Chapter

HIF Pathways in Clear Cell Renal Cancer

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Abstract

Clear cell renal cancers (ccRCC) are characterized by inactivation of the VHL (von Hippel–Lindau) tumor suppressor. Work leading to the 2019 Nobel Prize for Physiology or Medicine has shown that this is central to cellular oxygen-sensing, orchestrated by the HIF (hypoxia-inducible factor) transcription factors. These regulate hundreds of genes that underpin many hallmarks of cancer, including angiogenesis, cellular energetics, cell proliferation, resisting cell death, and avoiding immune destruction. However, HIF also promotes processes that are detrimental to cancer cells. Therefore, the overall consequence of HIF pathway activation is a balance of these influences. We explore how variations in the HIF pathway during tumorigenesis alter this balance to promote ccRCC formation.

Keywords: cancer, kidney, renal, clear cell, von Hippel Lindau, VHL, hypoxic, hypoxia-inducible factor, HIF

1. Introduction

Kidney cancer is the seventh most common malignancy in the Western world. In 2018, there were approximately 400,000 new kidney cancer cases and 180,000 kidney cancer-associated deaths worldwide [1]. The underlying causes of kidney cancer are complex and incompletely understood, although genetic factors (both inherited and somatic genetic mutations) are known to drive the disease. Additionally, certain lifestyle choices (such as smoking and a high protein diet) increase the risk of developing kidney cancer, consistent with its prevalence in the Western world. Unless surgically resectable, kidney cancer is largely incurable, and the 5-year survival rate for those with metastatic disease is only about 10% [2]. Systemic anti-cancer therapies, including those that inhibit the vascular response or enhance patients' immune response to the malignancy, have offered some hope [3]. However, these treatments confer limited efficacy and a considerable burden of toxicity. Therefore, there is a pressing need to better understand the drivers of kidney cancer in order to identify novel therapeutic strategies.

2. Histological subtypes of renal cancers

The most common form of kidney cancer is clear cell renal cell carcinoma (ccRCC), which arises from the adult renal tubular epithelium and accounts for approximately 75% of all kidney cancer cases. This subtype is termed as such due to the characteristic 'clear' cytoplasm of malignant cells observed histologically. This is

caused by accumulation of excess glycogen and lipid in the cytoplasm (due to highly dysregulated metabolic pathways), which are dissolved by the tissue fixation process [4]. Other less common subtypes of adult renal cancers that also arise from the tubular epithelium include papillary RCC (types 1 and 2); chromophobe RCC; and oncocytoma. Each subtype is associated with different histological features, genetic drivers, and clinical behaviors. Rarely, cancers can arise from other cell types in the adult kidney, including transitional epithelial cells of the ureter and renal pelvis (giving rise to transitional cell carcinoma) and various mesenchymal cell types (e.g. interstitial cells, giving rise to renomedullary interstitial cell tumors). Although childhood kidney cancer is generally rare, the most common form is Wilms tumor, which originates in developing tubular cells during fetal development [5].

It should be noted that even within a specific renal cancer histological subtype there is evidence for substantial heterogeneity, which has initiated efforts to further refine subtype classification based on additional features. Recent studies have found that ccRCC can be further stratified based on architectural, cytological and microenvironmental features, and that these features can predict patient outcome and response to therapy [6]. The underlying cause of this variation remains to be determined but could be due to certain genetic or epigenetic differences between ccRCC tumors. Consolidation of histological and molecular heterogeneity in ccRCC will be important for disease subclassification, as well as better understanding ccRCC biology, going forward.

3. VHL syndrome

Each kidney cancer subtype is associated with its own monogenic cancer syndrome [7]. Studying these rare family kindreds has provided unique insight into the genetic mechanisms underlying both inherited and sporadic cancers. In particular, clear cell renal cancer is associated with VHL syndrome, which is an autosomal dominant disorder, affecting 1 in 32,000 individuals, caused by heterozygous germline mutations of the VHL gene [8, 9]. As well as ccRCC, VHL syndrome is associated with a limited number of other tumors types, including hemangioblastomas of the retina and the central nervous system; pheochromocytomas; pancreatic lesions; endolymphatic sac tumors and epididymal cysts [8, 9]. VHL syndrome can be further sub-divided according to which of these different tumor types develop in individuals within the kindred [10, 11]. Four distinct patterns have been identified: type 1 VHL disease, which is associated with hemangioblastoma and ccRCC; type 2A, which is associated with hemangioblastoma and pheochromocytoma; type 2B, which is associated with hemangioblastoma, pheochromocytoma and ccRCC; and type 2C, which is associated with pheochromocytoma alone. Each of these subtypes is linked to particular types of VHL mutation, which have been shown to have different downstream biological effects [12–17].

4. The VHL gene

The human VHL gene was first identified following classical linkage analysis of families with VHL syndrome and was cloned in 1993 [18]. In humans it is located on the short arm of chromosome 3 (3p25) and has three exons that encode a protein of 213 amino acids, with a molecular weight of around 30 kDa (termed p30). However, the gene also contains a second translation start site at codon 53, leading to the generation of a shorter protein of approximately 19 kDa (termed p19), which appears to retain canonical activity [19]. As a consequence, oncogenic mutations, most

typically single-nucleotide variants (SNVs) or short insertion/deletions (indels), are restricted to codons 53–213 in exons two and three.

VHL acts as a tumor suppressor gene [20, 21]. ccRCC and other cancer types are associated with inactivating mutations of VHL, which lead to loss-of-function of the gene product (termed pVHL). Although autosomal dominant at the level of the individual, both alleles of the VHL gene must be inactivated in a cell for cancer to develop, in line with Knudson's two-hit hypothesis [22, 23]. Since VHL syndrome is caused by germline VHL mutation, all cells of the affected individual harbor this mutation. The remaining wild-type (WT) allele is somatically inactivated in the tumor progenitor cell, which then multiplies to form the cancer [20, 21]. Typically, somatic inactivation of the WT allele occurs as a result of an arm-level loss of chromosome 3p (**Figure 1**), although promoter hypermethylation or a second SNV/indel may also cause complete loss of functional VHL in the cell. Furthermore, since the cells of patients with VHL syndrome only require one somatic mutation to become functionally deficient in VHL, it is a relatively common event, accounting for the high tumor penetrance in these individuals. Indeed, over the course of their lifetime, these individuals often develop multiple tumors and close examination of their organs often reveals the presence of numerous synchronous tumors. However, VHL mutation is only associated with the very limited range of cancers outlined above, despite it being ubiquitously expressed. Therefore, VHL only appears to act as a tumor suppressor gene in very few tissues. Indeed, even within the kidney, ccRCCs appear to develop from a subset of proximal tubular cells [24]. It is assumed that somatic mutations in the wild-type copy of VHL do occur in other cell types, but it is not known whether these cells are eliminated by other tumor suppressor mechanisms, or simply fail to progress to overt cancer.

Importantly, VHL is also inactivated in the vast majority (approximately 90%) of sporadic ccRCC tumors, which occur in patients without a germline mutation in the VHL gene [25]. In order to develop cancer, these individuals require two somatic

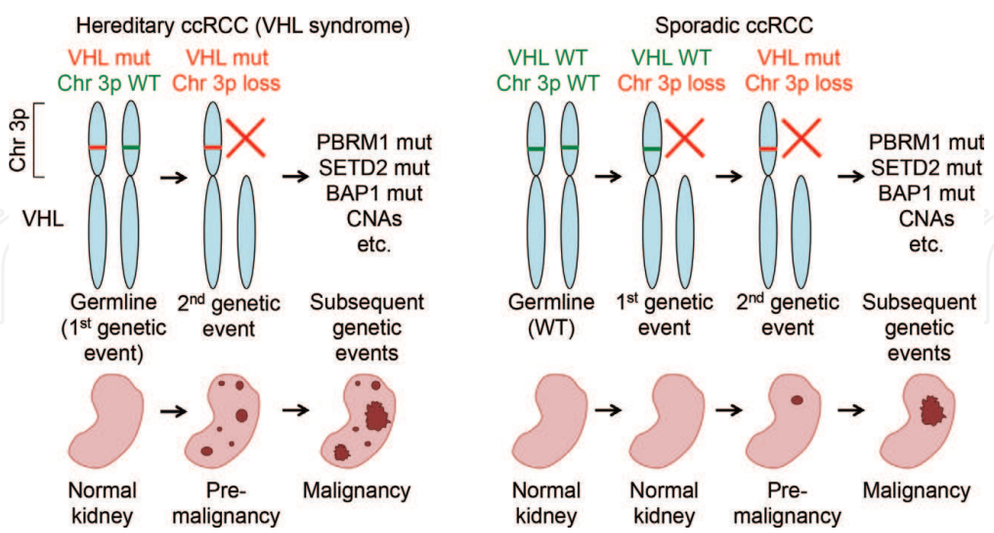

Figure 1.
VHL inactivation in ccRCC. Individuals with VHL syndrome are predisposed to ccRCC (termed hereditary ccRCC) as a result of a heterozygous germline VHL mutation. The second, wild-type allele is subsequently inactivated by somatic loss of chromosome 3p, resulting in biallelic VHL inactivation. On the other hand, in sporadic ccRCC, two somatic events are required for biallelic inactivation. Typically, one copy of chromosome 3p is lost followed by inactivation of the second VHL allele through mutation or promoter hypermethylation. Although the ordering is reversed, the same genetic aberrations are observed in both sporadic and hereditary ccRCC. However, because only one somatic event is required for biallelic VHL inactivation in patients with VHL syndrome, this is a much more likely event and occurs in multiple cells within the kidney, causing many pre-malignant lesions and multiple ccRCC tumors. chr= chromosome; CNAs= chromosomal copy number alterations; mut= mutation; WT= wild-type.

events affecting both copies of the VHL gene in the same cell (**Figure 1**). As a result, this occurs much less frequently, accounting for the much lower overall prevalence of ccRCC in the general population of about 1%. However, in contrast to VHL syndrome, the order of events is typically reversed, with loss of chromosome 3p frequently occurring first and the remaining copy then being inactivated by single-nucleotide substitution (SNV) or small insertions or deletions (indels) [26].

Of note, although biallelic VHL inactivation is required for ccRCC (and other tumors) to develop, it does not appear to be sufficient on its own (**Figure 1**). Mitchell et al. have estimated that in sporadic ccRCC, VHL inactivation predates tumor formation by a number of years or even decades [26]. Consistent with this, examination of the kidneys from patients with VHL syndrome has identified multiple isolated VHL-defective cells, which may be present as single cells or small non-invasive cysts [27, 28]. Furthermore, *in vitro*, inactivation of VHL leads to cellular senescence rather than unrestricted proliferation [29, 30]. Therefore, it is thought that additional gene mutations are required for these early VHL-defective lesions to develop into mature ccRCC. Indeed, more recently, additional somatic mutations have been identified in ccRCC [25, 31–33]. Most notable among these are inactivating mutations in the PBRM1 (polybromo 1), SETD2 (SET domain-containing 2) and BAP1 (BRCA-associated protein 1) tumor suppressor genes, mutation of which typically follows loss of VHL. Importantly, these three genes also reside on the short arm of chromosome 3. As a result, the loss of chromosome 3p frequently observed in both familial and sporadic ccRCC can simultaneously result in copy loss of all 4 of these ccRCC-associated tumor suppressor genes; VHL, PBRM1, SETD2 and BAP1.

5. Function of pVHL

Following identification and cloning of the VHL tumor suppressor gene, its sequence did not immediately suggest a function for the protein. However, early immunoprecipitation experiments indicated that pVHL forms a complex with elongin B and elongin C [34]; cullin 2, a member of the Cdc53 family of proteins [35]; and the RING-box protein Rbx1 [36, 37]. Importantly, the binding of pVHL to elongins B and C could be blocked by specific ccRCC-associated mutations in the VHL gene, strongly suggesting that these two proteins contribute to the tumor suppressor activity of VHL [34]. The subsequent identification of mutations in the TCEB1 gene, which encodes elongin C, in ccRCC tumors that have wild-type VHL further emphasizes the importance of this complex in ccRCC formation [25, 32, 38].

Elongins B and C, cullin 2 and Rbx1 are all components of an E3-ligase complex that adds polyubiquitin chains to specific proteins and thus targets them for degradation by the proteasome [39, 40]. This suggested that pVHL might act as the recognition component of a pVHL ligase complex. In a separate line of work, dysregulation of the hypoxia-inducible factor (HIF) transcription factors had been identified in VHL-defective ccRCC cells [41]. It was subsequently shown that pVHL directly interacted with HIF, leading to polyubiquitination and subsequent proteasomal degradation of its alpha-subunits [42, 43]. Again, the pVHL-HIF interaction could be blocked by specific ccRCC-associated mutations in VHL, leading to overexpression of HIF and underlining the importance of HIF in the development of ccRCC [42]. Importantly, this interaction was not only altered by pathogenic VHL mutations but was also regulated in an oxygen-dependent manner [44, 45]. This indicated that the pVHL-HIF interaction was integral to the mechanism of cellular oxygen-sensing.

The central role of HIF in ccRCC biology has been further underscored in numerous studies. In particular, in xenograft and transgenic mouse models of

VHL-defective ccRCC, tumor growth is dependent upon the presence of HIF [46–51]. Specifically, tumor growth is dependent on the DNA binding activity of HIF, which is required for it to transactivate its target genes [48]. Thus, HIF and its associated transcriptional response are key mediators of tumorigenesis in ccRCC.

In addition to HIF, pVHL can interact with a number of other proteins, although the biological significance of these interactions is incompletely understood [52]. Some of these interactions can lead to ubiquitination of other proteins aside from HIF. For example, pVHL has been reported to interact with and ubiquitinate two de-ubiquitinase enzymes (VDU1 and VDU2) leading to their degradation [53, 54]. In turn, VDU2 but not VDU1 may de-ubiquitinate HIF-1α, potentially providing another level of control to the HIF pathway [55]. In addition, pVHL can bind to and ubiquitinate two subunits of the RNA polymerase 2 complex, POL2RA (RPB1) and POL2RG (RPB7) [56–58]. Importantly, the pVHL-RPB1 interaction was shown to be oxygen-dependent, involving a mechanism similar to that regulating pVHL interaction with HIF [58]. Similarly, the erythropoietin receptor (EPOR), which lies downstream of the canonical HIF-target gene, erythropoietin (EPO), may also be bound and ubiquinated by pVHL in response to oxygen [59]. pVHL can also interact with and ubiquitinate the regulatory domain of atypical protein kinase C (PKC), a serine–threonine kinase that has roles in cell polarity and cell growth, leading to its degradation [60–62]. Again, this interaction may be regulated by oxygen [62]. Similarly, an oxygen-dependent interaction between pVHL and sprouty homolog 2 (SPRY2), which modulates the action of receptor tyrosine kinases, has been reported [63]. Taken together, these findings indicate that pVHL may contribute to oxygen signaling more extensively than simply through regulation of HIF.

pVHL may also play a non-canonical role in extra-cellular matrix assembly, independently of HIF. Specifically, pVHL can interact directly with the alpha-chain of collagen 4 and is important in maintaining the collagen 4 network [64, 65]. This molecule is heavily hydroxylated, and as will be explained below, hydroxylation is important in the recognition of HIF-alpha (as well as collagen 4) by pVHL. Importantly, this interaction can be dissociated by ccRCC-associated VHL mutations. Similarly, fibronectin co-immunoprecipitates with pVHL, and consistently the extracellular fibronectin matrix produced by VHL-defective ccRCC cells is also disrupted [66]. However, the contribution of this phenomenon to cellular oxygen sensing and ccRCC tumorigenesis is still unclear.

6. Oxygen-dependent regulation of HIF by pVHL

The importance of pVHL in the regulation of the HIF transcription factors, and the cellular transcriptional response to altered levels of oxygen, has provided tremendous insights into the mechanisms of cellular oxygen sensing. HIF was first discovered in the quest for transcriptional regulators of the erythropoietin gene (EPO), encoding the master regulator of red blood cell production [67]. It later emerged there were three HIF isoforms, HIF-1, HIF-2 [68, 69], and HIF-3 [70], each composed of a common, constitutive β-subunit (HIF-1β, also known as ARNT – aryl hydrocarbon receptor nuclear translocator) and a regulated alpha-subunit (HIF-1α, HIF-2α and HIF-3α respectively). HIF-1α is ubiquitously expressed at the mRNA level, thus HIF-1α protein is capable of being stabilized in all tissue types. HIF-1α is thought to drive core, canonical cellular responses to low oxygen levels (hypoxia) [71], including the metabolic switch to anaerobic glycolysis. The expression of HIF-2α mRNA and HIF-3α mRNA is more cell-type-specific and thus these transcription factors are thought to drive more specialized responses to hypoxia [69, 70, 72].

HIF-2α expression is generally more restricted to particular mesenchymal cell types, including endothelial cells in which it was first identified, hence its alias endothelial PAS domain-containing protein 1 (EPAS1) [69]. However, HIF-2α is also expressed in some epithelial malignancies, including ccRCC. HIF-3α expression is restricted to a select few cell types and can be alternatively spliced to yield several transcript variants [70]. The biological functions of HIF-3α have not been well-explored, although it is thought to antagonize the transcriptional responses of HIF-1α and HIF-2α [73–75].

HIF isoforms are all basic helix–loop–helix/Per-ARNT-SIM (bHLH–PAS) transcription factors, belonging to a much larger family that includes the oncogenic MYC proteins [76]. Each possess an N-terminal bHLH DNA-binding domain and two protein–protein interaction PAS domains responsible for dimerization. In addition, the three HIF-α isoforms each contain oxygen-dependent degradation domains (ODDDs), responsible for regulating protein abundance [77]. However, only HIF-1α and HIF-2α possess the C-terminal transactivation domains (C-TAD) [78].

In the presence of oxygen, HIF-α subunits are hydroxylated on two residues in the ODDD domains by a family of prolyl hydroxylase enzymes (PHD1, PHD2 and PHD3) [44, 45, 79]. These hydroxylated residues are recognized and bound by pVHL (in a complex with elongin B, elongin C and cullin 2) leading to its rapid ubiquitination and proteasomal degradation (**Figure 2**). Thus, when oxygen is abundant, HIF-α levels are low. However, since oxygen is a rate-limiting substrate for this reaction, HIF-α is stabilized in hypoxia. Inactivation of VHL in ccRCC cells will also block HIF from being degraded, leading to constitutive activation of HIF and its target genes, even in cells that are well-oxygenated. Accordingly, activation of both HIF and HIF target genes are hallmarks of ccRCC.

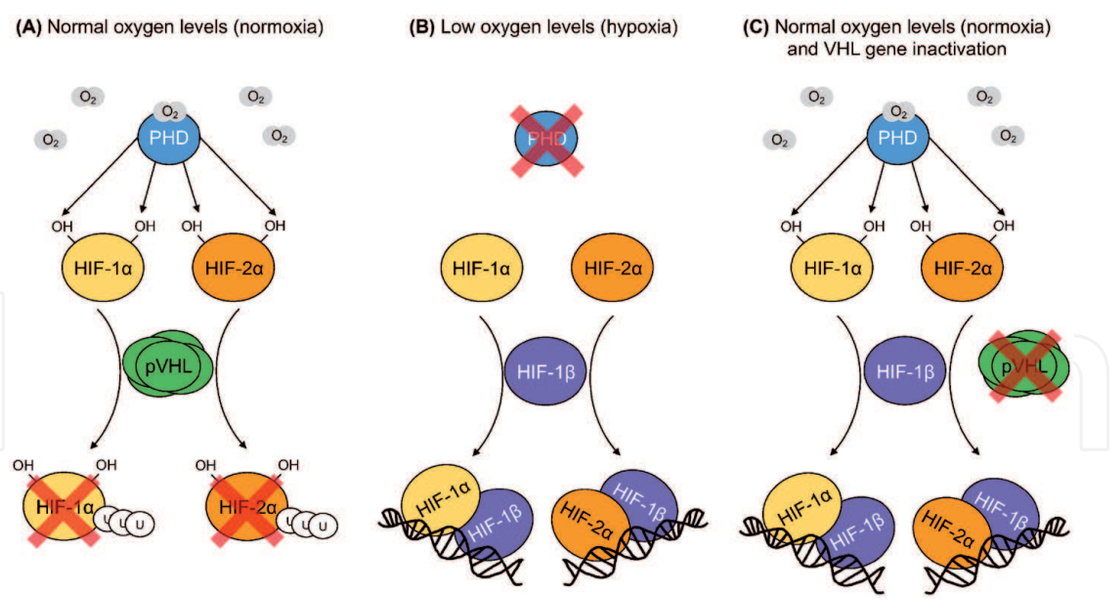

Figure 2.
Regulation of HIF by PHD enzymes and pVHL E3 ligase. (A) In normal oxygen conditions (normoxia), the oxygen-dependent PHD enzymes (PHD1, PHD2 and PHD3) hydroxylate both HIF-1α and HIF-2α transcription factor isoforms. This causes HIF proteins to be recognized and ubiquitinated by the pVHL E3 ubiquitin ligase complex, which targets them for rapid degradation via the proteasome. (B) In low oxygen conditions (hypoxia), PHD enzymes are inactive due to the lack of their oxygen substrate. Therefore, HIF-1α and HIF-2α are not hydroxylated and are not targeted for degradation by pVHL. Due to their stabilization, they are able to dimerize with their obligate binding partner HIF-1β. This allows them to bind to DNA and upregulate their target genes. (C) When the VHL gene is inactivated (as is the case in ccRCC and some other cancers), pVHL is either not expressed or is dysfunctional. Therefore, pVHL is unable to recognize HIF-1α and HIF-2α, even in the presence of oxygen when they are hydroxylated by PHD enzymes. This causes inappropriate stabilization of HIF-1α and HIF-2α, which then dimerize with HIF-1β and upregulate their target genes, regardless of oxygen levels.

In addition, HIF-1α and HIF-2α can be further modified at an additional site in the C-terminal TAD by an asparaginyl hydroxylase, termed factor inhibiting HIF 1 (FIH-1) [80, 81]. Similar to the PHD enzymes, FIH-1 activity is oxygen-dependent, but asparagine hydroxylation does not prompt recognition by pVHL. Instead, asparaginyl hydroxylated HIF is unable to bind to the transcriptional co-factor, CREB binding protein (CBP)/p300, which facilitates transcriptional activation at a subset of HIF-target genes [80–82]. Therefore, two distinct mechanisms act to control HIF activity and expression in an oxygen-dependent manner, one of which is blocked by VHL inactivation. In the context of ccRCC, this has two consequences. Firstly, FIH-1 may facilitate residual hypoxic regulation of HIF despite constitutive HIF stabilization [83]. Secondly, the transcriptional response to VHL inactivation in normoxic cells may not precisely mimic the transcriptional response to hypoxia.

7. The HIF transcriptional response

Once stabilized, both HIF-1α and HIF-2α, in complex with HIF-1β, are able to bind chromatin at either gene promoters or promoter-distant enhancers that contain one or more 5′-RCGTG-3′ recognition motifs, termed hypoxia response elements (HREs) [84, 85]. These short motifs are highly numerous across the genome and only a small proportion of accessible motifs are occupied by HIF, indicating that additional factors are involved in HIF DNA-binding [85]. HIF-binding sites may lie several hundreds of kilobases from the target promoter, interacting with it through chromatin looping, which can make it difficult to identify the transcriptional target of any given binding site. Therefore, much effort has been directed at determining both direct and indirect targets of the HIF transcriptional pathway in multiple settings, including in VHL-defective ccRCC cells, using both transcriptomic assays such as RNA-seq and assays of chromatin binding such as ChIP-seq [85–89].

These sequencing studies indicate that HIF acts as a gene activator rather than a repressor; causing the induction of hundreds to thousands of genes and triggering massive pathway activation [90–93]. These genes mediate diverse cellular functions including angiogenesis, erythropoiesis, glycolysis and the cell cycle [77, 94, 95]. This triggers a physiological response that enables cells to survive in low oxygen conditions. For example, HIF-dependent angiogenesis increases blood supply to oxygen-starved tissue; HIF-dependent erythropoiesis improves systemic oxygen delivery; HIF-dependent glycolysis allows cells to generate ATP in the absence of oxygen; and HIF-dependent cell cycle arrest can allow cells to conserve energy and reduce oxygen consumption.

Importantly, HIF-binding sites and HIF-regulated genes are highly cell-type specific. Thus, whilst HIF may regulate many hundreds of genes in any given cell type, only a small, core set of well-described genes are regulated in the majority of tissues [90, 93]. Furthermore, although both HIF-1α and HIF-2α share the same binding motif and their binding sites often overlap, HIF-1α tends to be more prevalent at gene promoters whereas HIF-2α is more prevalent at promoter-distant enhancers [90, 92]. In addition to this binding site specificity, post-DNA-binding mechanisms likely contribute to transcriptional selectivity between the two isoforms [96], such that specific genes may be regulated by either HIF-1α or HIF-2α only, even when both isoforms are bound [50, 97] For example, cyclin D1 (CCND1), transforming growth factor alpha (TGFA), vascular endothelial growth factor A (VEGFA), glucose uptake transporter 1 (SLC2A1/GLUT1), the MYC oncogene, and the stemness-related transcription factor OCT4/POU5F1 are specifically induced by HIF-2, whilst BCL2-interacting protein 3 (BNIP3) and carbonic anhydrase 9 (CA9) are positively regulated by HIF-1 [97–102].

Although primarily a physiological response, the HIF pathway is also relevant to the pathophysiology of cancer and many HIF target genes are central to the hallmarks of cancer described by Hanahan and Weinberg [103]. These include genes with prominent roles in angiogenesis, glycolysis, cell proliferation, cell invasion and immune evasion among other oncogenic processes (**Figure 3**). Indeed, HIF is activated in many types of solid tumor, largely as a result of intra-tumor hypoxia and is almost universally associated with a poor prognosis [104].

In particular, HIF promotes the metabolic switch from oxidative phosphorylation to anaerobic glycolysis by inducing a range of target genes, including those encoding transmembrane proteins that import glucose into the cell (SLC2A1/GLUT-1 and SLC2A3/GLUT-3) as well as multiple catalytic enzymes in the glycolytic pathway [71]. Oxidative phosphorylation is oxygen-dependent, therefore switching to oxygen-independent glycolysis allows hypoxic cancer cells to generate energy. However, glycolysis causes accumulation of byproducts in the form of acidic metabolites, which can be toxic to cancer cells. Therefore, HIF also upregulates genes encoding transmembrane proteins that rebalance intracellular pH to promote cancer cell survival. For example, the HIF target genes CA9 and CA12, encoding carbonic anhydrases, generate alkaline sodium bicarbonate ions in the extracellular space [105]. Sodium bicarbonate can then be imported into cells by ion channels to counteract intracellular acidity. Furthermore, once a tumor outgrows its blood supply and becomes hypoxic, HIF induces genes encoding pro-angiogenic secreted factors, such as VEGFA and placental growth factor (PGF), that serve to transmit extracellular signals and stimulate blood vessel production [106]. This increases delivery of nutrients and oxygen to cancer cells, enabling the tumor to further expand. Furthermore, HIF has recently been found to upregulate genes that help cancer cells evade destruction by the immune system. One such example is CD274,

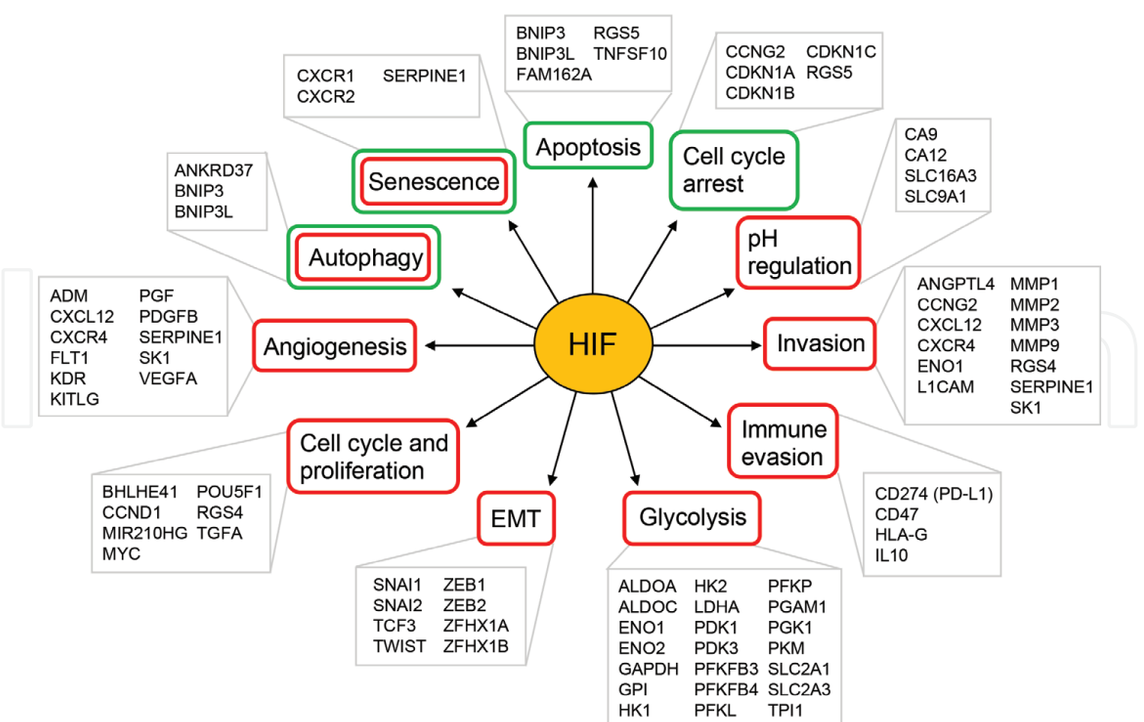

Figure 3.
HIF target genes that promote or restrict tumorigenesis. HIF regulates hundreds to thousands of target genes, which mediate diverse and sometimes conflicting cellular processes. For example, such processes can either promote or restrict tumor growth. Those that are typically considered tumor-promoting processes are depicted in red, whereas those that are typically considered tumor-suppressive are depicted in green. Cellular processes that can be either tumor-promoting or -suppressive (depending on the context) are depicted in red and green. Exemplar HIF target genes involved in each process are listed. Note that whilst some HIF target genes appear to be consistent across cell types and conditions, others are context-dependent.

encoding the transmembrane protein termed programed death ligand 1 (PD-L1), which is expressed in cancer cells [107]. PD-L1 interacts with its receptor termed programed cell death protein 1 (PD-1), which is expressed on the cell surface of T cells. The PD-L1/PD-1 interaction prevents T cell-mediated killing of cancer cells, therefore HIF may exacerbate this oncogenic mechanism.

However, since HIF evolved to mediate physiological responses to hypoxia, not all HIF target genes are advantageous in a cancer setting. Paradoxically, although HIF activates many pro-tumorigenic target genes, there are also anti-tumorigenic HIF targets (**Figure 3**). These may represent in-built tumor suppressor mechanisms that counterbalance oncogenic target genes when HIF is activated in response to physiological hypoxia. Tumor suppressive HIF target genes include BNIP3 and BNIP3L, which are pro-apoptotic proteins. BNIP3 and BNIP3L can promote either cell death or autophagy in response to hypoxia, depending on the context [108]. Furthermore, some HIF target genes may not influence cancer pathogenesis whatsoever and may represent genes that are only important in other contexts. This is epitomized by VHL loss in the earliest stages of ccRCC formation, which causes HIF activation in an inappropriate context (i.e. causing a cellular response to hypoxia when the cell is not hypoxic). In this setting, HIF causes a change in cell state that is unwarranted since the cell is exposed to normal oxygen levels. Therefore, many activated HIF target genes may confer no survival advantage or may even result in a "fitness penalty" to the cell in this context. Taken together, the overall consequences of massive HIF pathway activation in ccRCC will be a balance of many positive, neutral and negative effects [109]. The contribution of each effect may change during cancer pathogenesis as a result of subsequent somatic mutation, epigenetic events or changes in the tumor microenvironment allowing cancer cells to escape the long prodromal dormancy that occurs following VHL inactivation. Alternatively, the poise of the HIF transcriptional pathway may be partially pre-set prior to VHL inactivation due to cell-type specific differences in HIF target genes. In turn, this could render specific cell types particularly susceptible to VHL inactivation. Furthermore, genetic differences between individuals might alter specific HIF target genes, thus making that individual more or less susceptible to developing kidney cancer.

Activation of contrasting and aberrant pathways as part of large transcriptional programs is an emerging theme in cancer biology. For example, MYC, like HIF, has transcriptional targets with both oncogenic and tumor suppressive properties [110, 111]. Therefore, HIF activation in ccRCC serves as a model for studying large transcriptional cascades in cancer more generally.

8. Modulation of the HIF response during the pathogenesis of ccRCC

Early evidence to support the pleiotropic nature of the HIF pathway in kidney cancer came from the observation that HIF-1α and HIF-2α have opposing actions on tumor growth in ccRCC xenograft models. Whilst HIF-2α promotes tumor growth, HIF-1α has the opposite effect and restricts tumor growth [46, 48, 50, 51]. Furthermore, expression of HIF-2α target genes in ccRCC tumors correlates with poor patient prognosis, whereas HIF-1α targets genes are associated with improved survival [91].

Commensurate with this, HIF isoform expression appears to switch from HIF-1α to HIF-2α during the development of kidney cancer [28, 112]. In renal tubule epithelial cells, including proximal tubular cells from which ccRCC is derived, HIF-1α mRNA is highly expressed, whereas HIF-2α mRNA is undetectable [28]. Conversely, HIF-2α mRNA (and protein) is highly expressed in ccRCC, possibly as a result of downregulation of DNMT3a and resultant promoter demethylation of the EPAS1 gene that encodes

HIF-2α [113]. Furthermore, ccRCCs often downregulate HIF-1α through loss of copy number, deletion, truncation or transcript downregulation [25, 31, 32, 112, 114]. Given the tumor-suppressive function of HIF-1 and the oncogenic function of HIF-2, the shift from HIF-1α in the ccRCC cell of origin to dominant HIF-2α expression in overt ccRCC would favor a more oncogenic phenotype. However, even within the transcriptional repertoire of each isoform there are genes with heterogenous associations with prognosis, suggesting that other selective pressures, effective at the level of individual HIF target genes, may also be operating [91].

Indeed, suppression of individual HIF target genes with anti-tumorigenic properties has been reported in ccRCC. The pro-apoptotic gene BNIP3 is a canonical HIF target gene in many cell types. However, rather than being increased by constitutive HIF in ccRCC cells, its expression was found to be lower than in normal kidney cells. This is most likely as a result of epigenetic modification of the BNIP3 gene locus involving histone deacetylation [115].

In this respect, it is notable that of the many somatic mutations that co-occur with VHL inactivation in ccRCC, very few occur within HIF-target genes. However, to date, the majority of ccRCC sequencing efforts have focused on the coding genome or have targeted genomic regions of interest. Therefore, the majority of HIF binding sites (which are usually intergenic) have not been extensively examined and further studies may reveal somatic mutation of these sites in the future. However, epigenetic modifiers such as PBRM1, SETD2 and BAP1 are recurrently mutated in these tumors [25, 31, 32, 116–120]. PBRM1 encodes a subunit of the chromatin remodeling PBAF SWI/SNF complex; SETD2 encodes a histone methyltransferase; and BAP1 encodes a histone deubiquitinase. Interestingly, parallel evolution has been reported with respect to these mutations, whereby multiple mutations in the same gene are present in different cells of the same tumor [32]. This emphasizes their importance in driving ccRCC, as well as illustrating their temporal occurrence (i.e. subsequent to VHL mutation). Although the interaction between these ccRCC-associated somatic mutations and the HIF pathway remains unclear, PBRM1 inactivation enhances some aspects of the HIF response [121] and reduces the tumor-suppressor activity of HIF-1α, although the mechanisms are unknown [122]. Recurrent mutations are also found in genes within the PI3K/AKT/mTOR pathway, which is a master regulator of RNA translation. Expression of both HIF-1α and HIF-2α protein are differentially dependent on mTOR, with HIF-1α being regulated by both the mTORC1 and mTORC2 complexes, whilst HIF-2α is dependent solely on mTORC2 [123]. Therefore, HIF isoforms may be differentially affected by mutations in this pathway.

In addition, other oncogenic transcription factors activated in ccRCC may modulate the HIF response. For example, MYC activity is enhanced in ccRCC [124, 125] and synergizes preferentially with HIF-2, whilst antagonizing HIF-1 [102, 126]. In this way, MYC augments the switch from HIF-1 to the more oncogenic HIF-2 isoform. Importantly, MYC itself is a transcriptional target of HIF in ccRCC cells [127], providing a mechanism whereby stabilization of HIF following inactivation of VHL preferentially amplifies the HIF-2 transcriptional pathway in these cells.

9. Variation in the HIF pathway pre-disposes to renal cancer

As discussed above, genetic and epigenetic events occur somatically in ccRCC following VHL inactivation, allowing the HIF transcriptional output to adapt to a more oncogenic phenotype, thereby promoting tumor formation. However, differences in the HIF pathway that exist prior to VHL inactivation can also affect the ability of cells to form cancer. Indeed, it is highly likely that cell-type differences in the HIF pathway contribute to the tight tissue-specificity of VHL-associated cancer,

despite the almost universal operation of the VHL-HIF pathway in different mammalian cell types. Potentially, cell-type-specific components of the HIF pathway might favor tumorigenesis in permissive cell types, inhibit tumorigenesis in non-permissive cell types, or a combination of both (**Figure 4**). The exact mechanism underlying this tissue specificity remains to be determined, although elucidation of HIF target genes in cells permissive to VHL-associated cancers (compared to that in non-permissive cells) will be key in future studies. Of note, the G1/S-phase cell-cycle regulator cyclin D1 (CCND1) has been found to be a HIF-2 responsive gene, which is not regulated by HIF-1 and is unique to ccRCC cells [50]. Furthermore, CCND1 is required for ccRCC cell growth in mice [128]. This indicates that CCND1 and likely other tissue-specific HIF target genes may render certain cell types receptive to tumorigenesis upon VHL inactivation.

As well as being affected by somatic alterations and cell-type-specific features, the HIF pathway can also be modified by inherited genetic variants. Polymorphisms that predispose individuals to kidney cancer have been studied, and several of these have been shown to affect HIF target genes. Such variants have been identified by genome wide-association studies (GWAS), which compare the genome sequence of renal cancer patients with healthy control individuals [129–135]. Although these variants likely only account for about 5% of kidney cancer heritability [129], a disproportionately high number of these susceptibility loci overlap with cis-acting components of the HIF pathway [136]. This indicates that specific aspects of the HIF pathway are under genetic selection during the development of kidney cancer.

Many of these RCC-susceptibility loci lie in intergenic regions and so the functional target of these polymorphisms is not immediately apparent. However, several susceptibility loci overlap with, or lie adjacent to, HIF-binding sites [136]. In-depth analysis of chromatin looping and HIF-dependent gene regulation has identified a number of HIF target genes associated with these loci [127, 136–138]. At each locus, the renal cancer susceptibility polymorphism affects both HIF binding and expression of the HIF-target gene, either by generating a second HRE

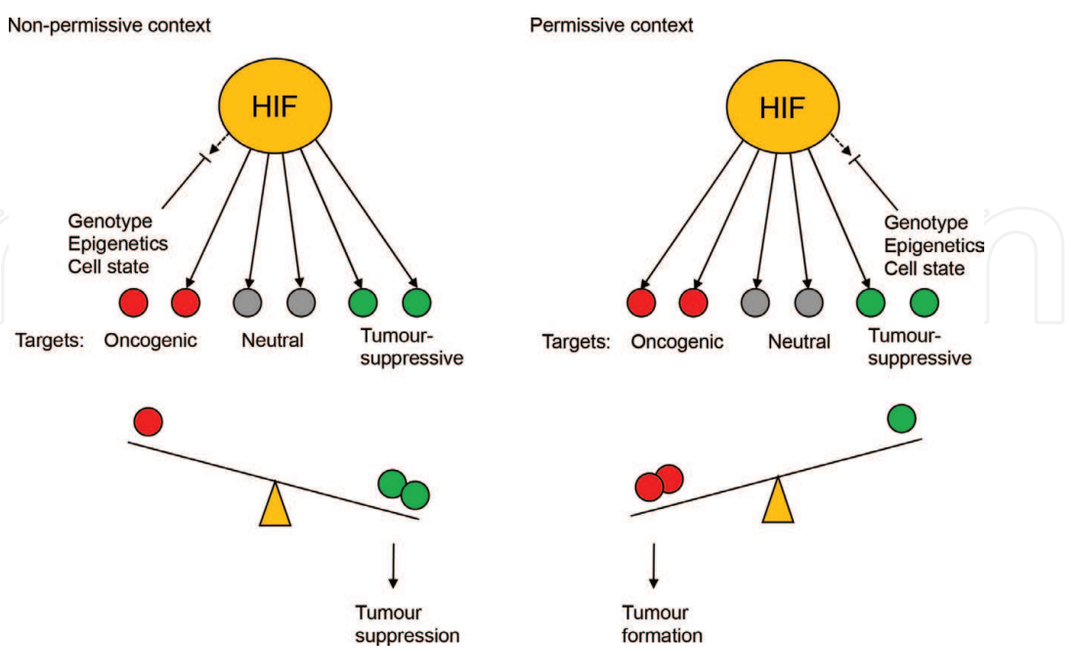

Figure 4.
Rebalancing the HIF pathway to favor tumorigenesis. HIF target genes include those that promote tumor growth (depicted in red), restrict tumor growth (depicted in green) and those that do not influence tumor growth (depicted in gray). Depending on the context (i.e. in a permissive or non-permissive context), activation of the HIF pathway may or may not be conducive to tumorigenesis. Features that could 'tip the balance' in a HIF-activated cell include genetic mutations, epigenetic features and the cell state (e.g. the underlying gene expression program).

motif or by altering chromatin accessibility. Most notable are polymorphisms at the 11q13.3 locus, which affect HIF-2-dependent expression of cyclin D1 (CCND1) [137]; polymorphisms at the 8q24.21 locus, which affect HIF binding and expression of the oncogenic transcription factor MYC [127]; and polymorphisms at the 12p12.1 locus, which alter HIF-1 dependent expression of the basic helix–loop–helix transcription factor BHLHE41 (also known as DEC2) [138]. Furthermore, RCC-susceptibility polymorphisms have been identified at the 2p21 locus, lying in the first intron of the EPAS1 gene that encodes HIF-2α, although whether these affect HIF-2α expression remains unclear [130, 139]. Importantly, each renal cancer susceptibility locus affects a single component of the HIF pathway. This directly implicates these genes in the pathogenesis of kidney cancer. Furthermore, it helps distinguish them from HIF target genes with neutral effects on RCC susceptibility that might be simply co-activated as part of large pathway upregulation. Therefore, these analyses have highlighted specific 'driver' genes that may provide attractive targets for future therapeutic approaches or as biomarkers that might predict tumor behavior.

10. Therapeutic implications of HIF pathway activation in ccRCC

In the absence of a surgical cure, the outlook for patients with clear cell renal cancer is poor, with a median survival of just 2 years. However, over recent years a number of systemic anti-cancer therapeutic strategies have emerged, which are beginning to alter the outcome for some of these patients.

10.1 Anti-angiogenic therapies

One strategy has focused on angiogenesis inhibitors to treat metastatic ccRCC. Whilst all tumors require a blood supply to obtain sufficient oxygen and nutrients to grow, ccRCC (and other VHL-dependent cancers such as hemangioblastoma) are particularly rich in blood vessels. Indeed, VEGFA, a master regulator of angiogenesis, [98, 99] is a direct transcriptional target of HIF and is highly expressed in ccRCC cells [41, 140]. Early anti-angiogenic strategies targeted VEGFA using the monoclonal antibody bevacizumab, with limited efficacy [141]. However, several other HIF target genes also encode pro-angiogenic factors, such as PGF, adrenomedullin (ADM) and plasminogen activator 1 (PAI-1), as well as the VEGF receptor, FLT1. These likely act in concert with VEGFA to orchestrate a robust angiogenic phenotype in the context of HIF activation. Therefore, rather than targeting individual factors, more recent strategies have used small-molecule receptor tyrosine kinase inhibitors (TKIs) to block the overarching angiogenic pathways [142]. However, while effective in some individuals, other tumors may fail to respond, likely reflecting heterogeneity in gene expression between tumors. Furthermore, the duration of response may be limited, possibly reflecting intra-tumor heterogeneity and the growth of resistant subclones.

10.2 Immunotherapy

In recent years, immune checkpoint inhibition via targeting PD-L1 and CTLA-4 has emerged as an effective treatment for advanced ccRCC. This is despite the relatively low mutational burden seen in this type of cancer, which often correlates with sensitivity to immunomodulatory therapy in other cancer types. Whilst HIF has multiple effects on the immune response [143], it is of particular interest that PD-L1 has been found to be transcriptionally regulated by HIF in ccRCC cells [107, 144, 145].

Therefore, it is possible that HIF-mediated activation of PD-L1 may underlie the sensitivity of ccRCC to inhibition of this pathway.

10.3 mTOR inhibitors

Historically, mTOR inhibitors have been used in the treatment of metastatic kidney cancer and remain part of the modern armamentarium [146, 147]. Inhibition of mTOR will negatively impact translation of HIF-alpha subunits, while preferential blockade of mTORC1 or mTORC2 may alter the balance of the two isoforms. Given the oncogenic role of HIF-2α in ccRCC and the selective regulation of HIF-2α by mTORC2, mTORC2 inhibition may provide a more targeted therapeutic approach in the future.

10.4 HIF-2 inhibitors

The finding that HIF-1α and HIF-2α have opposing effects on the pathogenesis of ccRCC initiated efforts to generate isoform-specific inhibitors. This led to the development of small molecule inhibitors that specifically prevent HIF-2α dimerizing with HIF-1β, thereby blocking HIF-2α -dependent transcription without affecting HIF-1α activity [148]. These inhibitors would be predicted to have greater efficacy compared to targeting both isoforms simultaneously, whilst reducing off-target side-effects. Indeed, investigation of these compounds as potential ccRCC treatments, both in animal models of ccRCC and early clinical trials, have yielded promising results [149–151]. Therefore, these compounds could provide another strategy for treating metastatic ccRCC.

11. Conclusions

Inactivation of the VHL tumor suppressor gene is the hallmark of clear cell renal cancer and leads to the upregulation of wide-spread hypoxia pathways, orchestrated by the transcription factor HIF. Whilst HIF proteins activate many genes that are central to the "hallmarks of cancer", other HIF-target genes may restrict cancer progression and the overall consequence of HIF pathway activation is a balance of these effects (**Figure 4**). Both genetic and epigenetic genetic events, occurring before or after VHL loss and HIF activation, can alter this balance to promote tumorigenesis.

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