A Clearer View of the Molecular Complexity of Clear Cell Renal Cell Carcinoma

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Abstract

The von Hippel–Lindau (*VHL*) tumor suppressor gene is mutated as an early event in almost all cases of clear cell renal cell carcinoma (ccRCC), the most frequent form of kidney cancer. In this review we discuss recent advances in understanding how dysregulation of the many hypoxia-inducible factor α–dependent and –independent functions of the VHL tumor suppressor protein (pVHL) can contribute to tumor initiation and progression. Recent evidence showing extensive inter- and intratumoral genetic diversity has given rise to the idea that ccRCC should actually be considered as a series of molecularly related, yet distinct, diseases defined by the pattern of combinatorial genetic alterations present within the cells of the tumor. We highlight the range of genetic and epigenetic alterations that recur in ccRCC and discuss the mechanisms through which these events appear to function cooperatively with a loss of pVHL function in tumorigenesis.

PATHOLOGY OF ccRCC

According to the *World Cancer Report 2014*, kidney cancer was the ninth most common cancer in men and the fourteenth most common in women worldwide in 2012 (1). There were an estimated 143,000 deaths from kidney cancer in 2012, representing the sixteenth most common cause of death from cancer worldwide. Incidence and mortality rates have been increasing in many countries. Smoking tobacco and being overweight or obese are established risk factors for kidney cancer. Two other factors, hypertension and acquired cystic kidney disease requiring dialysis, also increase the risk of renal cancer. Most renal carcinomas are sporadic, but 2–4% of cases are associated with inherited tumor syndromes, each of which predisposes patients to develop a different type of renal cell carcinoma (RCC). The von Hippel–Lindau (VHL) tumor syndrome is the most common inherited tumor syndrome. Other hereditary tumor syndromes with development of different renal cancers include hereditary papillary renal cell carcinoma (HPRCC), Birt–Hogg–Dubé (BHD) syndrome, hereditary leiomyomatosis renal cell carcinoma (HLRCC), succinate dehydrogenase renal cell carcinoma (SDH-RCC), tuberous sclerosis (TS), and Cowden's disease. These tumor syndromes are caused by alterations of other genes, including *MET*, *FLCN*, *FH*, *SDHB*, *SDHD*, *TSC1*, *TSC2*, and *PTEN*.

Morphologically, renal cancers are a heterogeneous class of tumors. The World Health Organization's current 2004 renal cancer classification system combines histological and genetic characteristics and recognizes some variations of renal cancers, which has clinical implications. About 70% of adult renal cell carcinomas are classified as clear cell renal cell carcinomas (ccRCCs); 10–15% are papillary RCCs; about 5% are chromophobe RCCs; and less than 1% are collecting duct RCCs (2). Recently, the International Society of Urological Pathology proposed an updated classification of renal tumors (3) that recognizes five entities as new, distinct epithelial tumor subtypes: tubulocystic RCC, acquired cystic disease–associated RCC, clear cell papillary RCC, MiTF-translocation RCC, and hereditary leiomyomatosis RCC syndrome–associated RCC. Each of these renal cell tumor subtypes has distinct molecular characteristics and different prognoses, and each may also respond differently to novel therapies. All new classification proposals use the name clear cell RCC instead of the designation conventional renal cell carcinoma, a term that was proposed in the Heidelberg tumor classification (4).

The most frequent renal tumor subtype is ccRCC, which is characterized by a specific morphology and genetic background (5). Macroscopically, these tumors have a typical yellow surface due to the high lipid content of the cells, which includes cholesterol, natural lipids, and phospholipids (**Figure 1*a***). Histologically, ccRCC is composed of cells with clear or eosinophilic cytoplasm contained within a characteristic, delicate vascular network (**Figure 1*b***), but the cells are architecturally diverse. The name clear cell reflects the fact that the cytoplasm is commonly filled with lipids and glycogen, which are dissolved during routine histological processing, creating a clear cytoplasm surrounded by a distinct cell membrane; however, many tumors contain larger populations of cells with eosinophilic cytoplasm. Some ccRCCs have a cystic appearance (**Figure 1*c***). This may be due to the presence of necrosis or genuine neoplastic cysts. Cases with complete cystic appearance and without a solid tumoral component are defined as multilocular cystic renal cell carcinomas, and these *VHL*-mutant tumors account for approximately 5% of all ccRCCs (6, 7). In line with the minimal tumor burden present in these neoplasms, patients with multilocular cystic RCCs have a better prognosis than those with solid ccRCCs. This specific tumor type, the presence of renal cysts in VHL disease, and the cystic appearance combined with a solid component in many sporadic ccRCCs have supported a newer concept of cyst-dependent and cyst-independent pathways in the initiation of ccRCC tumors (**Figure 2**) (8, 9).

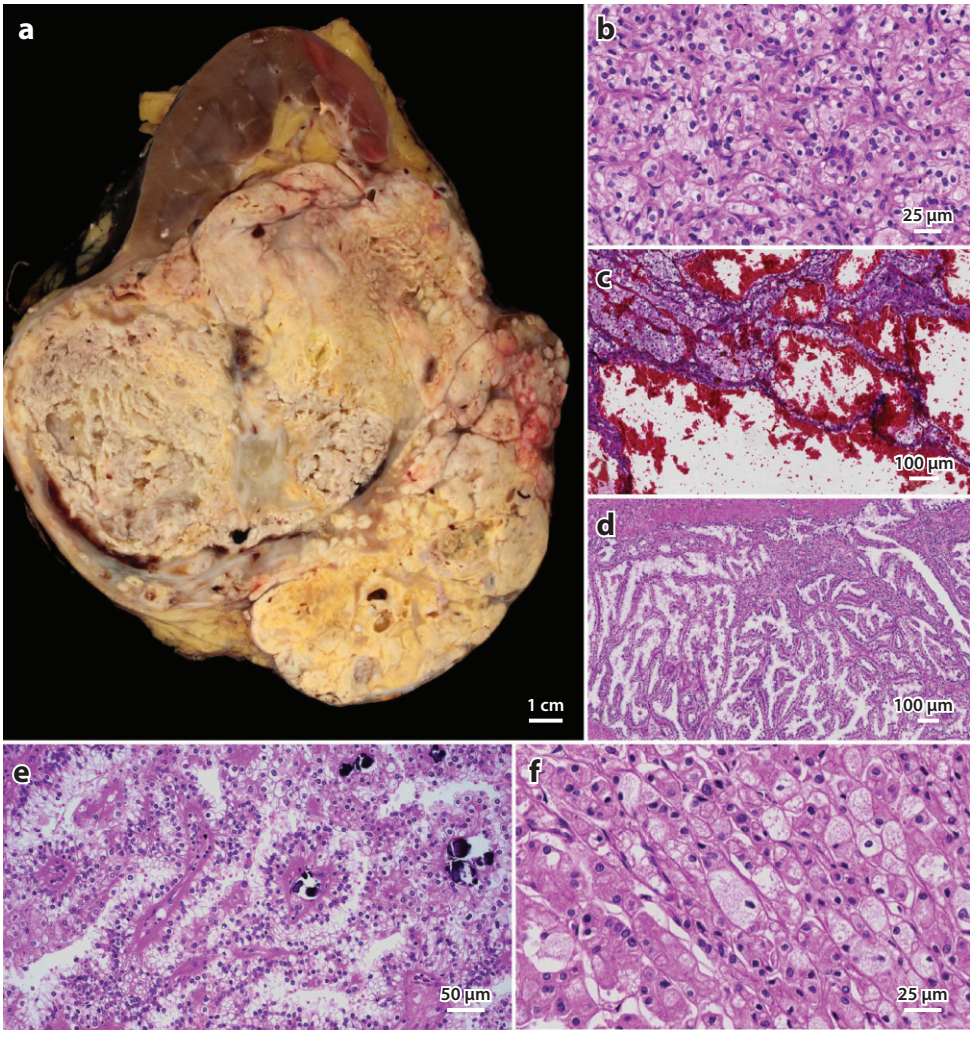

Figure 1

Macroscopy and histopathology of renal cell carcinomas with clear cells. (*a*) Clear cell renal cell carcinoma. Typical tumor cross-section with a characteristic yellowish cut surface due to the lipid content of the cells. There are focal areas with pseudocystic growth due to tumor necrosis. The prognosis is poor because of the large tumor size, presence of necrosis, and invasion of perirenal fat. (*b*) *VHL* mutant clear cell renal cell carcinoma showing typical clear cytoplasm of tumor cells within a characteristic, delicate vascular network and a solid growth pattern. The low nuclear atypia (Fuhrman nuclear grade 2) indicates a favorable prognosis for the patient. (*c*) *VHL* mutant clear cell renal cell carcinoma with histologically characteristic cystic growth. Cysts in this tumor are filled with blood. (*d*) *TFE3* translocation renal cell carcinoma. The tumor cells have clear cytoplasm. Architecturally, the tumor cells show papillary growth and psammoma bodies. *VHL* mutation is absent in this tumor subtype, but the tumors are characterized molecularly by a t(X;11) translocation. (*e*) Clear cell papillary renal cell carcinoma. Tumor cells of this novel renal cancer subtype have clear cytoplasm and a Fuhrman nuclear grade 1, but show a papillary growth. The relevance of *VHL* alterations is controversial in this tumor subtype. Most tumors of this subtype have no *VHL* mutations. (*f*) Chromophobe renal cell carcinoma. The tumor cells of this subtype have an oncocytic, sometimes clear, cytoplasm with a characteristic growth pattern and a different blood vessel pattern than seen in clear cell renal cell carcinoma. Chromophobe renal cell carcinomas are wild type for *VHL*.

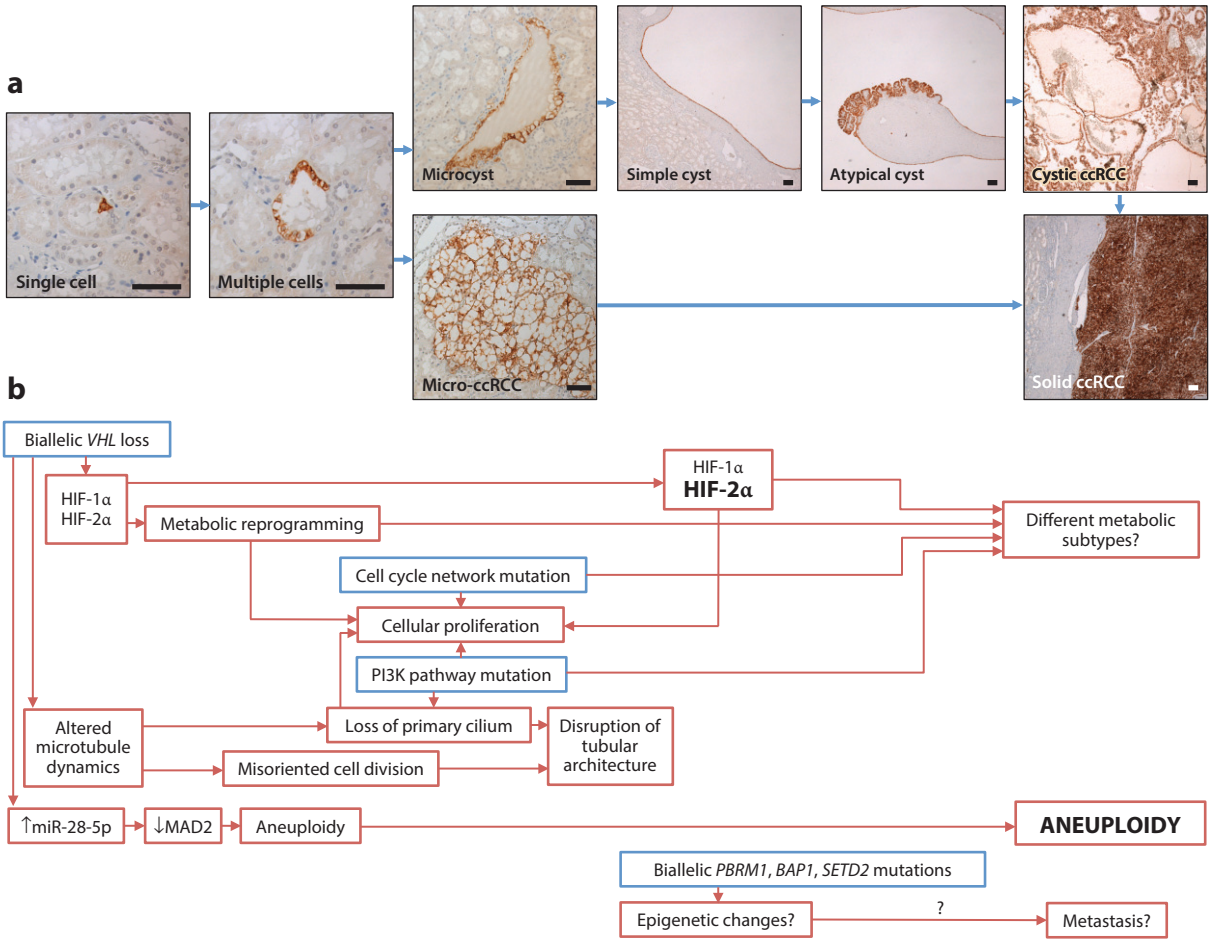

Figure 2

Proposed model of development of ccRCC. (*a*) Examples of different types of *VHL*-null cellular lesions in kidneys of patients with von Hippel–Lindau syndrome that have been identified by immunostaining using an antibody against carbonic anhydrase IX, the protein product of a gene that is strongly induced by HIF-α. Based on the morphological appearances and frequencies of occurrence of these lesions, as well as insights from mouse genetic studies, a model of disease initiation and progression can be proposed. Single *VHL*-null cells initially progress to small, multicellular lesions in the context of morphologically normal kidney tubules. The subsequent breakdown of tubular architecture caused by defects in the plane of cell division and/or loss of the primary cilium can lead to microcystic lesions that progress to simple cysts lined by an epithelial monolayer; these then progress to atypical cysts that are lined by multilayered, disorganized epithelial cells. We speculate that these atypical cystic lesions may represent precursors of cystic ccRCC. Alternatively, ccRCC may arise via a noncystic precursor lesion that we term micro-ccRCC. Growth of these lesions would be predicted to result in solid ccRCC. Scale bars = 50 μm. (*b*) Schematic outline of some of the genetic alterations (*blue boxes*) and molecular or cellular consequences of these mutations (*red boxes*) that are proposed to contribute to the formation and progression of ccRCC. The positions of the boxes speculatively reflect the timing of the occurrence of these events in relation to the progression model shown in panel *a*. Not all of the indicated alterations are found in all tumors. Different combinations of these alterations, as well as of other genetic alterations that are not shown in this figure, give rise to different molecular subtypes of ccRCC. Abbreviations: ccRCC, clear cell renal cell carcinoma; HIF-α, hypoxia-inducible factor α; PI3K, phosphoinositide 3-kinase.

THE VON HIPPEL–LINDAU TUMOR SUPPRESSOR

The most frequent familial renal carcinoma syndrome is the autosomal dominant VHL syndrome. VHL disease is characterized by the development of capillary hemangioblastomas of the central nervous system and retina, pheochromocytoma, and pancreatic and inner ear tumors. The typical renal manifestations are kidney cysts and ccRCC. VHL patients do not develop other types of RCC. Histological examination of macroscopically normal renal tissue from patients with VHL syndrome shows several hundred, often thousands, of small foci of clear cell tumors and cysts, which appear to be precursor lesions of ccRCC (**Figure 2**).

The *VHL* tumor suppressor gene was initially identified by positional cloning as the 3p25-26 gene that is altered in families with the von Hippel–Lindau familial cancer syndrome (10). *VHL* was subsequently also shown to be biallelically genetically altered in the majority of sporadic cases of ccRCC (11). In fact, as molecular analyses have become more sophisticated and rigorous, it has emerged that ccRCC represents a rare example of a solid tumor entity in which in almost every case of the disease there is biallelic alteration of a single common tumor suppressor gene. For example, in a recent study of 240 sporadic ccRCCs, 94% of tumors exhibited the loss of one copy of chromosome 3p (harboring the *VHL* locus), and 98% of these 3p tumors with a loss of heterozygosity also displayed inactivating mutations or hypermethylation of the remaining *VHL* allele (12). Thus, *VHL* was found to be biallelically inactivated in 92% of sporadic ccRCCs in the study. Therefore, the genetics of familial and sporadic ccRCC clearly indicate that genetic alterations causing the loss of function of *VHL* must play a central pathogenic role in the evolution of this tumor type.

Given the broad morphological spectrum of ccRCC and these recent comprehensive molecular characterizations, the designation ccRCC can now be considered to represent a concept that describes a tumor type with prominent vessel formation and tumor cells that are architecturally and cytologically diverse, but that share a characteristic molecular background. Other tumor entities—e.g., chromophobe RCC and some novel tumor entities—including clear cell papillary renal cancer or translocation carcinomas also have tumor cells with clear cytoplasm, but they lack *VHL* mutations and/or 3p losses (**Figure 1*d–f***). Therefore, the presence of *VHL* deletion is sometimes used as a diagnostic criterion for ccRCC, but the absence of *VHL* mutation or deletion does not exclude the presence of a ccRCC (see below) (13).

The protein encoded by the *VHL* gene, pVHL, acts as an adaptor protein to recruit different effector proteins to different target proteins. It thereby regulates a number of different biochemical activities and controls a variety of cellular processes. These include targeting the hypoxia-inducible factor α (HIF-α) transcription factors for oxygen-dependent ubiquitin-mediated proteolytic degradation (14), regulating microtubule stability (15), maintaining the primary cilium (16), activating p53 (17), controlling neuronal apoptosis (18), suppressing epithelial to mesenchymal transition (EMT) (19, 20), suppressing cellular senescence (21, 22), suppressing aneuploidy (23), secreting extracellular matrix components (24, 25), controlling growth factor receptor internalization (26), regulating canonical WNT signaling (27), ubiquitinating RNA polymerase II (28), degrading β2-adrenergic receptors (29), and regulating nuclear factor (NF) κB activity (30). A large number of different somatic *VHL* mutations have been identified in sporadic and hereditary ccRCC that have been shown to have a range of different effects on different activities of the pVHL protein. It is easy to imagine that alterations in some or all of these activities as a result of the loss of pVHL function could be relevant to tumorigenesis. In this review we discuss the current knowledge and recent advances in understanding of which of the many functions of pVHL contribute to the separate processes of tumor initiation and tumor progression. Although in many tumor types these distinct processes are governed by different genetic events, in ccRCC

it appears that the loss of pVHL function contributes to both tumor initiation and to progression to metastasis.

VHL MUTATION IS NOT SUFFICIENT FOR ccRCC FORMATION

The functional importance of pVHL as a tumor suppressor protein in ccRCC was established by studies showing that the reintroduction of wild-type *VHL* into *VHL*-null ccRCC cell lines was able to suppress the growth of these cells as tumor xenografts (31, 32). The absence of pVHL function is clearly necessary for the growth of fully transformed ccRCC cell lines in this setting. However, several findings illustrate that the transformation of normal kidney cells to ccRCC requires more than just the loss of *VHL* function. The study of kidneys of patients with inherited *VHL* mutations has been highly informative. Detailed immunohistochemical analyses of regions of normal histology in these kidneys using an antibody against the HIF-α transcriptional target carbonic anhydrase IX allows cells to be detected in which pVHL function has been homozygously lost due to inactivation of the wild-type *VHL* allele (**Figure 2**) (8, 33, 34). These studies have demonstrated that kidneys from VHL patients probably contain many tens of thousands, if not hundreds of thousands, of single cells or small multicellular clusters that are null for pVHL function. In this context, it becomes apparent that pVHL-deficient cysts and ccRCC arise infrequently in comparison with the total frequency of *VHL* mutant cells in these kidneys, indicating that the loss of pVHL function does not automatically result in a proliferative or survival advantage that initiates tumor formation. These observations may offer one explanation for the failure of numerous efforts to generate autochthonous mouse models of ccRCC based on deletion of *Vhl* in the mouse kidney. Homozygous germline deletion of *Vhl* causes lethality during embryogenesis due to the incorrect formation of the placental vasculature (35), and *Vhl* heterozygous mutant mice are not predisposed to develop kidney tumors, even when subjected to mutagenic stress (36). The generation of different strains of mice carrying loxP-flanked *Vhl* alleles has allowed the conditional homozygous deletion of *Vhl* generally in kidney cells using the *Actb-Cre* driver (37, 38), in proximal tubules using *Pepck-Cre* (39), in all nephron segments using *Pax8-Cre* (40, 41) or *Ksp1.3-Cre* (34), and in the thick ascending limb using *Thp-Cre* (42). None of these mice developed renal tumors. Thus, *Vhl* deletion alone in a variety of different kidney epithelial cells in mice is insufficient to cause ccRCC, apparently mimicking the situation in the kidneys of human patients with VHL.

Two explanations may potentially account for these observations in humans and mice. First, it is possible that only a specific, rare type of kidney cell, or subset of cells, can be transformed by the loss of pVHL to form cysts or ccRCC. Therefore, the loss of the remaining wild-type allele in most kidney cell types in patients with VHL would not cause transformation. We speculate that this putative and unidentified cell type has perhaps not yet been targeted in mice. A second, and more likely, explanation is that specific combinations of additional genetic alterations arise rarely in *VHL*-null cells, and these cooperate with the loss of *VHL* function to cause the initiation of tumor formation. It appears likely that it is the combination of the right set of cooperating mutations in the right cell type that is the trigger for formation of ccRCC. Recent progress toward understanding these two unresolved issues is discussed below.

THE CELLULAR ORIGIN OF ccRCC

The spectrum of cell types from which ccRCC can originate remains unclear. Although it is often stated in the literature that ccRCC arises from proximal tubular epithelial cells, there is evidence to suggest that epithelial cell types from other nephron segments may also have the capacity to give

rise to some cases of ccRCC. The evidence supporting a proximal tubular origin for many cases of ccRCC includes the finding that most, but not all, tumors exhibit positive immunoreactivity for various proteins that are normally expressed specifically by proximal tubular cells, including CD10, villin, renal cell carcinoma antigen, intercellular adhesion molecule 1, and multidrug resistance protein 2 (43–48). Further supporting a proximal tubular origin for ccRCC, most ccRCCs stain positively for the proximal tubule marker tetragonolobus lectin and negatively or weakly for the distal tubule markers peanut lectin (43) and Ksp-cadherin (49–51). Importantly, supervised transcriptional cluster analysis identified that ccRCCs exhibit an mRNA expression profile that is highly similar to microdissected proximal tubules but differs from the expression profiles of other microdissected nephron segments (52). However, ccRCCs also often express proteins that are normally specifically expressed by the distal tubule, including cytokeratin 19, CD24, and α3-integrin (43, 48). Some ccRCCs also show a variable pattern and intensity of staining for galectin-3, which is normally expressed by distal tubule and collecting duct cells but not by proximal tubule cells (53). These studies collectively show that ccRCCs can express or coexpress a variety of markers of different tubular segments. There are several potential interpretations of these observations. In light of recent understanding that there exist RCC tumors with clear cells that are not true ccRCC, some of the tumors in previous studies might have been misdiagnosed as ccRCC and may in fact actually have been other forms of RCC that originated from tubular segments other than proximal tubules. It is also not possible to exclude the possibility that the expression of certain markers, or sets of markers, may be either gained or lost as a result of the processes of cellular dedifferentiation and proliferation that occur during tumor formation. The expression pattern of ccRCC may in fact partly reflect the expression pattern of a regenerating tubular epithelium or potentially of more primitive cells that are found during earlier stages of kidney development. Indeed, careful studies of kidney biopsies from patients with VHL disease have demonstrated that clusters of *VHL*-null cells in the context of normal kidney tubules exhibit cellular dedifferentiation, which is illustrated by a gain in immunoreactivity for the mesenchymal marker vimentin (33), a frequent molecular feature of ccRCC (48, 54, 55) and marker of regenerating tubular cells (56). Interestingly, ccRCCs also frequently display sarcomatoid differentiation and overexpress periostin, a protein that induces EMT and invasion (57). These factors are both predictors of poor patient outcome (57). *VHL* mutant cells in the kidneys of patients with VHL disease also display a loss of expression of epithelial markers including E-cadherin and Tamm–Horsfall protein (19, 33). Thus, the loss of epithelial identity in an apparent EMT is one of the earliest consequences of the loss of *VHL* function in human kidneys. Interestingly, this is not the case in mice. Deletion of *Vhl* in kidney tubule epithelial cells does not cause acquisition of vimentin expression or loss of expression of E-cadherin or other proteins that are markers of differentiated renal epithelial cells (34, 58); this suggests that there may be species-specific differences in the function of the *VHL* tumor suppressor gene in humans and the *Vhl* gene in mice. It is possible that this lack of EMT may be one factor that precludes the development of *Vhl* mutant ccRCC tumors in mice.

It also appears that the loss of function of *VHL* has different effects on different tubular segments. It has been shown in the kidneys of patients with familial VHL disease that multicellular foci of *VHL*-null cells are almost always associated with distal tubules, whereas the majority of proximal tubular cells lacking pVHL function remain as single cells (33). It appears that there is a differential proliferative advantage conferred to the distal nephron segments over the proximal nephron segments as a result of the loss of *VHL* function, raising the notion that cystic lesions or ccRCCs may potentially arise from distal tubules. Although the analysis of kidney biopsies from VHL patients does not allow for true tracing of the origin or fate of cells over time, it does offer the advantage of allowing the identification of many early, pretumorigenic, unicellular, multicellular, and cystic lesions. By expanding the analyses of the number of patient biopsies and including many

of the markers described above, or by including new markers, it may be possible to gain further insights into the sequence of cellular and molecular changes that occur at the earliest stages of tumor formation. For example, demonstrating that a *VHL*-null cell in a distal tubule segment could gain proximal tubule markers, or that a *VHL*-null cell in a proximal tubule segment could gain distal tubule markers, would go some way toward addressing the confusions outlined above relating to the simultaneous expression of markers of several different tubule segments in ccRCC.

NOT ALL ccRCCs ARE CREATED EQUAL

Studies during the past decade, including very recent systematic analyses of large cohorts of ccRCC tumors using a variety of genomic scale technologies (12, 59), have highlighted that there are distinct molecular subclasses of ccRCC in terms of mRNA and microRNA (miRNA) expression profiles, the spectra of underlying genetic alterations, and protein expression patterns. For example, unsupervised cluster analysis has identified four different mRNA expression profiles and four different miRNA expression profiles that predict patient survival (59), highlighting the idea that the expression levels of many hundreds of different genes could impact the biological behavior of ccRCCs. Several recurring gene mutations, amplifications, deletions, and methylation-mediated gene silencing also occur at different frequencies and make up further distinct genetic subsets of ccRCC (12, 59–63). As described below, these genetic alterations fall into several distinct functional groups that affect cellular signaling pathways or regulatory mechanisms that likely cooperate with pVHL-regulated functions to contribute to the genesis or progression of ccRCC. Thus, it is becoming ever more apparent that ccRCC cannot be simply considered as a single histological entity but rather represents a collection of molecularly related, yet distinct, diseases. The ongoing challenges for researchers, pathologists, and clinicians will be to determine how each of these different molecular forms of ccRCC behave and how best to diagnose and treat them. In the following sections we focus on recent insights into the nature of some the cellular alterations that are mediated by the loss of *VHL* and also on the genetic alterations that appear to cooperate with the loss of *VHL* to drive tumor formation and progression.

HIF-1α AND HIF-2α CONTRIBUTE DIFFERENTLY TO ccRCC

Perhaps the biggest breakthrough in terms of advancing the molecular understanding of the causes of ccRCC was the discovery that pVHL functions as the recognition subunit of an E3 ubiquitin ligase complex, containing elongin B, elongin C, RBX1, and CUL2, that targets the HIF-1α and HIF-2α transcription factors for oxygen-dependent ubiquitin-mediated proteolytic degradation. Biallelic inactivation of pVHL function results in the constitutive stabilization of HIF-1α and HIF-2α, which can induce the expression of many overlapping, as well as distinct, transcriptional targets. In this review, we use HIF-α as a collective term for both HIF-1α and HIF-2α, in particular for those activities where the two isoforms appear to function equivalently. Readers are referred to several excellent reviews that describe the molecular details of this pathway and the many and varied cancer-relevant consequences of constitutive HIF-α activation that arise from inactivation of *VHL* in ccRCC, including angiogenesis, metabolic alterations, EMT, acquisition of stem cell–like properties, invasion, and metastasis (64–67).

In the context of the pVHL-E3-ubiquitin ligase complex, a long-standing mystery concerning ccRCC has recently been partly solved. Biallelic inactivation of *VHL* was known to be present in 70–90% of ccRCC cases, raising the question of the genetic driver mutation(s) in the missing 10–30% of cases. Sato and colleagues found that of the 8% of ccRCCs that were wild type for pVHL, 42% (8 of 19) harbored biallelic inactivation of *TCEB1*, which encodes elongin C (12). Similar to the effect of *VHL* mutation, these *TCEB1* mutations functionally impaired the degradation of

HIF-α, causing constitutive HIF-α activity. The fact that *TCEB1* mutations are always mutually exclusive with *VHL* mutations argues that there is a strong selective pressure in more than 95% of ccRCCs to inactivate the normal control of the degradation of HIF-α subunits. Although there have been no reported mutations of other important known components of the HIF-α degradation machinery in ccRCC, these, or mutations in other unexpected players that regulate HIF-α degradation, may still be waiting to be discovered in the remaining *VHL* wild-type tumors.

The genetic data suggesting that HIF-α activation is a common oncogenic driving force in ccRCC tumor formation are supported by studies of human *VHL*-deficient ccRCC cell lines that demonstrated that HIF-2α is necessary for the growth of these cell lines as xenografts in immunodeficient mice (68–70). A significant body of recent evidence has extended these initial observations and argues that there are differences in the roles of HIF-1α and HIF-2α in ccRCC development and progression. HIF-1α and HIF-2α appear to have opposing effects on cellular proliferation. Tumor xenografts of mouse embryonic stem cells or transformed mouse fibroblasts containing a deletion of the *Vhl* gene grew poorly in comparison with their *Vhl* wild-type counterparts (71, 72), suggesting that the loss of pVHL actually confers a growth disadvantage. Part of this growth disadvantage may be due to activation of HIF-1α, which exerts a negative effect on the proliferation of mouse embryo fibroblasts in cell culture (73) and on the growth rate of human ccRCC tumor xenografts (69). HIF-1α opposes the activity of the cell cycle-promoting protein MYC through direct binding to MYC itself (74), competitive binding to the MYC cofactors SP1 and MAX (73), upregulation of the transcription of the MYC inhibitor MXI-1, and promotion of proteasome-dependent degradation of MYC (75). In contrast to the inhibitory effects of HIF-1α, HIF-2α promotes the proliferation of mouse embryo fibroblasts (73), promotes tumor formation in ccRCC xenografts (68–70), and enhances MYC activity (73), resulting in upregulation of cell-cycle promoting genes such as *CYCLIN D1* and *CYCLIN D2*, and promotion of cell cycle progression. In summary, when considering HIF-1α and HIF-2α activities purely in the context of cell-cycle control and cellular proliferation (and excluding all other potential tumor promoting activities of these proteins) it appears that HIF-1α acts to restrain tumor development and HIF-2α promotes tumor development.

Consistent with the idea that the balance of HIF-1α and HIF-2α activities dictates cellular proliferation of *VHL*-null tumor cells, early lesions in the kidneys of patients with VHL disease tend to express predominantly HIF-1α, whereas more advanced lesions tend to express higher levels of HIF-2α (69). Moreover, sporadic cases of ccRCC that express both HIF-1α and HIF-2α display reduced MYC activity and lower frequencies of proliferating tumor cells in comparison with ccRCC cases that express only HIF-2α (76). Several mechanisms have been proposed that may contribute to this shift in the HIF-1α/HIF-2α balance during ccRCC progression. Single copy loss of the chromosomal locus harboring the *HIF1A* gene occurs frequently in ccRCC and predicts poor outcome (77), and ccRCC cell lines frequently express HIF-2α but do not express functional HIF-1α due to biallelic genetic alterations at the *HIF1A* locus (78). In addition to direct genetic alteration of the *HIF1A* locus, differential posttranscriptional and posttranslational effects on HIF-1α and HIF-2α may potentially play a part in the development of ccRCC. The hypoxia-associated factor (HAF) is an ubiquitin ligase that targets HIF-1α but not HIF-2α for proteolytic degradation: HAF binds to HIF-2α and enhances its ability to transactivate genes (79, 80). Thus, HAF can act as a molecular switch to promote the activity of HIF-2α over HIF-1α. The expression of HAF in relation to HIF-1α and HIF-2α protein expression levels in ccRCC has not yet been reported. Both miR-30a-3p and miR-30c-2-3p have been shown to specifically bind and inhibit the expression of *HIF2A* mRNA; it has also been shown that the expression levels of these miRNAs alter the balance of the expression of HIF-1α and HIF-2α in human ccRCC tumors (81). HIF-1α and HIF-2α expression levels in ccRCC cell lines have also been shown

to be differentially dependent on the activities of the mammalian target of rapamycin complex 1 (mTORC1), the mammalian target of rapamycin complex 2 (mTORC2), and the AKT1, AKT2, and AKT3 kinases (82). Because mutational activation of the phosphoinositide 3-kinase (PI3K)–mTOR pathway occurs frequently in ccRCC (see below), it is possible that this may also contribute to the differential regulation of the expression of HIF-1α and HIF-2α during the progression of some ccRCC tumors. Finally, in various cell-culture settings the expression of HIF-1α and HIF-2α appears to be mutually suppressive; increasing HIF-1α protein abundance decreases HIF-2α protein abundance, and vice versa (69, 83). Several mechanisms have been proposed to explain these observations including alteration of the relative translation rate of *HIF1A* mRNA (84) or the binding of HIF-1α or HIF-2α to a reverse-hypoxia response element in the *HIF1A* promoter region to effect epigenetic silencing of *HIF1A* mRNA expression (85).

Despite all of the evidence that HIF-1α and HIF-2α contribute to ccRCC progression, numerous mouse studies argue that activation of HIF-α is not sufficient for the formation of ccRCC. Deletion of *Vhl* (see discussion above) or combinatorial deletion of *Phd1*, *Phd2*, and *Phd3* (86) in mouse kidney epithelial cells results in constitutive HIF-1α and HIF-2α stabilization and in activation of HIF-α target genes but does not cause tumor formation. Moreover, transgenic expression of constitutively active, nondegradable mutants of HIF-1α or HIF-2α alone, or in combination, in kidney epithelial cells also failed to induce tumor formation beyond the stage of simple cysts or small dysplastic lesions that could be envisaged as ccRCC precursor lesions (41, 87, 88). One possible interpretation of all of these findings is that the activation of HIF-1α and HIF-2α alone or together is insufficient for tumor formation, but may provide an initial permissive environment that facilitates tumor formation once other cooperating genetic alterations arise. The balance of HIF-1α and HIF-2α activities may then act to modify the tumor phenotype as the tumor grows and progresses.

ccRCC IS A DISEASE OF HIF-α-MEDIATED METABOLIC REPROGRAMMING

The accumulation of fat and glycogen in the cytoplasm of tumor cells prevents staining by eosin, resulting in the so-called clear cell histology and giving rise to the eponymously named clear cell renal cell carcinoma. Analyses of the kidneys of patients with VHL disease (33) and the kidneys of genetic knockouts of *Vhl* in mice (34) have demonstrated that this phenotype is apparent in nontumorigenic pVHL-deficient cells, indicating that it is not a consequence of tumor formation but reflects a change in cellular metabolism that is present before tumor initiation. This histological phenotype turns out to be the tip of the iceberg when considering the range of metabolic alterations that are now known to occur as a consequence of the loss of pVHL function. In recent years, it has become apparent in a teleological sense that the general purpose of altered metabolism in cancer cells is to provide the cell with an adequate supply of all of the biosynthetic precursor molecules, reducing equivalents (NADH, NADPH), and energy (ATP) that are necessary for generating the macromolecules (nucleotides, fatty acids, amino acids, etc.) that are required for rapid cell division (89). We now know that HIF-1α and HIF-2α act as central metabolic regulators, functioning in many different ways to coordinate cellular metabolic pathways to ensure the supply of the many precursors of anabolic metabolism (90). Constitutive activation of HIF-α leads to upregulation of the expression of glucose transporters and almost all glycolytic enzymes (91), causing an increase in total glycolytic flux in ccRCC cells. HIF-α activation also induces the expression of pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A, which act to divert glucose-derived carbon (pyruvate) away from mitochondrial oxidation to be released from the cell as lactate (92, 93). This Warburg-like metabolic shift is reinforced by the fact that ccRCCs express low levels of

fructose-1,6-bisphosphatase 1, which has the effect of enhancing glycolytic flux and removing the inhibition of HIF-α nuclear transcriptional activity (94). HIF-1α also inhibits MYC activity, consequently lowering PPAR gamma coactivator (PGC)-1β expression, leading to a reduction of mitochondrial biogenesis and, consequently, mitochondrial respiration (75). High levels of glucose uptake also lead to increased flux through the pentose phosphate pathway (95). Although it has not yet been directly demonstrated, it is tempting to speculate that this effect might be mediated in ccRCC cells, at least in part, by HIF-1α-dependent upregulation of the expression of pyruvate kinase-M2 (96), which is a low-activity form of the pyruvate kinase enzyme that acts to decrease the flow of pyruvate to lactate, resulting in a piling-up of upstream glycolytic intermediates, which allows glucose-6-phosphate to be diverted into the pentose phosphate pathway (97). The increased pentose phosphate pathway flux provides the cell with the reducing equivalent NADPH and with ribose-5-phosphate, the precursor of nucleotide synthesis. Pyruvate kinase-M2 also acts as a cofactor for HIF-1α that stimulates the transcription of HIF-1α target genes involved in glycolysis, representing a feed-forward mechanism that supports increased glycolytic flux (96). Another feature of glucose metabolism in ccRCC cells is that it is converted at a high rate into glycogen. This may be explained by recent findings that hypoxia induces the HIF-α-dependent upregulation of the expression of the numerous genes involved in glycogen synthesis (98, 99). At least in cell-culture settings, this hypoxia-induced accumulation of glycogen allows cells to survive periods of glucose starvation. Whether this applies to ccRCC remains to be investigated.

Although most cells that are grown in an adequate supply of oxygen preferentially use oxidative glucose metabolism as the major carbon source for lipid biosynthesis, the inhibition of pyruvate entry into mitochondria in pVHL-deficient cells, hypoxic cells, or mitochondria-deficient cells, prevents efficient lipogenesis via this pathway (100–102). Tumor cells frequently utilize glutamine as an important carbon source that can be converted via glutaminolysis to α-ketoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle that can undergo further mitochondrial oxidation to generate energy (ATP) and citrate, which serve as precursors for cytoplasmic lipid biosynthesis (89, 103). In pVHL-deficient cells, however, analogous to the effect of HIF-α in inhibiting the entry of glucose-derived carbons (pyruvate) into the mitochondria, HIF-α also inhibits oxidative glutamine metabolism by inducing the expression of SIAH2, which targets the 48-kDa splice variant of 2-oxoglutarate dehydrogenase, a subunit of the TCA enzyme α-ketoglutarate dehydrogenase, for proteolytic degradation (104). This has the effect of blocking the metabolism of glutamine-derived carbon via the oxidative TCA cycle. Instead, *VHL*-deficient cells utilize a recently discovered alternative pathway for lipogenesis that involves the cytoplasmic conversion of glutamine to form acetyl coenzyme A via a so-called reductive glutamine metabolism (100–102).

HIF-2α also contributes to metabolic adaptation by upregulating the expression of the amino acid transporter SLC7A5 (105). This activity is particularly important for ensuring the supply of amino acids under conditions of low amino acid availability, serving to maintain the activity of proproliferative mTORC1 signaling (105). Indeed, *SLC7A5* expression has been shown to be necessary for the growth of a human ccRCC cell line as a xenograft tumor (105).

Interestingly, ccRCC tumors frequently harbor alterations in several other oncogene and tumor suppressor pathways that function as key regulators of diverse aspects of cellular metabolism, including p53, MYC, and PI3K–mTORC1 (see below) (89, 103). Therefore, it is likely that the final metabolic phenotype of any given ccRCC cell results from combined inputs, not only from the status of HIF-1α and HIF-2α but also from a variety of oncogenic stimuli that are present in the mutational spectrum of that cell. It is probable that there are in fact several different metabolic subtypes of ccRCC, including alterations not only in the above-mentioned glucose and glutamine metabolic pathways but also in other major metabolic pathways that remain to be discovered. Indeed, a recent transcriptional analysis of metabolic gene expression in ccRCC

predicts alterations in the nucleotide, one-carbon, and glycerophospholipid metabolic pathways (106), although these predictions have yet to be functionally validated. Although it is tempting to speculate that the altered metabolism of ccRCC cells may provide several advantages to cells in terms of tumor formation—for example, the accumulation of fatty acids and glycogen may provide energy stores for the cell when nutrient availability is poor—this idea remains to be rigorously tested experimentally in vivo. An important corollary is whether alterations in cellular metabolism could be exploited for therapeutic intervention. In this respect, a high throughput screen of a large library of compounds identified that ccRCC cells lacking *VHL* are sensitive to inhibitors of glucose uptake (107), providing encouraging proof-of-principle evidence that metabolic alterations in ccRCC are potential therapeutic targets.

ACTIVATION OF THE PI3K–mTORC1 SIGNALING PATHWAY IN ccRCC

More than a decade ago, several studies showed that loss of heterozygosity of *PTEN* (108), reduction of expression of PTEN protein (109), or activation of AKT (110, 111), correlated with poor outcomes for patients with sporadic cases of ccRCC. Recent comprehensive genomic studies have complemented these early findings by showing that numerous genes whose protein products are components of the PI3K–mTORC1 signaling cascade are mutated in a largely mutually exclusive manner in approximately one-fifth of all ccRCCs (12, 59). These mutations include predicted loss-of-function mutations of negative regulators of the pathway including *PTEN*, *TSC1*, or *TSC2*, or activating mutations or amplifications of positive regulators of the pathway, including *PIK3CA*, *PIK3CB*, *PIK3CG*, *AKT1*, *AKT2*, *AKT3*, *RHEB*, *MTOR*, *RPS6KA2*, *RPS6KA3*, and *RPS6KA6*. The *SQSTM1* gene, which is frequently overexpressed as a result of common 5q copy number amplifications in ccRCC, has also been postulated to represent an alternative mechanism of mTORC1 activation (112). These findings argue that, for at least a subset of ccRCCs, there is a selective advantage obtained from a cooperating mutation that leads to dysregulation of the PI3K–mTORC1 signaling axis. Functional evidence supporting the notion that activation of the PI3K signaling pathway may cooperate with loss of *VHL* function to induce kidney cell proliferation and initiation of ccRCC comes from mouse studies showing that deletion of *Vhl* and *Pten* in kidney epithelial cells caused the formation of highly proliferative kidney cysts (34). Moreover, *VHL* mutant ccRCC cell lines are highly sensitive to mTORC1 inhibition (113), and human clinical studies have shown that drugs that inhibit mTORC1 exhibit good clinical activity as front-line and second-line therapy for ccRCC (114, 115).

CELL-CYCLE REGULATORS AND SENESCENCE IN ccRCC

Deletion of *Vhl* in primary and transformed mouse embryo fibroblasts induces, respectively, the onset of premature senescence or proliferation arrest (21, 22, 71). Deletion of *Vhl* sensitizes mouse cells in culture and in vivo to undergo senescence in response to oxidative stress (21), and senescence induced by loss of *Vhl* has been shown to be dependent on either pRB function (22) or p53 function (21, 58). These studies argue that the loss of *Vhl* function in primary mouse cells engages cell-cycle checkpoint and/or senescence pathways and has the effect of impairing cellular proliferation. Analogous to the known role of senescence as a cellular response that suppresses tumor progression in response to the loss of numerous other tumor suppressor proteins or in response to the activation of oncogenes, it may be speculated that this response could represent a barrier that must be overcome by additional mutations to allow the formation of ccRCC from *VHL*-null human kidney cells. Although no studies have determined whether the loss of *VHL* function also induces senescence in human kidney epithelial cells, it is noteworthy that mutually exclusive

alterations in genes encoding components of cell-cycle and senescence regulatory networks—including mutations or copy number deletions of *CDKN2A*, *TP53*, *RB1*, *ATM*, *CHEK2*, or *MDM2*, or copy number gains of chromosomal regions harboring *MYC* or *MDM4*—are found in about 40% of sporadic ccRCCs (12, 59). Codeletion of *Vhl* and *Trp53* in kidney epithelial cells in mice causes the formation of simple cysts, atypical cysts, and epithelial tumors, mimicking some of the ccRCC precursor lesions that are found in the kidneys of human VHL patients, thus providing strong functional evidence to support the idea that cooperation between the loss of pVHL function and genetic impairment of the cell-cycle regulatory machinery contributes to ccRCC development (58).

THE MICROTUBULE CONNECTION: PRIMARY CILIA AND MITOTIC SPINDLE ORIENTATION

Another major biological activity of pVHL is its function as a microtubule-interacting protein that can inhibit tubulin's intrinsic GTPase activity in vitro and regulate the dynamic behavior and direction of microtubule growth in cells (15, 116–118). Since different classes of tumor-derived pVHL mutants are differently compromised in their ability to regulate microtubule dynamics, it appears likely that this activity of pVHL contributes to tumor suppression (116). Human and mouse cells express both a long and a short pVHL isoform, derived from alternative translational initiation codons (119, 120). Although both isoforms are able to bind to microtubules, in human cells the long form of pVHL colocalizes predominantly with cytoplasmic microtubules, whereas the short form of pVHL localizes predominantly in the nucleus (15). Re-expression of the short isoform in *VHL*-negative cancer cells only partially rescues the dynamic behavior of microtubules (116). In line with this, genetic deletion of only the long form of pVHL in mice significantly affected cytoplasmic microtubule dynamics, yet the mice in this study did not present any obvious phenotypic abnormalities or develop tumors (121). This finding argues that the microtubule-stabilizing function of the long form of pVHL may become important in the context of other genetic alterations that arise during tumor formation or under conditions of specific cellular stress. In this context, the formation of kidney cysts in VHL patients provides a good example of how alterations in two different microtubule-associated activities of pVHL can contribute to the initiation of disease.

The first function relates to the role of pVHL in maintaining the structure of the primary cilium. The primary cilium is a microtubule-based, antenna-like organelle at the surface of the cell that functions to sense and transmit numerous mechanical and chemical signals (122). The fact that many inherited cystic kidney diseases are associated with mutations that impair the correct formation or function of the primary cilium has given rise to the idea that the primary cilium is a suppressor of cystogenesis, an idea that is well supported by many mouse genetic studies (123). pVHL localizes to the primary cilium and contributes to the stability of the axoneme (16), a microtubule-based scaffold with a characteristic structure in the cilium. Reintroduction of pVHL into pVHL-deficient human ccRCC cell lines has been shown to increase the frequency of ciliated cells, and knockdown of *Vhl* in immortalized mouse kidney epithelial cells reduced the frequency of ciliated cells (118, 124, 125). However, deletion of *Vhl* in primary cells in culture, or in kidney epithelial cells in vivo, did not result in a reduction in the frequency of cells with a primary cilium, indicating that pVHL is not absolutely necessary for the formation or maintenance of this organelle (9, 16, 34). These apparently contradictory findings were resolved by studies showing that pVHL becomes necessary for primary cilium maintenance in the context of inhibition of GSK3β and activation of ERK kinases (9, 16, 34). Supporting these findings, *VHL* mutant cystic lesions in VHL patients display hyperactivation of the PI3K signaling pathway, leading to inactivation of GSK3β

and reduced frequencies of ciliated cells (16, 34); additionally, mimicking this signaling and genetic environment by combined mutation of *Vhl* and *Pten* in mouse kidneys caused kidney cysts with reduced cilia frequency (34). Thus, via an HIF-α-independent microtubule stabilizing–dependent mechanism, the loss of pVHL function sensitizes cells to lose their primary cilia in the background of alterations in other signaling pathways, such as activation of the PI3K pathway, thus removing the cyst-suppressing function that is normally exerted by the primary cilium. Intriguingly, a recent study has provided another twist to the tale of the regulation of primary cilia by pVHL, with the identification of an HIF-1α-dependent mechanism that controls the formation of primary cilia (126), a cell biological process that is separate from the maintenance of cilia. This study demonstrated that ubiquitin-specific protease 8 functions as a deubiquitinase for HIF-1α, opposing the activity of pVHL-mediated ubiquitination. Ubiquitin-specific protease 8 has been shown to function in normoxia to maintain a basal level of HIF-1α, allowing repression of *Rabaptin 5* gene expression. Preventing high levels of Rabaptin 5 is important to inhibit it from activating Rab5, which would promote early endosome fusion (127), prevent endocytic cycling, and thereby impede ciliogenesis. Therefore, pVHL not only directly regulates cilia maintenance via stabilization of the microtubule-based axoneme but also participates in an HIF-1α-dependent endosome trafficking network that regulates cilia formation. It appears that there is still a lot that remains to be discovered about how these pathways, and potentially other cooperating signaling pathways, are dysregulated during the initiation and progression of ccRCC and whether they could be targeted therapeutically to prevent the onset of disease or to treat existing ccRCC.

A second microtubule-dependent function of pVHL that appears to be relevant to the initiation of cyst formation relates to its role in stabilizing astral microtubules during mitosis. The astral microtubule network functions as a link that anchors the mitotic spindle poles to integrin contacts on the cellular cortex, thereby controlling the positioning of the cell division plane to provide directionality to the process of cytokinesis (128, 129). Live cell imaging studies have demonstrated that pVHL-deficient cultured cells have a poorly developed astral microtubule network, resulting in metaphase arrays of chromosomes that rotate in all directions rather than being anchored in the correct plane (23). This spindle-tumbling phenotype results in mitosis at randomly oriented planes of cell division. Misorientation of cellular division in vivo has also been demonstrated in *Vhl*-null kidney cells that were induced to proliferate following a challenge of ischemic kidney damage (130). In a tubular epithelial structure, the consequences of failure to maintain the correct orientation of cell division are severe; cell divisions that are not oriented along the axis of the tubule are predicted to cause widening of the tubule, which is potentially an initiating stage of cyst formation, or to cause regions of aberrant tubular architecture. Indeed, when *Vhl* knockout kidneys were analyzed 4 months after induction of ischemic damage, small cysts and regions of dysplasia were frequently observed, providing important in vivo evidence that loss of this function of pVHL is likely to be important at the earliest stages of tumor formation (130).

In summary, pVHL, by regulating two independent microtubule-dependent processes—cilia maintenance and mitotic spindle orientation—guards against the breakdown of the normal tubular structure and the onset of cyst formation or the formation of other ccRCC precursor lesions.

pVHL GUARDS AGAINST ANEUPLOIDY AND DNA DAMAGE

As well as regulating the orientation of mitotic cell division, pVHL also has a function at an earlier stage in mitosis, namely to ensure the fidelity of the mitotic spindle checkpoint. This effect is independent of both pVHL's function in regulating microtubules and in regulating HIF-α. The loss of pVHL function leads to reduced protein abundance of MAD2 (23). MAD2 is a key component of the mitotic spindle checkpoint that prevents the onset of anaphase until all chromosomes

have been correctly bound by the microtubules of the mitotic spindle. Through an unknown mechanism, miR-28-5p expression is upregulated in pVHL-deficient cells, causing inhibition of the translation of *MAD2* mRNA, weakening the mitotic spindle checkpoint, and leading to an increased rate of chromosome missegregation and whole chromosome aneuploidy (23, 131). Importantly, this effect is observed not only in cultured human and mouse cells but also in *Vhl*-null mouse kidney epithelial cells that are proliferating in response to kidney damage in vivo (130). Administration of inhibitors of miR-28-5p suppressed this phenotype, providing strong functional evidence that the accumulation of aneuploidy due to changes in miR-28-5p and MAD2 expression is likely to be a very early event in the formation of *VHL* mutant tumors in humans. The degree of aneuploidy is predicted to increase during tumor progression as cells continually divide. Indeed, in sporadic human ccRCC tumors there is a positive correlation between low protein levels of pVHL and low protein levels of MAD2: Lower MAD2 levels were more commonly associated with higher grade and highly aneuploid tumors (23), and miR-28-5p expression levels correlate linearly with aneuploidy (131). It remains to be experimentally tested whether aneuploidy represents a driving force in the initiation or progression of ccRCC or, analogous to other tumor types, whether it may act to restrain tumor progression in the background of other tumor-driving mutations (132).

In addition to pVHL's role in protecting against aneuploidy, it also has been reported to function via an HIF-α-independent mechanism to regulate the repair of damaged DNA. In response to DNA double-strand breaks, suppressor of cytokine signaling 1 induces K63 ubiquitination of pVHL, which has been shown to be necessary for efficient execution of the DNA damage response and homologous recombination repair (133). Loss of this function of pVHL in ccRCC may lead to an increased rate of DNA mutation that could potentially contribute to tumor evolution.

THE 3p TUMOR SUPPRESSOR GENE CLUSTER

Consistent with a major role of *VHL* in initiating ccRCC are the frequent loss of chromosome 3p in sporadic ccRCC, the mapping of the *VHL* gene to 3p25-26, and the observation that ccRCC in patients with VHL disease and sporadic cases share the presence of *VHL* mutations. Interestingly, the results of several large-scale genomic sequencing studies have recently demonstrated that the 3p locus also harbors at least three additional ccRCC tumor suppressor genes, all of which have been implicated in regulating chromatin structure or modifying histones. Sequencing of more than 3,500 candidate genes in about 100 ccRCCs identified truncating mutations in three genes implicated in chromatin modification in about 3% of RCCs, namely the histone 3 lysine demethylases *UTX/KDM6A* and *JARID1C/KDM5C*, and the histone 3 lysine methylase *SETD2* (134). Exome sequencing of 7 ccRCCs and verification of potentially recurring mutations in a total of 200 tumors revealed that the SWI/SNF chromatin remodeling complex gene *PBRM1* showed truncating mutations in 41% of ccRCCs (63). Intriguingly, *PBRM1* and *SETD2* are both found on chromosome 3p21. PBRM1 can regulate p53 transcriptional activity toward induction of senescence, and it increases expression of the cyclin/cyclin-dependent kinase inhibitor CDKN1A (p21) (135). Reduced expression of PBRM1 has been associated with poor prognosis, but it has not been associated with the presence or absence of *VHL* mutations (136). About 15% of ccRCCs also harbor loss-of-function mutations in another 3p resident gene, encoding the nuclear deubiquitinase BAP1, which also plays a role in chromatin remodeling (60). *BAP1* mutant tumors are typically high grade and are associated with poor outcome (12, 60). Interestingly, *PBRM1* and *BAP1* mutations are almost always mutually exclusive in ccRCC, and *SETD2* mutations frequently occur alone or together with *PBRM1* but do not occur with *BAP1* mutations (12, 59, 60). Thus, the four most frequently mutated genes in ccRCC (*VHL*, *PBRM1*, *BAP1*, and *SETD2*) all localize to chromosome 3p, which is subject to copy number loss in more than 90% of ccRCC tumors.

Aberrant DNA methylation and histone protein modification are frequently detected in ccRCC and represent an additional, potentially reversible, mechanism of tumor-suppressor gene inactivation. The *VHL* gene is epigenetically silenced by promoter region methylation in up to 20% of cases (137). It has been shown that the *RASSF1A* tumor suppressor gene on chromosome 3p21 is methylated in 30–50% of sporadic clear cell and papillary RCCs, suggesting that *RASSF1A* inactivation typically results from a combination of allelic loss and methylation in ccRCC (138, 139). Several studies have identified numerous genes that have a mean combined methylation/mutation rate of more than 20% in RCC (59, 62). Among these genes are two additional candidate tumor suppressor genes that are also localized on chromosome 3p, namely *TU3A* in 42% of ccRCCs and *DLEC1* in about 30% of RCCs. Thus, there may potentially be at least seven relevant ccRCC tumor suppressor genes located on 3p. Extensive studies will be required to define if and how each of these different combinations of gene losses of function caused by mutations or epigenetic silencing can cooperate with *VHL* mutation to cause tumor formation or progression, and whether these different molecular subclasses of ccRCC affect the response of tumors to different therapies.

OTHER GENETIC ALTERATIONS IN ccRCC

In addition to tumor-initiating pathways involving the loss of chromosome 3p, a variety of other genomic and epigenomic findings have been linked to the prognosis of ccRCC. Chromosome 14q allelic loss, and loss of 4p and 9p, have been associated with poor prognosis (140–142). *HIF1A* is a likely target of the 14q deletions. Many ccRCCs harbor a focal, homozygous *HIF1A* deletion that leads to absent protein production (78, 141). Copy number gains of chromosome 5q are harbored by up to 70% of ccRCCs (59, 143). Unbalanced translocations involving chromosomes 3p and 5q, resulting in the loss of chromosome 3p and gain of 5q, have been reported in kidney cancer. Recently, the relevant target on chromosome 5q has been identified as *SQSTM1* (112). The *SQSTM1* gene product, p62, is a multifunctional protein that serves as an adaptor molecule that facilitates the degradation of specific proteins by autophagy. Notably, the p62 protein interacts with a number of signaling molecules to enhance the activity of downstream effectors such as NF-κB and mTOR. The potential tumor suppressor gene *FHIT* is epigenetically silenced in about 50% of ccRCCs, and several WNT-pathway inhibitors have been reported to be epigenetically inactivated in ccRCC by methylation, e.g., SFRP proteins (*SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5*) and Dickkopf genes (*DKK1*, *DKK2*, and *DKK3*) (for a review, see 144). Finally, inactivating mutations of the *NF2* tumor suppressor gene have been identified in a small subset of ccRCCs, all of which were wild type for *VHL* (134).

METASTASIS AND TREATMENT OF ccRCC

The prognosis of ccRCC is closely correlated with the disease stage and the tumor differentiation grade. In the past, the Fuhrman grading system was a worldwide accepted grading system and was used for all renal tumor types. The Fuhrman grade is based on nuclear diameter, nucleolar prominence, and nuclear pleomorphism. Recently, grading systems relying solely on nucleolar prominence have shown a stronger association with patient outcome compared with those relying on Fuhrman grade for ccRCC and papillary RCC. In addition, studies have shown that Fuhrman grading is inappropriate for chromophobe RCC. The presence of histological tumor necrosis also influences clinical outcomes (3, 145–147). Metastatic ccRCC is detected in approximately 30% of patients at the time of diagnosis, and a similar percentage of patients subsequently develop metastasis. These carcinomas most commonly metastasize hematogenously via the vena cava, primarily to the lung. Retrograde metastasis may also occur along the paravertebral veins into the bones. These carcinomas are well known for metastasis to unusual sites and for late metastasis.

Metastasis to the brain has been reported in up to 18% of renal cancer patients (148). Loss of *VHL* appears to contribute to metastatic tumor phenotypes through activation of HIF-α and downstream targets, including matrix metalloproteinases, PAX2, CD10, and CXCR4 (149–152). Different tendencies for metastasis to specific organs also depend on intrinsic properties of the primary tumor and specific characteristics of the target organ. Such intrinsic tumor cell features include chemokine–chemoreceptor expression, including that of CCL2, CCL7, CCR1, and the CXCR4/CXCL12 system (148).

In patients with ccRCC confined to the kidney and its regional lymph nodes, nephrectomy or nephron-sparing tumorectomy is the primary treatment modality. In metastatic RCC, systemic treatment has been used with varying results. Cytotoxic and hormonal therapies have been of little benefit in ccRCC, but cytotoxic therapy with interferon-α and interleukin-2 was the only proven systemic therapy until 2007. The identification of critical biological targets in ccRCC—the VHL and mTORC1 pathways—has resulted in new categories of systemic therapy, including the use of tyrosine kinase inhibitors, monoclonal antibodies, and mTOR inhibitors, all aiming to suppress angiogenesis mediated by vascular endothelial growth factor (VEGF) (reviewed in 153). However, the therapeutic efficacy of these agents has proven to be rather modest, highlighting the need for the development of alternative therapeutic drugs.

GENETIC AND EPIGENETIC ALTERATIONS AS PROGNOSTIC AND PREDICTIVE MARKERS

An ongoing challenge in the age of personalized and molecularly targeted medicine will be to find ways of efficiently exploiting the ever-increasing understanding of the molecular causes of ccRCC to develop novel genetic and epigenetic markers to assist with diagnosis, to determine prognosis, and to predict the response to therapy. Biomarkers for potential prognostication of RCCs have been frequently proposed, but they have not entered clinical practice. New oncologic therapeutic options are rapidly becoming available for patients with metastatic ccRCC, but there are currently no predictive biomarkers that can be used to plan therapeutic management (154).

The activation of HIF-α-dependent angiogenic pathways provides the rationale for the use of targeted VEGF-directed therapies (reviewed in 155). Therefore, it is reasonable to speculate that the presence of *VHL* alterations is associated with a poorer prognosis for ccRCC patients and that the status of the *VHL* alteration can predict the response to drugs that modulate the downstream targets of the pVHL/HIF-α pathway, including sunitinib, sorafenib, temsirolimus, and bevacizumab. However, contrary to this hypothesis is the clinical observation that VHL-disease-associated ccRCCs seem to grow more slowly and are associated with an overall better prognosis than sporadic ccRCCs. Clinical findings demonstrated that most *VHL*-negative ccRCCs in VHL patients never metastasize (156). A number of studies have examined whether the presence or type of *VHL* mutation might have prognostic or predictive significance, and some studies have indeed reported that ccRCCs with loss-of-function mutations are associated with worse outcomes, but reviews of all previous clinical trials have showed that *VHL* mutation status does not correlate with disease outcome or response to agents targeting VEGF (reviewed in 157).

INTRATUMORAL HETEROGENEITY IN ccRCC

A major complication of developing effective therapies against cancers is that individual tumors are often genetically heterogeneous, meaning that different cell populations within the tumor can have different sensitivities to therapeutic agents. Intratumoral heterogeneity in ccRCC has been demonstrated using exome sequencing, chromosome aberration analysis, and ploidy profiling on multiple, spatially separated samples obtained from primary renal carcinomas and associated

metastatic sites (158). Examples of intratumoral mutational heterogeneity have been seen for multiple genes, including *PBRM1*, *BAP1*, *SETD2*, *PTEN*, *PIK3CA*, and *KDM5C*. These genes had apparently undergone multiple, independent, inactivating mutations in distinct clonal populations within a single tumor. It was concluded from this and other studies that intratumoral heterogeneity leads to an underestimation of the tumor's genomic landscape as it is portrayed in a single biopsy sample and that primary tumors and consecutive metastases may have different molecular alterations (159). Xu et al. (160) confirmed these findings on the single-cell level by using single-cell exome sequencing of 25 ccRCC cells. Intriguingly, the prevalence of a single tumor having multiple *VHL* mutations is reported to be up to 8% (161). Recently, a study using a next-generation sequencing approach reported several low-frequency single-nucleotide variants, including two independent clonal expansions with different *VHL* mutations in primary ccRCC and their matching metastases (162). It is expected that next-generation sequencing approaches will increase the prevalence of single tumors having multiple *VHL* mutations. Rechsteiner et al. (163) addressed the issue of the heterogeneity of *VHL* mutations and characterized *VHL* missense mutations in silico to predict the effect on protein structure and function. This study characterized three groups of missense mutations: (*a*) those with severe destabilization of pVHL; (*b*) those with no destabilizing effects on pVHL but with relevance for the interaction with HIF-α, elongin B, and elongin C; and (*c*) those showing pVHL functions comparable with wild type (163). This strategy of categorizing *VHL* missense mutations into driver and passenger mutations could be used to evaluate the value of *VHL* mutation status for predicting the response to therapeutic agents.

Epigenetic events are also likely to be heterogeneous within a tumor. Repressive chromatin modifications and DNA methylation have been recently shown to restrict the expression of metastasis-associated HIF-α target genes (164). Using a ccRCC cell line, this study showed that a subpopulation of cells expressed a metastatic gene expression program, giving rise to the idea that metastasis in ccRCC is based on an epigenetically expanded output of the tumor-initiating pathway that arises in a subset of cells. Whether this occurs stochastically or as a result of alterations in genes that control the cellular epigenetic status—such as *PBRM1*, *BAP1*, or *SETD2*—remains to be determined.

The fact that there is considerable genetic and epigenetic heterogeneity within primary tumors and metastases in individual patients suggests that future therapeutic planning will have to take these issues into account. Analyses of multiple biopsies from primary tumors and metastases will likely be required to determine the different mutational spectra that exist. Ultimately, it is hoped that therapeutic agents will be able to be developed that can exploit those genetic alterations that arose at the earliest stages of tumor formation (so-called trunk mutations) and that are present in all cells. In this respect, because genetic alterations of *VHL* are so prevalent in ccRCC and have always been found as trunk mutations in the tumors analyzed, there is still hope that drugs may be developed that exploit the many molecular and cellular alterations that result from the loss of pVHL-mediated functions.

SUMMARY POINTS

1. Biallelic loss of *VHL* function occurs as an initiating event in more than 90% of ccRCCs.
2. The VHL tumor suppressor protein pVHL functions as a tumor suppressor via HIF-α-dependent regulation of the cell cycle, angiogenesis, the epithelial to mesenchymal transition, metabolic reprogramming, invasion, stemness, and metastasis.

3. Additionally, pVHL functions as a tumor suppressor via HIF-α-independent regulation of primary cilia, mitotic spindle orientation, chromosome segregation, and DNA repair.
4. The 3p locus is lost in most ccRCCs and contains up to seven potential ccRCC tumor suppressor genes: *VHL*, *PBRM1*, *BAP1*, *SETD2*, *RASSF1A*, *TU3A*, and *DLEC1*.
5. Other mutational events, including activation of the PI3K–mTORC1 pathway and dysregulation of cell cycle regulatory networks, occur frequently in *VHL* mutant ccRCC tumors.
6. Tumors in ccRCC contain considerable genetic (and likely also epigenetic) intratumoral heterogeneity, indicating the parallel evolution of multiple tumor clones.

FUTURE ISSUES

1. An important area of research will be to elucidate the effects of different combinations of mutations on the initiation and progression of ccRCC.
2. It will be important to identify novel therapeutic agents that are effective against ccRCC cells that harbor specific genetic alterations.
3. To facilitate preclinical testing of therapeutic approaches, it will be necessary to generate autochthonous mouse models of ccRCC that reflect the different combinations of mutations that arise in human ccRCC.
4. Recent insights about the genetic basis of ccRCC will need to be integrated into routine clinical practice to inform the diagnosis of the disease, as well as the prognosis and therapeutic planning.

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12. Comprehensive genome-scale mutational and epigenetic analyses of ccRCC, demonstrating that there are numerous molecular subtypes.

14. Discovery that pVHL regulates the oxygen-dependent proteolytic degradation of HIF-α transcription factors.

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