peripheral blood were found to correlate with a poor prognosis in ccRCC [68]. Interestingly, elevated CEPCs were also detected in a case of sporadic ccRCC caused by somatic *VHL* inactivation [69]. Therefore CEPC mobilization observed in VHL tumors may result from both intrinsically higher motility of the EPCs or from stimulation emanating from the VHL tumors. In the latter case, VHL tumors can induce CEPC mobilization through Epo [70], which is expressed by ccRCC [42, 43]. In addition, ccRCC can also induce the mobilization of VEGFR1+ CACs, at least in part through VEGF [71].

4. VHL, HIF and the hematopoietic system

The role of HIF in the immune system has been extensively reviewed [25–28, 72]. We will therefore summarize only some of the most recent findings and highlight papers that directly address the role of VHL.

In the last decade it has become evident that HIF, and in particular HIF-1α, regulates hematopoietic stem cells, and both innate and adaptive immune cells [25–29, 72]. In contrast, the role of VHL in these cell lineages has been addressed only in a small number of studies. However, there is evidence that cells of the hematopoietic system are sensitive to even small changes in HIF-1α expression levels. Hence, in many cases, opposite phenotypes are observed in *HIF-1*α−/− and *VHL*−/− cells, and in some cases *VHL* haploinsufficiency is observed, with *VHL*+/− cells displaying a phenotype that is intermediate to that observed in *HIF-1*α−/− and *VHL*−/− cells [29, 30]. This is consistent with a model that implicates the importance of HIF-1α dosage: *HIF-1*α−/− cells display loss of function, *VHL*+/− cells intermediate gain-of-function due to moderate HIF-1α up-regulation, and *VHL*−/− cells hyper- or malfunctioning due to high-level HIF-1α over-expression.

It is now recognized that lymphatic organs as well as sites of inflammation are hypoxic [28, 29]. Cells of the hematopoietic system rely to a large extent on an anaerobic source of ATP: glycolysis [73–75]. Since it is the master transcription factor of glycolysis related genes, HIF-1α plays a prominent role in many lineages of the hematopoietic system [28, 73–75], and defects in *HIF-1*α deficient immune cells can be in part explained by decreased ATP levels [74]. Besides its role in regulating glycolysis, HIF-1α also regulates apoptosis, cell proliferation and differentiation of the hematopoietic lineages. For instance, it was recently shown that HIF-1α is necessary for the maintenance of quiescence in long-term hematopoietic stem cells (LT HPSC) [29]. In an induced conditional *HIF*-1α knockout (using Poly I:C induced Msx-Cre), LT HPSCs showed increased proliferation when experimentally challenged as well as upon aging. The increased proliferation led to senescence, and ultimately to an exhaustion of the LT HPSC pool. Consequently, *HIF-1*α−/− HPSCs failed to reconstitute the bone marrow in serial transplantations. In contrast, deletion of *VHL* in HPSCs led to increased quiescence and increased numbers of HPSCs, in particular LT-HPSCs. However, these *VHL* mutant HPSCs were not functional: they failed

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to reconstitute the bone marrow in transplantation experiments due to a combination of abnormal quiescence, homing defects and enhanced apoptosis [29]. The observed defects in *VHL*−/− HPSCs were HIF-1α dependent and cell autonomous. Interestingly, heterozygous *VHL* HPSC displayed an intermediate phenotype: deletion of one *VHL* allele led to increased quiescence and increased numbers of HPSCs; however, in contrast to *VHL*−/− HPSCs, *VHL*+/− HPSC were functional and reconstituted the bone marrow of lethally irradiated mice even more efficiently than wild-type HPSCs. Hence, HIF-1α levels in HPSCs are fine-tuned to maintain a balance between quiescence necessary for the maintenance of the HPSC pool, and proliferation necessary to replenish the hematopoietic system. The increased HPSC activity conferred by *VHL* heterozygosity may be a significant contributing factor in the progression of VHL disease, considering the hypervascularity associated with the VHL tumors and the potential contribution from inflammatory response (see below).

HIF-1α also plays an important role in lymphocytes. *HIF-1*α deficiency leads to an increase in B1 B cells, and a concomitant reduction in B2 B cells, the latter due to a lack of proliferation of pro-B cells [76]. The role of VHL in B cell differentiation has not been directly addressed, but epigenetic down-regulation of VHL has been recently reported in diffuse large B cell lymphoma and chronic lymphocytic leukemia [77–80].

The role of HIF-1α in T cells differentiation and activation is somewhat controversial, although conflicting findings may be in part explained by distinct roles of HIF-1α in different T cell subsets and immature vs. mature T cells. Neumann and colleagues reported attenuated T cell receptor (TCR) signaling in *VHL*−/− T cells [81]. This effect was HIF-1α-dependent, since it was not observed in *VHL*−/− *HIF-1*α−/− T cells. Hence, HIF-α expression appears to regulate TCR signaling negatively. Consistent with this, deletion of *VHL* in the thymus led to increased apoptosis of T cells at the double positive stage (a stage at which T cells are positively selected for a functional TCR) [82], resulting in a decrease in mature T cells. Furthermore, *HIF-1*α−/− T cells were reported to produce more inflammatory mediators such as IFNγ and TNFα- than wild-type T cells when stimulated *in vitro* [83]. Also, mice in which *HIF-1*α was deleted in T cells showed improved survival of sepsis due to T cell activation [84]. In contrast to these findings, which indicate that HIF-1α negatively regulates T cell survival and activation, hypoxia induced HIF-1α was found to inhibit activation induced cell death (AICD) in T cells [85]. However, this protective effect may be also explained by attenuated TCR signaling, since AICD is TCR dependent. On the other hand, it is difficult to reconcile the anti-inflammatory effects of HIF-1α discussed above with the recent finding that HIF-1α is essential for the induction of Th17 cells, a pro-inflammatory CD4 T cell subset [86]. CD4 T cells can differentiate into either Th17 or Treg T cells, depending on the cytokine environment [87]. RORγt and Foxp3 are key transcription factors in Th17 and Treg differentiation, respectively, and Foxp3 also inhibits RORγt [87]. Dang and colleagues [86] showed that HIF-1α tips the balance towards Th17 differentiation by inducing RORγt. Consistent with these findings, mice in which *HIF-1*α was conditionally deleted in CD4 T cells had reduced numbers of Th17 cells and were protected from experimentally induced encephalitis (a model for autoimmune disease). Therefore, *VHL*+/− with moderate increase in HIF-1α can potentially promote Th17-mediated inflammatory response.

The role of HIF in the myeloid lineage was addressed using a lysozyme M driven Cre, which led to efficient deletion in both macrophages and neutrophils [72, 74]. *In vivo* experiments using these conditional knockout mice show that both HIF-1α and HIF-2α have essential roles in myeloid-mediated inflammation. Myeloid *HIF* deletion protected mice from inflammation induced by chemical irritation, experimentally induced autoimmune disease and LPS induced sepsis [72, 74]. Conversely, mice with myeloid *HIF*

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deletion were impaired in controlling bacterial infections [88]. These studies also showed that HIF-1α and HIF-2α have distinct, non-redundant roles in innate immunity. In myeloid cells, HIF-1α is needed for ATP generation, granzyme synthesis and iNOS/NO production, and, in neutrophils, protection from apoptosis, whereas HIF-2α mediates cytokine production and up-regulates chemokine receptors involved in macrophage migration [25, 26, 74, 88]. Consistent with HIF over-expression upon *VHL* deletion, *HIF* and *VHL* myeloid knockout resulted in opposite phenotypes both *in vitro* and *in vivo* [74]. *VHL*−/− macrophages produced more granzymes and iNOS/NO, and were more efficient than wild-type macrophages at lysing phagocytosed bacteria *in vitro*. Furthermore, in a phorbol ester induced ear inflammation model, mice with myeloid *VHL* deletion displayed a significantly enhanced inflammatory response, although it was not investigated whether this phenotype was solely due to HIF over-expression [74].

5. VHL, inflammation and cancer

Based on the observation that HIF is essential for myeloid and Th17-mediated inflammation, it is now thought that hypoxia is a strong pro-inflammatory cue for immune cells [26–28, 86, 89]. Importantly, *VHL* haploinsufficiency, which is likely associated with partial up-regulation of HIF, is observed both in HPSCs and neutrophils (see above) [29, 30]. Also, in support of a link between VHL and inflammation, it has recently been reported that pulmonary hypertension, a complication of Chuvash polycythemia, is caused by lung fibrosis [90]. It is hence likely that the immune system of *VHL* heterozygous individuals is skewed towards the development of inflammatory responses. Such a pro-inflammatory environment may contribute to tumorigenesis. Tumor infiltrating macrophages, called tumor associated macrophages (TAMs), are thought to promote tumor growth by releasing pro-angiogenic and immunosuppressive cytokines. In support of this, a high degree of TAM infiltration correlates with poor prognosis in human cancers [65]. Nuclear HIF-2α was detected in TAMs of many human cancers [91], and conversely, myeloid *HIF-2α* deletion led to a reduction in TAM recruitment, and concomitantly, slower tumor growth [72]. It is tempting to speculate that *VHL* deletion (*VHL*−/− or *VHL*+/−) would lead conversely to faster tumor growth due to more efficient TAM recruitment and/or increased cytokine production by TAMs. However, alterations in other immune cells, such as Th17 T cells, could also promote tumorigenesis. It remains to be elucidated if immune cells contribute to the formation of tumors in VHL disease, and if so, which types of immune cells are relevant in this process.

Besides a direct role in the immune cells, *VHL* knockouts in mouse kidney epithelia have also been shown to induce an inflammatory response. A knockout strain specific for the podocyte in kidney exhibited glomerulomegaly and occasional glomerulosclerosis [92]. Interestingly, a *Pepck-Cre* driven (specific for proximal tubules) *VHL* knockout could be induced to develop renal fibrosis after subtotal nephrectomy of one kidney and complete removal of the other [93]. In addition, hypoxia and increased HIF-1α or HIF-2α activities have been linked to kidney fibrosis in mouse [94, 95]. *VHL* mutant ccRCC cells have been shown to over-produce such inflammatory cytokines as TNFα [96] and TGF-β [97], and to up-regulate NFkB [98, 99], the latter can in turn induce cytokine expression. The connection between *VHL* mutant cells and inflammation is worthy of more in-depth investigation, since prolonged inflammation can promote proliferation through the action of secreted cytokines, and importantly, can induce genetic changes in pre-cancerous cells by induction of reactive oxygen species or by oxidative inactivation of mismatch repair enzymes [100–103].

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6. VHL, glucose metabolism and insulin secretion

β-cells in the endocrine islets of the pancreas produce insulin and store it in secretory vesicels [104]. Glucose Stimulated Insulin Secretion (GSIS) is essential for glucose homeostasis and is induced by quantitative ATP generation after glucose uptake. A rise in the ATP/ADP ratio induces the closure of ATP sensitive KATP channels, leading to depolarization of the plasma membrane and Ca^{2+} influx. The Ca^{2+} influx in turn stimulates the excocytosis of insulin. Hence, insulin secretion is coupled to extracellular glucose concentration: a rise in extracellular glucose concentration results in increased intracellular ATP generation, and ultimately, in increased insulin secretion. Since HIF-1α up-regulation is known to affect glucose metabolism, the role of VHL in insulin-mediated glucose homeostasis has attracted extensive interest. β-cell (RIP2) or pan-pancreas (Pdx2) specific Cre mice have been used to delete *VHL* in β-cells [105–109]. Several studies reported that gross morphology of the pancreas was unaffected by β-cells specific or pan-pancreatic *VHL* deletion [105–108], although two studies suggested an age dependent decrease in β-cells [106, 107]. In contrast, a recent study reported development of precancerous lesions in pan-pancreatic *VHL* knockout mice, but not upon deletion of *VHL* in α-or β-cells [109]. These differences are at least in part due to differences in the mouse strains used in the studies (mixed C57BL/6 - Sv129 vs C57BL/6, BALB/C or A/J) [110]. Interestingly, the β-cells *VHL* knockout mice, but not the pan-pancreatic *VHL* knockout mice displayed also dwarfism [105, 107, 111]. Cantley and colleagues reported that the dwarfism was caused by RIP2-Cre activity in the hypothalamus, which led to reduced growth hormone (GH) production [105], whereas Shen and colleagues did not oberve a significant down-regulation of GH [109]. It is possible that these differences are due to the age at which the GH levels were assayed (3 months vs 6 monts). More importantly, several studies showed that *VHL* knockout in β-cells (pan-pancreatic or β-cell specific) led to impaired GSIS at high glucose concentrations (e.g., following glucose injection), and consequently glucose intolerance (impaired clearance of glucose from the blood), the hallmark of type-II diabetes [105, 106, 108]. Impaired GSIS and glucose intolerance were found to be HIF-1α dependent, since they were not observed in *VHL* and *HIF-1*α double β-cell knockout mice [105, 106]. In the cell, glucose can be used for aerobic or anaerobic ATP generation. In the aerobic pathway, glucose is processed to pyruvate, which is converted to acetyl-CoA and is channeled into the tricarboxy acid (TCA) cycle, generating electron donors for oxidative phosphorylation in mitochondria, with oxygen as the electron acceptor. In contrast, the less-efficient glycolytic ATP generation involves the processing of pyruvate to lactate. *VHL* deletion in β-cells led to HIF-1α-dependent upregulation of pyruvate dehydrogenase kinase 1 (PDK1), which inhibits the conversion of pyruvate to acetyl-CoA, preventing its entry into the TCA cycle, and Lactate dehydrogenase A (LDHA), which generates lactate from pyruvate. Hence, as described for other cell types, HIF-1α stabilization in β-cells induced a metabolic switch from ATP generation through oxidative phosphorylation to ATP generation through glycolysis. Although these studies disagreed on whether glucose uptake and basal insulin secretion were affected in *VHL* knockout cells, they demonstrated that the lack of oxidative glucose metabolism was the likely cause of the observed type-II diabetes phenotype. A link between *VHL* mutation and diabetes has not been established clinically. Nonetheless, the metabolic imbalance gleaned from the *VHL* functional studies may help clarify some aspects of the etiology of type-II diabetes. These models also serve as an *in vivo* confirmation of metabolic switch in the *VHL* mutant cells, which may be a relevant contributor to the suspected metabolic-stress-induced inflammatory response in cancer cells.

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7. Hematopoietic stem cells, hemangioblastoma and VHL-associated extramedullary hematopoiesis

HB is a highly vascularized tumor of the central nervous system that affect the retina, cerebellum, brain stem and spinal cord [112]. Although HB is a benign tumor, it is typically associated with large peritumoral cysts, which cause morbidity and mortality by exerting pressure on the surrounding neuronal tissue [112–114]. The exact etiology of HB is still unclear. HB consists of a vascular and a stromal component [112]. Somatic *VHL* inactivation was detected only in the stromal component [115–117]. Hence, although they are sometimes described as such, HBs are not endothelial tumors; rather, the overgrowth of endothelium is caused, at least in part, by proangiogenic factors synthesized by the tumor. However, HB tumor cells not only express Epo, VEGF and angiopoietin, but also the receptors of these cytokines (e.g., EpoR, Flk1/VEGFR2 and Tie2) [41, 118–120], suggesting that they act as paracrine and autocrine growth factors to promote both angiogenesis and tumor growth. We have also shown that heterozygous *VHL* mice exhibited elevated angiogenic activity in response to bFGF stimulation due to increased FGF receptor accumulation in the endothelial cells [20].

Most interestingly, there is evidence that HB tumor cells are derived from developmentally arrested, hemangioblast-like stem cells [119–122]. Hemangioblast is a structure that gives rise to the first endothelial and hematopoietic cells during embryogenesis (e.g., the extraembryonic yolk sack associated blood island) [123], and is thought to contain mesodermal stem cells that differentiate into both endothelial and hematopoietic cells, or, as recent findings suggest, sequentially into first endothelial, then hematopoietic stem cells, with a transient “hemogenic” endothelium [124, 125]. Similar to the hemangioblasts, tumor cells in hemangioblastoma express the early mesodermal marker Brachyury, as well as stem cell markers such as Flk1/VEGFR2, the transcription factor stem cell leukemia (scl) and CD133 [119, 121]. Furthermore, tumor cells isolated from hemangioblastoma give rise to both endothelial cells and hematopoietic cells when cultured *in vitro* [121] and foci of extramedullary hematopoiesis (EMH) are observed in advanced hemangioblastoma [120, 122]. Importantly, loss of heterozygosity is observed in the foci of EMH [120], confirming that they arise from *VHL*−/− cells. Since HB tumor cells can differentiate into endothelial cells *in vitro*, it is possible that *de novo* vasculogenesis contributes to the overgrowth of blood vessels observed in HB. Vortmeyer and colleagues detected fetal hemoglobin in HB-associated EMH foci, arguing that the *VHL*−/− cells found in HB contribute to primitive hematopoiesis [122]. The hematopoietic progenitor characteristics of these HB cells imply that they may originate from aberrantly mobilized HPSCs.

However, HB has also been reported to express neuronal markers such as neuron-specific enolase [126–129], neural cell adhesion molecule [129–131], and glial fibrillary acidic protein (GFAP), although the latter is controversial, with some studies suggesting that GFAP+ cells might correspond to entrapped astrocytes [96, 129, 132, 133]. Autopsy revealed widespread small ‘tumorlets’, which are thought to be hemangioblast precursors, in the roots of spinal nerves and the cerebellum of VHL patients [134–136]. Detailed histological and immunhistochemical analysis of tumorlets and HB suggests that HB progresses from slowly proliferating mesenchymal cells to highly proliferative epithelioid cells, the latter displaying the clear cell morphology typical for VHL cancers [122, 134, 136]. Since the topology of hemangioblastoma coincides strikingly with the expression pattern of scl in the developing CNS, it was suggested that hemangioblastoma might arise from dormant stem cells of neurectodermal origin [119]. However, the expression of hemangioblastic markers and the mesodermal marker Brachyury is difficult to reconcile with a neurectodermal origin. On the other hand, no Brachyury expression was detected in tumorlets, which are thought to be dormant precursors of HB [134]. Due to its hybrid

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neuronal-mesodermal phenotype, the origin of HB tumors remains unclear. Nonetheless, it may be worth exploring whether primitive HPSCs or hemangioblast stem cells are implicated in HB. The CNS localization could be explained by homing defects of HPSCs involving endothelial cell adhesion molecules or chemoattractant cytokines specifically expressed by the CNS vasculature. In support of this hypothesis, homing defects were observed in *VHL*−/− HPSCs [29]. Furthermore, EMH targeting the spleen is observed in the R/R polycythemic mouse [33, 35], despite normal bone marrow function. Interestingly, leukemic EMH has been linked to the CXCR4-SDF-1 chemotactic system [137]. This is particularly relevant since *CXCR4* is a HIF responsive gene that was shown to be up-regulated in *VHL* mutant cell lines [12], and is known to be expressed in ccRCC and HB [13]. Furthermore, CXCR4 was down-regulated in *HIF-1*−/− macrophages [72], indicating that HIF dosage regulates CXCR4 expression not only in cancer cells, but also in hematopoietic lineages. Since the CXCR4 chemotactic system is utilized in embryonic neuronal cell migration [138], it is possible that *VHL* mutant HPSCs may be aberrantly mobilized and ectopically localized to the future HB loci.

8. Perspectives

It has become increasingly clear that tumor progression is a systemic problem that involves, at the very least, the host immune and angiogenic responses. This is particularly true with the VHL disease. In the case of VHL patients, the host hematopoietic and immune systems are not simply responders to the growth of tumors but active contributors to the disease. This is suggested by the increasing body of evidence that links VHL function to the activity of HPSCs. Heterozygous *VHL* HPSCs and endothelial cells both show increased activities, suggesting that they may contribute to the hypervascular phenotype of the VHL tumors. Haploid-insufficient HPSCs may also promote tumor growth through increased inflammatory response or directly contributing multipotent cells to the tumor loci. In addition, *VHL* mutant tumor cells can secret a number of growth factors and cytokines that can also activate the inflammatory and angiogenic components of the primary tumors. We therefore argue that in designing new treatments for the VHL disease, a systemic approach including targeting the hematopoietic system and the inflammatory response should be considered.

Acknowledgments

The body of literature on VHL and HIF is very large, and many excellent papers were not cited due to space limitations. Also, since data on VHL are frequently published in papers whose main focus is HIF function, we cannot exclude that papers relevant to the topics of this review have been overlooked, and apologize if this should be the case. H.L.B. is a recipient of a NIH post-doctoral training grant (5T32HL007501-program director Dr. Adam Lerner). This work is also supported by a grant to T.H. from the National Institutes of Health, USA (R01CA109860).

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