State-of-the-science: An update on renal cell carcinoma

Eric Jonasch¹, Andrew Futreal¹, Ian Davis², Sean Bailey², William Y. Kim², James Brugarolas³, Amato Giaccia⁴, Ghada Kurban⁵, Armin Pause⁶, Judith Frydman⁴, Amado Zurita¹, Brian I. Rini⁷, Pam Sharma⁸, Michael Atkins⁹, Cheryl Walker⁸*, and W. Kimryn Rathmell²*

*Co-corresponding authors

¹MD Anderson Cancer Center, Houston, TX
²UNC Lineberger Comprehensive Cancer Center, Chapel Hill, NC
³UT Southwestern Medical Center, Dallas, TX
⁴Stanford University Medical Center, Stanford, CA
⁵University of Toronto, Toronto, ON
⁶McGill University, Montreal, Quebec
⁷Cleveland Clinic Taussig Cancer Center, Cleveland, OH
⁸University of Texas-Houston Medical Center, Houston, TX
⁹Beth Israel Deaconess Medical Center, Boston, MA

Communication: W. Kimryn Rathmell, MD, PhD
450 West Drive, CB 7295
UNC Lineberger Cancer Center
Chapel Hill, NC 27599
TEL: 919-966-8644
FAX: 919-966-8212
EMAIL: Rathmell@med.unc.edu

Keywords: Renal cell carcinoma, VHL, HIF, Epigenetics, Genetics, Tumor subtypes, Biomarkers

Running Title: Renal cell carcinoma
Abstract
Renal cell carcinomas (RCC) are emerging as a complex set of diseases with major socioeconomic impact and a continued rise in incidence throughout the world. As the field of urologic oncology faces these trends, several major genomic and mechanistic discoveries have altered our core understanding of this multitude of cancers, including several new rare subtypes of renal cancers. This review will examine these new findings, and place them in the context of the well-established association of clear cell RCC (ccRCC) with mutations in the von Hippel-Lindau (VHL) gene and resultant aberrant hypoxia inducible factor (HIF) signaling. The impact of novel ccRCC-associated genetic lesions on chromatin remodeling and epigenetic regulation is explored. The effects of VHL mutation on primary ciliary function, extracellular matrix homeostasis, and tumor metabolism are discussed. VHL proteostasis is reviewed, with the goal of harnessing the proteostatic machinery to refunctionalize mutant VHL. Translational efforts using molecular tools to understand discriminating features of ccRCC tumors and develop improved prognostic and predictive algorithms are presented and new therapeutics arising from the earliest molecular discoveries in ccRCC are summarized. By creating an integrated review of the key genomic and molecular biological disease characteristics of ccRCC and placing these data in the context of the evolving therapeutic landscape, we intend to facilitate interaction between basic, translational and clinical researchers involved in the treatment of this devastating disease, and accelerate progress towards its ultimate eradication.
Introduction

A rapid series of discoveries in ccRCC, bolstered by advances in genomic biology and the embrace of targeted therapy have ushered in a new era of biological investigation and therapeutic opportunity for this challenging disease. ccRCC is unresponsive to traditional chemotherapies, highly radiation resistant, and lacks the hallmark genetic features of solid tumors such as \textit{KRAS} or \textit{TP53} mutations. The unique tight association between ccRCC and mutations in the \textit{VHL} gene, and the resulting constitutive stabilization of hypoxia inducible factors (HIF-1$\alpha$ and HIF-2$\alpha$), have been a source of intense study over the past nearly two decades. Stemming directly from the studies of \textit{VHL} insufficiency is an enhanced understanding of the intricate relationship between this tumor type and the tumor endothelial vascular network, and the result has been the development of therapies which not only reduce tumor burden, but also have extended the natural life expectancy of patients with metastatic disease.

This review will examine recent developments poised yet again to produce a paradigm shift in our understanding of the biology of ccRCC and other tumors, as well as to generate a landscape ripe for development of new therapeutics. An international panel of experts provides a concise description of the most relevant developments in their field for ccRCC. Andy Futreal summarizes the discoveries arising out of the deep sequencing studies performed over the last few years, and Ian Davis and Cheryl Walker describe the impact of these mutations on cellular behaviour. The potential consequences of this finding are enormous, and provide an explanation for the source of tumor heterogeneity as well as a target for therapeutic intervention. Understanding of the \textit{VHL} gene and HIF signaling continues to evolve as well. Pathways are never as simple as they initially appear, and the intense focus on HIF-1$\alpha$ signaling associated with \textit{VHL} mutation has gradually shifted to a focus on HIF-2$\alpha$ as the offending culprit in this disease, with definitive evidence now available. Sean Bailey and William Kim describe these findings in more detail.

RCC is increasingly being recognized as a metabolic disease, and key lesions in nutrient sensing and processing have been detected. These metabolic
abnormalities provide protection for the tumor but also may provide a source of vulnerability and therapeutic opportunity. James Brugarolas and Amato Giaccia describe this important network. The same is true for the recently described abnormalities in extracellular matrix engendered by loss of VHL function, which are elucidated by Ghada Kurban and Arnim Pause. VHL is increasingly being recognized as an important regulator of the primary cilium, and by extension, of the cilia centrosome cycle. A better understanding of the role VHL plays in this pathway can potentially lead to insights in RCC carcinogenesis. Cheryl Walker provides a summary of this complex and intriguing function.

It has been well established that VHL mutations lead to malfolded, and poorly functioning VHL protein. A better understanding of VHL proteostasis may allow us to develop strategies to refold or otherwise refunctionalize point mutated, full length VHL. Eric Jonasch and Judith Frydman report on recent developments in this emerging field. Numerous biomarkers have emerged to clarify the presence of heterogeneity among tumors that can be exploited for prognostic value or intervention. Kimryn Rathmell reviews the emergence of molecular classification for RCC, and Amado Zurita describes prognostic and predictive biomarkers under development. Finally, the goal of all of this outstanding science is to allow us to offer patients better chances for survival and more healthy lives. The therapeutic options for ccRCC have evolved rapidly in the last six years, and continue unabated. Both targeted therapies directed at features uncovered in molecular and genetic studies, and improved opportunities to re-direct the immune system have significant potential to continue to improve the outlook for ccRCC. Brian Rini describes current and emerging molecularly targeted agents, and Pam Sharma and Michael Atkins review the exciting new developments in immunotherapy for RCC.

**GENETICS**

Renal cell carcinoma (RCC) is a collective term applied to a set of cancers arising in the epithelium of the renal tubules comprised of three main histopathological entities. Clear cell RCC (ccRCC) is the dominant histology, accounting for approximately 65% of reported cases, followed by papillary and
chromophobe RCC, accounting for approximately 15-20 and 5%, respectively. Other more rare subtypes make up the remainder of RCC cases including collecting duct, mucinous tubular, spindle cell, renal medullary, and MiTF-TFE translocation carcinomas.

Hereditary RCC, which accounts for around 4% of cases, has been a relatively dominant area of RCC genetics. Causative genes have been identified in several familial cancer syndromes that predispose to RCC including *VHL* mutations in von Hippel-Lindau disease that predispose to ccRCC[1], *MET* mutations in familial papillary renal cancer[2], *FH* (fumarate hydratase) mutations in hereditary leiomyomatosis and renal cell cancer that predispose to papillary RCC[3] and *FLCN* (folliculin) mutations in Birt-Hogg-Dubé syndrome that predispose to primarily chromophobe RCC[4]. In addition, germline mutations in the *TSC1/2* genes predispose to tuberous sclerosis complex where approximately 3% of cases develop ccRCC[5] and *SDHB* (succinate dehydrogenase type B) germline mutations in patients with paraganglioma syndrome give rise to increased risk of developing multiple types of RCC[6]. Moving away from rare monogenic disease to population-based RCC susceptibility, genome wide association study results from a recent study of almost 6000 RCC cases has implicated loci on 2p21 and 11q13.3 in RCC susceptibility[7]. 2p21 contains the *EPAS1* gene encoding a transcription factor operative in hypoxia-regulated responses while the other region has no known coding genes.

There has been, however, comparatively less progress in the elaboration of the somatic genetics of sporadic RCC. By far, the most studied somatically mutated gene is *VHL*, which follows the classic tumor suppressor gene paradigm of a germline cancer susceptibility gene also manifesting as being somatically mutated in the sporadic form of cancer type[8]. *VHL* is somatically mutated in up to 80% of ccRCC[9]. The majority of these mutations are protein-terminating mutations with loss of the wild-type allele via large-scale loss of heterozygosity of chromosome 3p. There is a further small proportion (5-10%) of cases with no apparent somatic mutations that apparently methylate the locus and thus are functionally *VHL* null[9-11]. Along a similar theme of congruence of germline and somatic genetics, albeit
with a diminished magnitude of effect, there are dominantly activating kinase domain MET mutation reported in 4-10% of sporadic papillary RCC[2]. Conversely, somatic mutations in FLCN in chromophobe RCC are rare[12] and somatic FH mutations in sporadic papillary renal cancers were not found [12-14]. Similarly, somatic mutations of TSC12 and SDHB were not identified in sporadic RCC [13, 14]. Recently however, somatic mutations in TSC1 have been found in sporadic ccRCC [15]. TSC1 mutations occur in 5% of ccRCCs and may predict for extraordinary sensitivity to mTORC1 inhibitors clinically [15].

Further investigation of RCC somatic genetics has included evaluation of cancer genes important in other adult epithelial cancers. Taking all histologies combined, the COSMIC database reports somatic point mutations in TP53 in 10% of cases, KRAS/HRAS/NRAS combined ≤1%, CDKN2A 10%, PTEN 3%, RB1 3%, STK11/LKB1 ≤1%, PIK3Ca ≤1%, EGFR 1% and BRAF ≤1% (http://www.sanger.ac.uk/ genetics/CGP/cosmic/). MYC has been reported to be amplified in papillary RCC [16] and rare cases of RCC have been reported with EGFR amplification [17]. Focusing on the most prevalent histology, ccRCC, the contribution of cancer genes commonly mutated in other tumor types provides limited insight into what additional somatic genetic events are contributing to pathogenesis.

With this as a background, systematic approaches have been undertaken to elaborate the somatic genetics of ccRCC. A screen of 3,544 protein coding genes via PCR-based exon re-sequencing in 101 cases of ccRCC identified several new cancer genes in RCC [18, 19]. Remarkably, four out of five genes with robust statistical support for being new cancer genes encode proteins involved in histone methylation/demethylation. Truncating mutations were identified in KDM6A/UTX, SETD2 and KDM5C/JARID1C which encode an histone 3 lysine 27 (H3K27) demethylase, an histone 3 lysine 36 (H3K36) methyltransferase and an histone 3 lysine 4 (H3K4) demethylase, respectively. MLL2, an H3K4 methyltransferase, was also mutated at a significant rate. These data implicate deregulation of histone H3, known to be a major regulator of euchromatin/transcription, as a new area of RCC biology for exploration. Of note and further confirming the utility of large-scale
systematic approaches, *NF2* truncating mutations were unexpectedly identified in a significant proportion of the small subset of ccRCC that are *VHL* wildtype. Taken together, however, these genes are mutated in less than 15% of ccRCC suggesting the existence of additional cancer genes.

A subsequent study has moved to solution capture and sequencing of the coding exons of 20,000 protein coding genes utilizing next-generation sequencing technologies to more comprehensively investigate ccRCC somatic genetics. This work identified a second major somatically mutated cancer gene in ccRCC and thus substantially reshaped the field of RCC genetics. Truncating mutations in the *PBRM1* gene were identified in a remarkable 41% (92/227) of ccRCC [20]. *PBRM1* encodes the Baf180 protein, a chromatin targeting subunit of the SWI/SNF chromatin remodeling complex implicated in multiple chromatin/transcriptionally mediated processes through interaction with histone H3 [21, 22], reinforcing the striking theme of deregulated chromatin in ccRCC biology. Of note, *VHL*, *SETD2* and *PBRM1* are all located on chromosome 3p, thus providing a likely explanation for the near pathognomonic loss of 3p seen in ccRCC. Indeed, half of all cases with a demonstrable *VHL* point mutation in this series have a *PBRM1* truncating mutation and 9/9 cases with a *SETD2* mutation also have concurrent *VHL* and *PBRM1* mutations. This work has framed important new areas for ccRCC basic and clinical research.

Recent work performing deep sequencing on samples from a variety of locations from individual large tumors and metastatic lesions shows that considerable heterogeneity exists within these tumors suggesting a branched pattern of evolution [23]. Mutational events, such as the VHL mutation among others were ubiquitous to all samples, however, certain mutations were present only in either the primary tumor or the metastatic lesions, and many mutations were private. Of particular interest, different phylogenetic branches demonstrated distinct *SETD2* mutations, indicating a convergent pattern of selection for certain genotypic events. More work to understand this process, and the implications for biomarker development is needed.
Given the findings of these recent studies, it is a certainty that other RCC cancer genes and driver mutations remain to be identified. To this end, there are international efforts underway (ICGC www.icgc.org and TCGA cancergenome.nih.gov) to sequence large numbers of RCC at the whole genome level, coupled with transcriptomic and epigenomic analyses. This work is proceeding at pace, and thus the comprehensive structure of RCC somatic architecture will be revealed in the coming few years.

**EPiGENETICS**

Together with long-standing insights into HIF deregulation through VHL loss, these findings suggest that RCC development may represent a nexus of epigenetic and transcriptional deregulation, and exploration of epigenetic modification could reveal critical biological properties and offer clues to novel therapeutic approaches.

**Genetic alterations in epigenetic regulators**

As described above, high throughput genetic studies of RCC have identified recurrent mutations in genes encoding several epigenetic regulators. Mutated genes have been implicated in chromatin regulation through nucleosome repositioning and histone tail modification. *PBRM1*, which was found to be mutated in nearly 40% of human RCC [20, 24], is a component of the Polybromo BRG1-associated factor complex (PBAF, SWI/SNF-B). PBAF, like SWI/SNF, functions as a nucleosome remodeler and has been shown to be involved in transcriptional regulation [25-27]. Less common mutations were also identified in two methyltransferases, *SETD2* and *MLL2*, and two demethylases, *UTX* (KDM6A) and *JARID1C* (KDM5C) [18] (Figure 1). Deletion of 3p, a common finding in ccRCC associated with the loss of VHL at 3p25 can also affect *SETD2* and *PBRM1*, both at 3p21 [28]. SETD2 mediates the trimethylation of H3K36 [29], a histone mark that is placed during transcription and may be important for maintaining faithful transcription [30, 31] whereas MLL2 mediates H3K4me3, a mark associated with active transcription. UTX demethylates H3K27me3 [32-34], a histone mark associated with repressed chromatin. Interestingly, UTX associates with MLL2 [32, 35], suggesting that demethylation of repressive marks is linked to placement of marks associated with transcriptional
activation. JARID1C demethylates H3K4 [36]. The finding of mutations in *MLL2* and *JARID1C*, which act oppositely on the same residue, suggests that the genomic effects of mutations in these genes are likely to be complex (Figure 1). Although some mutations may result in widespread epigenetic variation, the effect of others may demonstrate preference for specific regions of the genome [37].

**HIF- and hypoxia-mediated epigenetic regulation**

The hypoxia response pathway has previously been shown to have a direct effect on histone modification. HIF has been shown to activate several chromatin demethylases, including JMJD1A (KDM3A), JMJD2B (KDM4B), JMJD2C (KDM4C) and JARID1B (KDM5B), all of which are directly targeted by HIF [38-41]. Re-expression of pVHL in *VHL* deficient cell lines increased H3K4me3 levels associated with decreasing levels of JARID1C, a target of HIF2α [24]. Silencing JARID1C in *VHL*-deficient tumor cells augmented tumor growth in a xenografted mouse model suggesting that JARID1C acts as a tumor suppressor. In contrast, hypoxia may increase methylation through HIF independent mechanisms. Like HIF prolyl hydroxylase (EGLN3), histone demethylases are members of the dioxygenase superfamily, which require oxygen as well as iron and 2-oxoglutarate for activity [42, 43]. In a manner analogous to stabilization of HIF via decreased hydroxylation, hypoxia was shown to suppress JARID1A (KDM5A) activity resulting in increased H3K4me3 levels [44]. This suggests the hypothesis that loss of demethylases (and by analogy increased histone methylation) is part of a “hypoxia phenotype” which is selected for in RCC. This “hypoxia phenotype”, which is mimicked by *VHL* loss, would also be mimicked by loss of histone demethylase activity, which as noted above, is a high-frequency event in RCC.

Chromatin organization also influences HIF function. Studies of HIF, induced under conditions of hypoxia, demonstrated preferential targeting of HIF to previously nucleosome-depleted chromatin regions [27]. Moreover, the co-expression of SWI/SNF components BRG1, BAF170 and BAF57 augmented HIF activity from a HIF responsive reporter [26]. This study demonstrated that BRG1, but not BRM silencing, decreased HIF responsiveness, suggesting that PBAF may be more critical for HIF function than SWI/SNF.
To what extent mutations of epigenetic regulators influence chromatin or HIF targeting remains unknown. Because of the direct influence of hypoxia on demethylase activity, it is likely that the relationship between epigenetic variation and HIF targeting will differ under conditions of hypoxia in primary cells and in the context of specific epigenetic alterations in tumor cells. Altering the activity of an individual epigenetic regulator that functions as part of a complex may result in pleiotropic effects resulting from alterations in the stoichiometry of active complexes.

In addition to epigenetic regulation through histone tail modification, DNA methylation in RCC has been well recognized. Through studies of tumors, urine and RCC-derived cell lines, hypermethylation of several tumor suppressor genes have been demonstrated. RASSF1 may be hypermethylated in over half of RCC with less common hypermethylation of VHL and CDKN2A [10, 45-48]. Additional studies have identified methylation and silencing of other genes include TIMP3 and secreted frizzled-related protein 2 ([49, 50] [51]). The use of genome-wide assays of methylation and studies of differential methylation will likely identify many more loci that are methylated in ccRCC [52, 53], however, the relationship between DNA hypermethylation and histone modification in the context of RCC remains unclear.

**Therapeutic implications**

Epigenetic differences may predict variation in patient outcome. Global decreases in H3K4 methylation and H3K18 acetylation have associated with decreased patient survival [37, 54]. Since epigenetic alterations and transcriptional deregulation are central to RCC, employing agents with predicted epigenetic influences may have an effect on disease outcomes. In preclinical studies, treatment with the histone deacetylase inhibitor vorinostat augmented the activity of the mTOR inhibitor temsirolimus to induce apoptosis in xenografted RCC cell lines [55]. However, a phase II trial of a different HDAC inhibitor, panobinostat, in patients with refractory metastatic RCC failed to demonstrate an objective response [56]. A more precise understanding of the role of epigenetic alterations could indicate other targetable strategies.
HIFs AND HIF TARGET GENES

The hypoxia-inducible factors (HIF) are a family of transcription factors that contain a basic helix-loop-helix domain and function in a heterodimeric complex [57]. There are three known HIFα subunits (HIF-1α, HIF-2α, HIF-3α) which heterodimerize with their binding partner ARNT (HIF-1β) to transcriptionally regulate target genes containing hypoxia response elements (HREs). HIF-1α and HIF-2α, are best characterized and are known to regulate transcriptional programs associated with cellular and physiological adaptation to hypoxia such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), and carbonic anhydrase 9 (CA9), amongst others [58]. While there is significant overlap in genes that are transcriptionally activated by HIF-1α and HIF-2α, each HIF family member is thought to also transactivate unique target genes [59]. For example, HIF-1α has been linked to regulating genes in pathways associated with glycolytic metabolism such as, SLC2A1 (GLUT1), LDHA and autophagy BNIP3 while HIF-2α is uniquely responsible for transcriptionally activating genes associated with proliferation and de-differentiation, TGFα, CCND1 (Cyclin D1), and Oct4, respectively.

VHL regulation of HIF

An important realization in understanding the molecular pathogenesis of VHL deficient RCC was that under conditions of normoxia, the pVHL complex binds to and polyubiquitinates HIFα subunits, resulting in their targeting and destruction by the proteasome[57]. The interaction between HIF and pVHL is mediated by an enzymatic, post-translational hydroxylation of HIF on conserved prolyl residues by a family of HIF prolyl hydroxylases (PHDs or EGLNs). In keeping with the notion that regulation of HIF is an important function of pVHL, the majority of disease associated VHL mutations are predicted to abolish the interaction between pVHL and HIF[60]. Moreover, studies in mice suggest that HIF activation (in particular HIF-2α) mediate the majority of the phenotypes seen in the setting of VHL loss [61-63].

Role of HIF in RCC
Early in vitro and cell line xenograft studies suggested that while HIF-2α is both necessary and sufficient for the growth of transformed RCC cell lines [64-66] HIF-1α is not[67], indicating that HIF-1α is expendable for RCC growth. However, it appears that HIF-1α is not merely dispensable in the context of RCC but actually functions as a tumor suppressor gene. Several lines of evidence support this hypothesis. First, targeted exon sequencing of RCC has demonstrated (albeit rarely) inactivating mutations in HIF-1α, while copy number analysis RCC cell lines and primary tumors suggest that the HIF-1α locus is frequently lost along with the long arm of chromosome 14 (14q) [18, 68]. Secondly, while all VHL defective clear cell renal cell carcinomas appear to overexpress HIF-2α, about one third of these tumors appear to lack HIF-1α expression as well [69]. Finally, functional studies in vitro and in vivo suggest that over expression of HIF-1α in VHL wild type cells restrains tumor growth while suppression of HIF-1α in VHL deficient cells enhances tumor growth [68, 70]. Together these studies show support for HIF-1α as tumor suppressor gene in renal cancer development and HIF-2α as a key driver for renal cancer progression.

While there are a number of reasons to explain the contrasting properties of HIF-1α and HIF-2α in RCC pathogenesis, one intriguing observation is that HIF-1α and HIF-2α have opposing roles on the regulation of c-Myc activity. Specifically HIF-1α acts to suppress c-Myc activity while HIF-2α promotes the transactivation or transrepression of c-Myc specific target genes [59, 69, 71]. In keeping with this notion, RCC tumors that exclusively express HIF-2α have increased proliferation rates. Furthermore, intriguingly, a subset of clear cell RCC tumors appear to have copy number amplification of 8q24, where c-Myc resides [72].

VHL PROTEOSTASIS

Two pVHL isoforms exist in the cell, a 213 amino acid, 30 kilodalton form, and a 160 amino acid 19 kilodalton version[73]. In order to function, pVHL must fold to its native conformation. Proper folding and functionality of pVHL requires its tight association with elongin B and elongin C to give rise to a VHL-elongin BC
complex (herein VBC). Failure of pVHL to fold and to interact with elongin BC results in misfolding and proteolytic degradation of pVHL [74]. This section summarizes pVHL protein homeostasis (also called proteostasis), and how disease causing mutations affect pVHL stability and functionality.

Molecular chaperones are essential mediators of protein folding and quality control of most proteins in the cell. Following synthesis on ribosomes, folding of functional pVHL protein is the result of a complex interplay between nascent pVHL and cellular chaperones. Nascent pVHL is shuttled from the ribosomal machinery with the assistance of heat shock protein 70 (HSP70)[75]. pVHL is then folded into its tertiary structure via association with the chaperonin complex TRiC (for TCP-1 Ring Complex), also called CCT (chaperonin containing TCP-1)[75-78]. This heterooligomeric complex consists of two stacked rings with a central chamber where unfolded polypeptides bind and fold. TRiC is responsible for folding a number of key proteins that, like pVHL, are also subunits of oligomeric complexes [76, 77, 79, 80]. Hsp70 likely functions to stabilize non-native forms of pVHL, while TRiC/CCT facilitates pVHL folding, which is coupled to its incorporation into assembly of VBC [81-83]. Upon VHL binding to elongin BC to form mature VBC, pVHL is released from TRiC [75] (Figure 2).

Binding of VHL to TRiC occurs at amino acids 114-119 and 148-155, called Box 1 and Box 2, respectively[84]. Both motifs, located in adjacent strands of the beta-domain, harbor tumor-causing mutations that disrupt association with TRiC and lead to misfolding of newly translated pVHL. Mutations that block pVHL incorporation into a well folded VBC complex appear to result in destabilization and lower intracellular levels of pVHL, although residual functionality is maintained in some cases [85]. Further analysis of how specific mutations affect the interaction of pVHL with chaperones and chaperonins provides insight into targetable mechanisms of pVHL protein destabilization. Disease causing mutations in TRiC Box 1 and Box 2 binding sites [84, 86, 87], prevent association of pVHL to TRiC, resulting in a malfolded protein, and the absence of a mature VBC complex in the cell. Disease causing mutations also occur in the amino acid 155-181 elongin C binding region[86, 87]. This class of mutants can bind to TRiC, but cannot stably
bind to elongin BC. Loss of elongin C binding capacity appears to prevent pVHL release from TRiC [84], resulting in a lack of mature VBC complex.

Failure to generate a properly folded pVHL or a mature VBC complex will result in pVHL degradation through the ubiquitin-proteasome system. Chaperones are also involved in this quality control process [84, 88]. pVHL degradation specifically requires another chaperone, Hsp90, which does not participate in pVHL folding [88]. The identification of two distinct pathways of chaperone interactions for pVHL, one leading to folding and one to degradation, suggests that the fate of pVHL may be controlled by a hierarchy of chaperone interactions. Understanding the mechanism of how destabilized pVHL mutants are targeted for proteasomal degradation may lead to strategies of refolding and stabilization of pVHL that is functional and competent to complex with elongins B and C. Bortezomib and MG132 are capable of increasing levels of VHL, and a cell-based Prestwick compound screen identified several compounds that up-regulate point mutated VHL [89]. Efforts to analyze functional consequences of pVHL upregulation using these compounds, as well as an expanded screening effort are underway.

In summary, our evolving understanding of proteostasis allows new therapeutic approaches to be developed for VHL disease. Recalibrating the interaction between point-mutated pVHL and the chaperones and chaperonins may alter disease phenotype and provide benefit for patients with lesions possessing either germline or sporadic VHL mutations.

**RCC: ONE OF THE “CILIOPATHIES”**

Together with Polycystic Kidney Disease (PKD), Tuberous Sclerosis Complex (TSC) and VHL Syndrome are considered ‘ciliopathies’ [90]. In PKD, TSC2 and VHL deficiency, renal cysts develop following loss of gene function, often as preneoplastic lesions. One of the hallmarks of cysts is dysfunctional primary cilia. All cells possess a single primary cilium, a non-motile organelle composed of a central microtubule axoneme anchored by the basal body, surrounded by the ciliary membrane (Figure 3). In the kidney, the primary cilium projects from the apical surface of renal epithelial cells into the kidney lumen, where it responds to fluid flow and acts as a
chemo-, osmotic, mechano-sensor of the environment. Loss of primary cilia results in dysregulated cell signaling, cystogenesis in the kidney and several other organs, and is one of the hallmarks of many types of cancer, including RCC.

Several cell signaling pathways linked to tumorigenesis either localize specifically to the primary cilium and/or are spatially regulated by this organelle, including Wnt, Hedgehog and platelet-derived growth factor (PDGF) signaling [90, 91]. In addition to aberrant signaling, the microtubule organizing center (MTOC) that forms the foundation for the primary cilium, the basal body, also functions in the cell during mitosis as the centrosome [92]. The fact that this MTOC must shuttle between functioning as a basal body for the primary cilia and a centrosome for the mitotic spindle means that the cilia-centrosome cycle must be tightly coupled to cell division to maintain genomic stability. The cilia-centrosome cycle is important for maintaining genomic stability. Centrioles of the basal body, which serve as the microtubule organizing center (MTOC) for the ciliary axoneme, also serve as the MTOC for the mitotic spindle. There they function as the centrosome, which is comprised of a pair of centrioles responsible for spindle formation during mitosis. Thus, the centrioles serve two distinct and mutually exclusive functions in the cells, serving as the MTOC for either the mitotic spindle (during M phase) or the primary cilium (during G0/G1). The fact that this MTOC shuttles between these two functions means that the cilia-centrosome cycle must be tightly regulated to guarantee the fidelity of centrosome replication, spindle formation and genomic stability. Importantly, defects in cilia-centrosome cycle checkpoints have the potential to cause inappropriate centrosome replication, supernumerary centrosomes, and ultimately aneuploidy. Interestingly, it has recently been shown that ,VHL localizes to the mitotic spindle in mammalian cells and causes spindle misorientation and chromosomal instability when defective or absent [93]

	pVHL, TSC and PKD-associated proteins also share a common function: regulation of the structure and function of the primary cilium. These “renal cystoproteins” have been localized at the primary cilium where they exert a variety of cellular responses [94, 95]. For instance, PKD-1 plays a critical role at the primary cilium where it is involved in ciliary mechanotransduction. Several studies
have indicated that VHL is also involved in the biogenesis and function of the primary cilia [94, 96, 97] and biallelic inactivation of this gene is associated with loss of cilia [98]. Consistent with this observation, RCC of the clear cell type, associated with loss of pVHL, shows markedly reduced cilia formation when compared to papillary carcinoma [99]. In addition, pVHL binds to microtubules [93, 100], and co-localizes with the acetylated tubulin in the cilia where its mobility is dependent on its association with Kif3A [101]. Studies linking TSC2 deficiency to ciliary defects are more recent where loss of TSC2 is linked specifically to the development of aberrant primary cilia. This abnormal ciliary phenotype is also associated with loss of TSC1, which localizes to the basal body [102].

REGULATION OF THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is a complex structural component that surrounds the cells and provides support. It is composed of proteoglycans, hyaluronic acid and glycoproteins such as fibronectin and many types of collagens [103, 104]. Disruption of its regular architecture has been associated with tumor growth, angiogenesis, and metastasis. pVHL plays an important role in the regulation of the ECM. It was shown to interact directly with fibronectin and collagen IV, resulting in their assembly into the ECM and suppression of tumorigenesis, angiogenesis and cell invasion [105-109]. Most pVHL mutants fail to bind and degrade HIF-α, however all pVHL mutants tested to date fail to bind fibronectin and collagen IV and lose the ability to assemble an ECM [105-111]. The interaction of pVHL with fibronectin is mediated by pVHL neddylation which acts as a molecular switch conferring selectivity to fibronectin binding over CUL2 [109, 112], while its interaction with collagen IV is dependent on endoplasmic reticulum (ER) hydroxylation [107]. The VHL-collagen IV interaction was shown to occur at the ER membrane, with pVHL binding to a 70 kDa fragment of the collagen IV amino terminus that protrudes out of the ER into the cytosol [107]. The mechanistic significance of these interactions is still not clear but it was shown that pVHL did not affect fibronectin and collagen IV production or secretion nor resulted in collagen IV proteosomal degradation [106, 107].
The role of pVHL in ECM regulation is independent of its role in HIF-α regulation. Indeed, it was shown that inactivation of the VHL-ECM assembly pathway results in tumors that are highly vascularized, have a remodeled fibronectin and collagen IV matrix and show increased invasive ability. Loss of the VHL-HIF-α regulation pathway resulted in tumors with high VEGF levels but with decreased angiogenesis, a tightly assembled fibronectin and collagen IV matrix and low invasive capacity. Therefore while both pathways cooperate in supporting tumorigenicity, ECM remodeling may promote angiogenesis by providing a path for blood vessels to infiltrate tumors [106].

Tumor cell invasion is dependent on adhesion and proteolytic remodeling of the ECM, both of which are influenced by pVHL activity. pVHL was shown to regulate adhesion molecules; its inactivation leads to downregulation of the adherens junction protein E-cadherin and stimulation of invasion in RCC [113-115]. Loss of pVHL function also leads to downregulation of tight junction proteins occludin and claudin, in an E-cadherin independent manner [116]. In these studies, disruption of both adherens and tight junctions were mediated by loss of the pVHL-HIF-α regulation pathway. In another study, pVHL was found to downregulate integrins in a HIF-α independent manner and this correlated with restoration of tight and adherens junctions [117]. Cells lacking pVHL also fail to form β1 fibrillar adhesions, possibly contributing to the increased cell motility and invasiveness seen in the absence of a functional pVHL [118].

VHL pathways also regulate matrix metalloproteinases (MMPs), a family of matrix-degrading enzymes involved in ECM turnover. RCC cell lines lacking pVHL showed increased invasiveness in growth factor-reduced Matrigel, overproduced MMPs 2 and 9 and displayed an extensive branching morphogenesis phenotype in response to hepatocyte growth factor/scatter factor as compared to those with wild-type pVHL[119]. Activation of MMPs upon loss of pVHL activity can be attributed to disruption of both VHL-ECM and VHL-HIF-α pathways. Loss of VHL-ECM pathway regulation in RCC cells resulted in increased cell invasiveness and activation of MMP-2 [106], and HIF-α was also shown to influence RCC cell invasiveness by regulating membrane type-1 MMP expression [120, 121].
Proteolytic remodeling of the ECM by MMPs was shown to expose cryptic sites in collagen IV, normally hidden within the triple helical structure, leading to loss of integrin α1β1 binding and a gain of binding to the αvβ3 integrin, resulting in stimulation of angiogenesis [122]. Antibodies directed towards collagen IV cryptic sites led to inhibition of angiogenesis, tumor growth and metastasis in vivo suggesting the importance of collagen IV matrix remodeling in these processes [122-125].

The role of pVHL in maintaining ECM integrity and suppression of tumorigenesis, angiogenesis and invasiveness is multi-faceted and complex. It may result from interplay between several mechanisms that still remain unresolved. It is possible that pVHL mediates fibronectin and collagen IV modification, allowing their proper assembly into the ECM. Loss of these interactions would lead to an aberrant ECM, activation of MMPs, ECM remodeling, release of ECM sequestered growth factors and stimulation of tumorigenesis, angiogenesis and invasion. Disruption of integrins and cell adhesion molecule regulation would further enhance the invasive RCC phenotype. Understanding the mechanisms of ECM regulation by pVHL could provide additional or alternate therapy for RCC patients distinct from tyrosine kinase inhibitors.

**RENAL CELL CARCINOMA AND METABOLISM**

An intimate link between metabolism and renal cancer was established by the discovery that genes encoding enzymes of the Krebs cycle suppress tumor formation in kidney cells [126, 127]. The Krebs cycle refers to 9 sequential enzymatic reactions implicated in oxidizing acetyl-CoA generated from glucose, fatty acids and amino acids to CO₂ (Figure 4). This cycle is essential to the process of mitochondrial ATP generation. Succinate dehydrogenase (SDH), a complex of 4 different polypeptides (SDHA-D) that is also involved in electron transfer, catalyzes the conversion of succinate to fumarate. Heterozygous germline mutations in SDH subunits predispose to pheochromocytoma/paraganglioma and mutations in SDHB and SDHD have also been associated with renal cell carcinoma (RCC) [6, 128].
Fumarate hydratase (FH) catalyzes the next reaction of the Krebs cycle, the conversion of fumarate to malate. Heterozygous germline FH mutations cause hereditary leiomyomatosis and renal cell cancer (HLRCC), a syndrome characterized by cutaneous and uterine leiomyomas as well as RCC [3, 129]. RCCs occur in 20-50% of HLRCC families, are typically papillary-type 2 (pRCC-2) [130], and tend to be very aggressive [131].

The FH and SDH genes function as two-hit tumor suppressor genes [55, 127]. Loss-of-function mutations in the germline are usually accompanied by loss of heterozygosity (LOH) in the tumor causing the truncation of the cycle and the accumulation of intermediates [132, 133]. The accumulation of succinate or fumarate causes the inhibition of a family of 2-oxoglutarate-dependent dioxygenases normally implicated in HIF-α hydroxylation [134-136]. In the absence of this modification, HIF-α evades recognition by pVHL and accumulates, leading to increased HIF activity and tumor development [57]. In addition, the accumulation of succinate and fumarate results in the succination of proteins, including Keap1 [137, 138]. Keap1, is a component of an E3 ubiquitin ligase that targets NRF2 for degradation, and its succination blocks NRF2 degradation resulting in its accumulation and increased expression of stress-response and antioxidant genes [137-139].

Truncation of the Krebs cycle results in a compensatory increase in glucose uptake and glycolysis [132, 140-142]. Accordingly, HLRCC-associated pRCC-2 are intensely FDG-PET positive [142, 143]. Unlike other tumor cells, FH-deficient pRCC-2 cells are unable to grow in low glucose concentrations [142]. This dependency on glucose offers an opportunity for therapeutic intervention and we recently reported an attempt to treat an HLRCC patient with advanced pRCC-2 refractory to mammalian target of rapamycin complex 1 (mTORC1) inhibition with an inhibitor of glycolysis [143].

Metabolic derangements are also associated with mutations in VHL. Germline VHL mutations predispose to clear-cell RCC (ccRCC) [1], referred to in this manner because of the accumulation of lipid and glycogen which gives a clear appearance to
the tumor cells in the tissue after processing. By contrast to *VHL*, *SDH* and *FH* genes are seldom mutated in the sporadic setting [12, 13]. Interestingly, while *Vhl* mutations do not cause RCC in the mouse, disruption in the liver phenocopies the accumulation of lipid and glycogen observed in ccRCC [61, 144-148]. Thus, hepatocytes may serve as a model to study the role of *VHL* in metabolism. Acute *Vhl* disruption in hepatocytes results in a Hif-dependent inhibition of mitochondrial respiration [148]. Deprived of *Vhl*, glucose and ketone production by hepatocytes drops and the mice die within days [148]. While the relative contribution of Hif-1 and Hif-2 remains to be fully determined, Hif-2 may play an important role [147-149]. Should a similar inhibition of mitochondrial respiration occur in ccRCC, these tumors may be exquisitely sensitive to glycolysis inhibitors.

Vulnerabilities arising from *VHL* loss in ccRCC are also being exploited through synthetic lethal screens [150, 151]. This was illustrated genetically by screening *VHL*-deficient ccRCC cell lines with shRNAs against kinase targets. This screen identified several kinases synthetic lethal with *VHL* including cyclin-dependent kinase 6 (CDK6), hepatocyte growth factor receptor (MET), and dual specificity mitogen-activated protein kinase kinase 1 (MEK1) [151]. Small molecule inhibitors of CDK6 also proved to reduce the viability of *VHL*-deficient ccRCC tumor cells [151]. In addition to the shRNA approach, small molecule screening has also been fruitful in identifying new targets exhibiting enhanced cytotoxicity against *VHL*-deficient ccRCC. The compound STF-62247 significantly reduced the survival of *VHL*-deficient ccRCC in cell culture as well as in transplanted tumors in immunodeficient mice. STF-62247 induces autophagy and disrupts Golgi trafficking, which in *VHL*-deficient cells leads to cell death [150]. From the same screen, a second compound, STF-31, was identified that also exhibits enhanced cytotoxicity against *VHL*-deficient ccRCC. STF-31 inhibits glucose uptake by the Glut-1 transporter and induces necrotic cell death in *VHL*-deficient ccRCC [152]. This small molecule provides evidence that targeting glucose metabolism directly in *VHL*-deficient ccRCC could provide a therapeutic gain clinically.

Another pathway implicated in RCC pathogenesis and which plays an important role in metabolism is the mTORC1 pathway (Figure 5). mTORC1 is the
target of two FDA approved drugs, temsirolimus and everolimus, and is a master
regulator of cell growth. mTORC1 integrates environmental and cellular cues with
the cell growth machinery [153]. Signals from energy stores [154], oxygen [155] and
growth factors [156] are largely transduced to mTORC1 through a protein complex
formed by the proteins tuberous sclerosis complex 1 and 2 (TSC1/TSC2). By
contrast, nutrients regulate the subcellular localization of mTORC1 [157]. Only in
the presence of nutrients is mTORC1 receptive to signals funneled through
TSC1/TSC2 [160, 161]. The best characterized function of mTORC1 is in promoting
protein translation, a process mediated, at least in part, by the phosphorylation of
S6K and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [158, 162,
163]. However, mTORC1 plays also an important role in suppressing autophagy
[159, 163] and regulating mitochondria [164]. In addition, several transcription
factors are regulated by mTORC1. mTORC1 regulates HIF-1 [165-170] coupling
thereby trophic functions to angiogenesis. mTORC1 also regulates sterol regulatory
element binding protein1 (SREBP1), a master regulator of lipogenesis [171, 172].
Finally, we recently reported that mTORC1 regulates the transcription factor EB
(TFEB)[173], a controller of lysosome biogenesis [174]. Interestingly, the TFEB
gene is translocated in a subset of RCCs [175, 176] and the regulation of TFEB by
mTORC1 may provide opportunities for therapeutic intervention.

DEFINING NEW MOLECULAR SUBTYPES OF RCC

It has become increasingly clear that ccRCC is an incredibly heterogeneous
disease. We have learned only in recent years how thoroughly distinct are the
differences between ccRCC and non-clear cell histologies, that they in fact may be
best served to be considered as different diseases, with distinct biology, prognosis,
and response to treatment [177-180, 181 , 182 ]. Indeed, even within the less
common papillary type RCC, two distinct subtypes emerge, papillary types 1 and 2
[183-188]. These two papillary subtypes are associated with distinct familial
syndromes: hereditary papillary RCC (associated with type 1 papillary RCC), caused
by germline mutations in the Met proto-oncogene [2], and hereditary
leiomyosarcoma and RCC (HLRCC, associated with type 2 papillary RCC) [129], in
this case fumarate hydratase, are discussed above. The underlying genetic events in sporadic versions of these two histologically defined subtypes are undergoing investigation.

Within the category of clear cell RCC, heterogeneity has also been widely appreciated, despite studies revealing an increasingly tight connection with mutation of \( VHL \). As discussed above, \( VHL \) mutation provides a permissive setting for the deregulation of HIF family members, notably HIF-1\( \alpha \) and HIF-2\( \alpha \) [189]. Tumors can be classified, therefore, into tumors expressing both factors (H1H2) or those expressing only HIF-2\( \alpha \) (H2), or a smaller number that produce a functional pVHL [69]. These definitions demonstrate distinct patterns of gene expression and signal transduction, and suggest that HIF profile may be important for selecting therapy. This method of subclassifying ccRCC tumors has been hindered by assay inconsistency for the highly labile HIF proteins. Future studies may be best served to consider a transcriptionally based instrument for assigning H1H2 vs H2 status.

Indeed, ccRCC provides an outstanding tumor model for expression-based analyses, and numerous groups laid the groundwork for defining the heterogeneity of this tumor classification based on transcriptional measurements. Studies based on supervised gene expression profiling of primary tumors vs metastases, early vs late recurrences, or short vs long survival have consistently shown differentially expressed genes [179, 190, 191]. Recently, Rini et al reported on a transcriptional profile indicative of poor risk for recurrence developed from paraffin embedded specimens [192], which indicates that expression-based biomarkers are ready for translation to the clinic for prospective evaluation.

In parallel, several groups have performed unsupervised analyses to determine if inherent subtypes exist within ccRCC that can be defined based on purely molecular means [193, 194]. Two primary subgroups are found in relatively equal abundance in unselected tumors, suggesting that ccRCC may be represented by two major subclassifications, termed ccA and ccB in recent analyses [195]. The ccA and ccB subclassifications share many similarities to the gene sets identified in good risk or poor risk tumors described above, respectively, in particular gene sets.
involved in local invasion, and epithelial to mesenchymal transition. Moreover, when the clinical outcomes are examined, ccA cases display a long median survival of 8.6 years, whereas their ccB counterparts display median survival of only 2 years (p=0.002). The advantages these emerging strategies of subclassification include the potential to assign the profile of an individual tumor; capture of molecular information which is tied to genetic events which may be critical for selection of targeted therapy; and prognostic models which also consider clinically intermediate disease categories. A recent validation by meta-analysis confirmed the presence of these ccA and ccB subtypes, but further identified a subset defined by gene expression indicative of a wild type VHL and variant histology consistent with the newly described clear cell papillary subtype [196, 197].

In spite of the hurdles ahead, it seems likely that molecular strategies to classify individual tumors are on the horizon (Figure 6). In fact, the emerging data from clinically supervised strategies to find risk-associated biomarkers, and molecularly driven strategies to identify patterns within unselected tumors suggest that these two differing top-down, and bottom-up approaches are converging on the same conclusion: That ccRCC is composed of two dominant subgroups, which are closely aligned with clinical outcome. How this information will enable physicians and patients to make wise decisions in the management of ccRCC, or eventually select optimal pharmaceutical therapy remains to be seen, but in the light of many emerging targeted therapies, such information is likely to be highly valuable.

**BIOMARKERS**

The modern emergence of therapeutic options based on the increased understanding of the genetics and molecular biology of the RCC group of diseases has intensified the need for biomarkers to accurately assess prognosis, identify patients likely to benefit from therapy and specific drugs or classes of drugs, and understand mechanisms of resistance. Here we present a succinct overview of the most recent advances in the development of biomarkers for RCC, in particular for the clear cell subtype. Although some are promising, it is important to note that none of these biomarkers are available for clinical testing.
In clinically localized ccRCC, the emphasis has been placed on biomarkers of prognosis expressed in tumor tissue. Some have been found independently prognostic, such as the HIF-1α-regulated hypoxia marker carbonic anhydrase IX [198], the antiapoptotic protein survivin [199-202], the cell proliferation protein KI-67 [203-206], and the immune inhibitory family of ligands B7-H [207-209], but their clinical value is still in question due to lack of independent and prospective validation. IMP3 (one of the insulin-like growth factor II mRNA binding proteins), whose immunohistochemical expression in tumor cells was found associated with short metastasis-free and OS, is a rare exception since findings were subsequently validated in an independent patient cohort [210, 211].

Cytogenetic and gene expression profiling studies have also shown some potential to deliver prognostic information in non-metastatic ccRCC. In mostly small cohorts of patients, specific chromosomal abnormalities have been linked to good (5q gain) or poor prognosis (9p, 14q loss) [212-214]. However, the relation of 9p loss with poor outcome including prognostic value for small renal masses has been repeatedly observed [215, 216], making this a logical candidate to incorporate to available prognostic algorithms. A number of potential biomarkers related to tumor development and progression have additionally emerged from gene expression analyses, several of which have identified gene signatures associated with significant survival differences in patients [217-219]. However, as with the immunohistochemical and cytogenetic markers, these signatures have not yet been validated. Work to validate these gene signatures to predict risk of recurrence is ongoing.

In patients with advanced ccRCC, the availability of effective treatments targeting the VEGF and mTOR pathways has shifted the focus towards the search of biomarkers, predominantly in tumor tissue but also blood, capable of predicting therapy response and resistance. While the analysis of VHL gene status has not resulted in consistent data to support either a prognostic or predictive value [220-224], the activation state of the HIF subunits [69] and multiple HIF-responsive genes are being examined. One HIF target, VEGF, and other angiogenesis-related and
tumorigenic factors in serum or plasma have been evaluated across multiple clinical trials of targeted agents in RCC. It has been established that higher baseline VEGF levels are associated with worse tumor stage and grade, performance status, and overall prognosis [225-230]. Moreover, in a phase III trial of sorafenib vs placebo, patients with VEGF in the highest concentration quartile obtained greater relative benefit from sorafenib than those with lower concentrations [230]. However, studies addressing whether VEGF is a predictive marker for identifying RCC patients likely to benefit from VEGF-targeted therapies have yielded inconsistent results [225, 230, 231]. Preliminary evidence supports the premise that proteomic plasma profiling of cytokines and angiogenic factors (CAFs) in plasma or serum may be used to develop prognostic and predictive biomarkers, and potentially also contribute to molecularly improve RCC classification [232]. Using this approach, two broad groups of metastatic ccRCC patients were identified, one predominantly expressing angiogenesis/hypoxia-related markers and a second one showing an alternative expression of inflammatory markers. Regarding clinical benefit by VEGF inhibitors, a recent study in plasma samples collected in subsequent phase II and III studies of pazopanib identified low concentrations of IL-8, HGF, OPN and TIMP-1 with improved PFS on pazopanib [233]. IL-8 had previously been implicated in resistance to sunitinib [234]. Unfortunately, no biomarkers predictive of differential benefit between available and active drugs in RCC have been validated. In a randomized phase II study of sorafenib vs sorafenib in combination with interferon that yielded no differences in PFS, a candidate 6-CAF signature consistent of markers in the angiogenic/hypoxia group (OPN, VEGF, collagen-IV, soluble CAIX, TRAIL, soluble VEGF receptor-2) predicted for distinct PFS in the 2 arms [232]. Results of similar analyses in larger patient sets are eagerly awaited.

**IMMUNOTHERAPY**

The ability of some renal tumors to evoke an immune response and the possibility that this may lead to spontaneous regression of metastatic renal cell carcinoma (RCC) in some patients have spurred the idea of developing immunotherapy as an effective treatment for patients with RCC [235-237]. Various
immunotherapeutic strategies have been tested with many showing some evidence of activity [238, 239]. Established therapies consist of cytokines such as interferon alpha (IFN) and interleukin 2 (IL-2). IFN was reported to provide a survival benefit in a meta-analysis [240]. HD IL-2 has produced tumor responses in ~10-20% of patients with some patients achieving long-term response off treatment [241-245]. The FDA approved HD IL-2 as treatment for metastatic RCC in 1992 based on Phase II data [241]. However, both IFN and HD IL-2 are associated with substantial toxicities, which have limited their use [246, 247]. In addition, due to the emergence of novel VEGF and mTOR targeted therapies, which are easier to administer, better tolerated and proven to provide clinical benefit in Phase III clinical trials [248, 249], the use of IFN and HD IL-2 as treatment for metastatic RCC has diminished. However, a subset of patients clearly exists who develop significant clinical benefit from immunotherapy. Efforts are ongoing to understand the mechanisms of action and identify predictors of response to cytokine therapies such as IFN and HD IL-2 in an attempt to better select patients for treatment. In addition, novel immunotherapeutic strategies are being developed due to advances in the field of basic immunology, which have provided strong scientific and pre-clinical data to enable successful immunotherapy trials.

Improved understanding of various mechanisms by which T-cell activation can be positively or negatively regulated (Figure 7) has led to the development of agents which can enhance anti-tumor T cell responses. The first such agent, and prototype, is anti-CTLA-4 antibody, which laid the foundation for the development of other immune checkpoint agents such as anti-PD-1 antibody.

Upon engagement of T cell receptor with antigen bound by MHC (signal 1) and co-stimulation provided by CD28 interacting with B7-1 and B7-2 (signal 2), T cells become activated to produce cytokines and proliferate [250, 251]. However, T cell activity must be regulated in order to prevent damage to normal cells and tissues. Therefore, when T cells are turned ‘on”, a series of signals within the cells also generate an “off” mechanism. This “off” switch is known as CTLA-4. CTLA-4 acts to limit T cell responses [252, 253]. The understanding of how CTLA-4 functions led to the idea that an antibody that blocks CTLA-4, thereby temporarily
disengaging the “off” switch, would allow for enhanced T cell responses against tumors. This idea was validated in pre-clinical models [254, 255] and then tested in clinical trials [256-258]. Two Phase III randomized clinical trials were completed and documented a survival benefit for patients with metastatic melanoma who were treated with the anti-CTLA-4 antibody known as ipilimumab (Bristol-Myers Squibb)[259, 260]. Based on these data, ipilimumab was recently FDA-approved in March 2011 as treatment for patients with metastatic melanoma. Since anti-CTLA-4 targets a molecule expressed on T cells, as opposed to a molecule on tumor cells, this therapy is potentially applicable to multiple tumor types.

Anti-CTLA-4 has been evaluated in patients with metastatic RCC. In a Phase II trial, two cohorts of patients with advanced RCC received two different dosing schedules of ipilimumab: a 3mg/kg loading dose followed by either 1mg/kg or 3mg/kg maintenance doses every 3 weeks [261]. Of the 21 patient receiving the 1mg/kg maintenance dose, one patient (4.7%) experienced a partial response. Of 40 patients treated with the 3mg/kg maintenance dose, 5 (12.5%) experienced partial responses. Importantly, responses were observed in patients who had failed prior HD IL-2 suggesting that there is no clear cross resistance. Given its recent FDA approval for melanoma, ipilimumab will likely be investigated further in patients with RCC.

PD-1 is another receptor that is expressed on activated T cells [262]. Interactions with PD-1 and its ligands (PD-L1 and PD-L2) can act to inhibit T cell responses. PD-L1 was shown to be over-expressed in many RCC and greater expression was associated with worse prognosis [263]. MDX-1106 is a monoclonal antibody directed against PD-1 which was recently assessed in Phase I trials including many patients with advanced RCC[264, 265]. Anti-tumor activity was seen in a patient with RCC in the initial trial involving a single dose of the PD1antibody[264]. In a subsequent study [265] , MDX-1106 was administered in doses of 1, 3, and 10mg/kg given every 2 weeks. Of 16 patients with RCC treated at various doses, 5 patients (31%) achieved objective responses, including one complete response. This promising activity coupled with a mild toxicity profile has prompted the initiation of a phase II trial MDX-1106 in patients with advanced RCC.
Over the next several years, agents such as ipilimumab and MDX-1106 will likely be assessed, possibly with cytokines and other therapies, in various sequences and combinations with the goal of achieving higher rates of durable responses than is possible with currently available therapies.

**MOLECULARLY TARGETED THERAPY**

The biology of RCC as elucidated above has led to the development of multiple agents targeting elements of the relevant VEGF and mTOR pathways [266]. Table 1 outlines that major phase III trials of targeted therapy in RCC that have led to regulatory approval of several agents. There are several points to be made about key discriminating features of these agents. VEGF-targeted therapy produces more robust RECIST-defined objective response rates than cytokine therapy, on the order of 30% to nearly 50% for the most active agents. Within the class of VEGF receptor inhibitors, the response rate can vary from 10% to nearly 50%, with the higher rates observed with the drugs that more potently inhibit the VEGF-Receptor. It is also recognized that anti-tumor activity, especially of VEGF-targeting agents, is not entirely captured by size changes alone, as tumor necrosis (reduced perfusion on a contrast-enhanced CT scan) is felt to be indicative of drug effect and may or may not be accompanied by tumor size reduction. mTOR-targeted therapy in general produces more modest response rates of 2-10%, although to date studied in different populations than the VEGF-targeted therapies [267, 268]. When considering the percentage of patients who have at least some tumor burden reduction on therapy (i.e. including patients with 1%-29% reduction not meeting the arbitrary 30% reduction required for a RECIST-defined response), VEGF-targeted therapy shrinks tumors in about 75% of patients while mTOR-targeted therapy shrinks tumors in about 50-60% of patients.

Progression-free survival is generally doubled with targeted therapy compared with placebo/cytokines. Again, here we see important differences among the VEGF-R inhibitors, with the biochemically more potent agents producing a PFS of approximately 11 months in untreated patients, compared to 5 months for the biochemically weaker agent, sorafenib. Axitinib is the most biochemically potent,
but to date results are only available in previously-treated patients [269]. Of note, in the subset of patients not exposed to prior VEGF-targeted therapy in the AXIS trial (the cytokine-refractory subgroup), the median PFS was over 12 months. In regards to PFS, the mTOR-targeting agents have been studied in unique patient circumstances, poor-risk for temsirolimus and VEGF-R TKI-refractory RCC for everolimus [267, 268]. The PFS in each was modest (approximately 5 months), but the effect of these agents front-line in good/intermediate risk patients awaits further study. In addition, the clinical activity of drugs is more robust in untreated patients than in cytokine-refractory, and even less in patients who have already failed targeted therapy. Last, overall survival in these trials is of note for several reasons. The front-line trials of VEGF-targeted agents have produced an OS of approximately 2 years, roughly double that of historical cytokine-treated controls. Nonetheless, no single trial (with the exception of the temsirolimus trial in poor risk RCC) has shown a statistically significant overall survival benefit (despite a numerical advantage in the median OS). This is largely felt to be due to the high percentage of patients who cross over from initial therapy on trial (placebo or cytokine) and receive one or more active targeted therapies at progression. The efficacy of such a sequential ‘salvage’ strategy has confounded interpretation of OS from these trials, although there is general consensus that targeted therapy has meaningfully extended the lives of metastatic RCC patients.

There is no consensus on the ‘best’ drug for initial therapy or the optimal sequence of agents. Ongoing trials are beginning to approach these questions, but the multitude of agents and the relative rarity of RCC make definitive trials not possible. Future research in targeted therapy in RCC is focused on such questions of relative toxicity/efficacy among agents, the importance of switching mechanism at progression, and biomarkers of response and resistance that may allow for improvement upon the current standard of an empiric sequence of monotherapies.

CONCLUSIONS

There has been a clear and important evolution in our understanding of RCC biology. We are now challenged with converting this newly acquired information
into actionable items that will alter our approach to prevention, diagnosis and management of RCC. Several new rare types of cancers are now recognized to occur in the kidney, which will both challenge the urologic oncology community to maintain up-to-date guidelines for the management of these tumors and provide new opportunities to develop effective personalized therapies.

By comparing genomic, transcriptomic and epigenetic data from precursor lesions and early ccRCC, we will be able to establish a roadmap of tumor ontogeny for this more common subtype. Use of material from patients with hereditary VHL disease will be essential to achieve this goal. These data, in conjunction with epidemiological and laboratory based studies, will allow the investigator to identify driver mutations and epigenetic changes, allowing development of markers that permit early identification of ccRCC. In the same manner, study of the cilia centrosome cycle and HIF regulation will build a mechanistic, molecular biological understanding of early cancer development, with resultant opportunities for therapeutic intervention.

In more advanced disease, the study of genomics, transcriptomics and molecular biology will provide investigators with insight into mechanisms of tumor progression, especially if performed in parallel with in vitro and in vivo models employing potential driver pathways identified in ccRCC or other rare variant cancers. The hope is that by understanding both the cause and the consequence of the complex interactions between genomic and epigenetic changes, and assigning significance to the output of these alterations, we will be able to replicate RCC tumor diversity, identify subgroups, and develop more specific therapeutic interventions. To achieve this goal will require coordinated interaction between high-throughput platform experts, molecular biologists and computational scientists capable of controlling and codifying the complex systems that arise from these collaborations. The recognition that the output of these changes is profoundly influence by host genomic and phenotypic characteristics, and that the tumor microenvironment varies as a function of these characteristics requires the development of precise tools to measure the tumor microenvironment.
Finally, the renaissance of tumor immunology was fueled by the recognition that tumors can take advantage of the innate regulatory pathways built into T-cells and other immune effectors. As we begin to understand the impact of tumor biology on T-cell regulation as well as on the recruitment of bone marrow derived immunological precursors, significantly better treatments will become available for patients with ccRCC in the next few years. Understanding the interface between evolving tumor biology and host genomic determinants of stromal end endothelial phenotype will further advance this field.

We are poised to make very significant advances in RCC research in the next few years. With the right team and the right tools, the potential for a truly personalized approach to treatment is within reach.

Acknowledgement
We would like to acknowledge Ruhee Dere for assistance in figure production.
### TABLE 1. Phase III Trials of Targeted Therapy in Metastatic Renal Cell Carcinoma

<table>
<thead>
<tr>
<th>Trial</th>
<th>Number of patients</th>
<th>Clinical setting</th>
<th>RR (%)</th>
<th>PFS (months)</th>
<th>OS (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF-Targeted Therapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>AVOREN</em> Bevacizumab + IFNa vs. IFNa[270]</td>
<td>649</td>
<td>First-line</td>
<td>31 vs. 12</td>
<td>10.2 vs. 5.5 (p&lt;0.001)</td>
<td>23.3 vs. 21.3 (p=0.129)</td>
</tr>
<tr>
<td><em>CALBG 90206</em> Bevacizumab + IFNa vs. IFNa[271]</td>
<td>732</td>
<td>First-line</td>
<td>25.5 vs. 13</td>
<td>8.4 vs. 4.9 (p&lt;0.001)</td>
<td>18.3 vs. 17.4 (p=0.069)</td>
</tr>
<tr>
<td>Sunitinib vs. IFNa[248]</td>
<td>750</td>
<td>First-line</td>
<td>47 vs. 12</td>
<td>11 vs. 5 (p=0.0001)</td>
<td>26.4 vs. 21.8 (p=0.051)</td>
</tr>
<tr>
<td><em>TARGET</em> Sorafenib vs. Placebo[272]</td>
<td>903</td>
<td>Second-line (post-cytokine)</td>
<td>10 vs. 2</td>
<td>5.5 vs. 2.8 (p&lt;0.01)</td>
<td>17.8 vs. 15.2 (p=0.88)</td>
</tr>
<tr>
<td>Pazopanib vs. placebo[273]</td>
<td>435</td>
<td>First line/second line (post-cytokine)</td>
<td>30 vs. 3</td>
<td>9.2 vs. 4.2 (p&lt;0.0001)</td>
<td>22.9 vs. 20.5 (p=0.224)</td>
</tr>
<tr>
<td><em>AXIS</em> Axitinib vs. sorafenib [269]</td>
<td>723</td>
<td>Second line (post-sunitinib, cytokine, bevacizumab or temsirolimus)</td>
<td>19 vs. 9 (p=0.0001)</td>
<td>6.7 vs. 4.7 (p&lt;0.0001)</td>
<td>Not reported</td>
</tr>
<tr>
<td><strong>mTOR-Targeted Therapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ARCC</em> Temsirolimus vs. Tem + IFNa vs. IFNa[249]</td>
<td>624</td>
<td>First line, ≥ 3 poor risk featuresa</td>
<td>9 vs. 5</td>
<td>3.8 vs. 1.9 for IFNa monotherapy (p=0.0001)</td>
<td>10.9 vs. 7.3 for IFNa (p=0.008)</td>
</tr>
<tr>
<td><em>RECORD-1</em> Everolimus vs. placebo[274]</td>
<td>410</td>
<td>Second line (post sunitinib and/or sorafenib)</td>
<td>2 vs. 0</td>
<td>4.9 vs. 1.9 (p&lt;0.0001)</td>
<td>14.8 vs. 14.5</td>
</tr>
</tbody>
</table>

* RCC renal cell carcinoma, RR response rate, OS overall survival, PFS progression free survival, VEGF vascular endothelial growth factor, IFNa interferon alpha,

a Including serum lactate dehydrogenase level of more than 1.5 times the upper limit of the normal range, a hemoglobin level below the lower limit of the normal range; a corrected serum calcium level of more than 10 mg per deciliter (2.5 mmol per liter), a time from initial diagnosis of renal-cell carcinoma to randomization of less than 1 year, a Karnofsky performance score of 60 or 70, or metastases in multiple organs.
FIGURE LEGENDS

Figure 1. A number of histone modifying genes are mutated in renal cell carcinoma. These include the H3K36 trimethylase SETD2, the H3K27 demethylase UTX/KDM6A, the H3K4 demethylase JARID1C/KDM5C and the SWI/SNF complex component PBRM1, shown in this cartoon to represent their relative activities on Histone H3.

Figure 2. VHL proteostasis involves the chaperone mediated translocation of nascent VHL peptide from the ribosome to the TRiC/CCT chaperonin, where folding occurs in an ATP dependent process. The VBC complex is formed while VHL is bound to TRiC, and the mature complex is then released. Three different classes of mutation exist: Class A mutations prevent binding of VHL to TRiC, and abrogate folding into a mature complex. Class B mutations prevent association of Elongins C and B to VHL. Class C mutations inhibit interaction between VHL and HIF1a.

Figure 3. Immunofluorescent images of primary cilia in VHL+ and VHL- cells using ciliary marker alpha-acetylated-tubulin (red) and centrosomal marker anti-pericentrin (green), counterstained for DNA with DAPI (blue). Left panel shows three color merge VHL+ cells; right panel demonstrates the absence of cilia in VHL- cells. Individual cells are color coded by various shades of gray; cilium (red) and associated basal bodies (green) are also segmented by automated image analysis.

Figure 4. Regulation of Prolyl Hydroxylases by Tricarboxylic Acid (TCA) Cycle Intermediates. Prolyl hydroxylases use TCA cycle intermediates to help catalyze the oxygen, iron and ascorbate dependent- addition of a hydroxyl side chain to a Pro402 and Pro564 of HIF alpha subunits, leading to VHL binding and degradation. Defects in either fumarate hydratase or succinate dehydrogenase will drive up levels of fumarate and succinate, which competitively bind prolyl hydroxylases, and prevent HIF prolyl hydroxylation. This results in higher intracellular HIF levels.
Figure 5. HIF regulation and mTOR pathway connections. Hypoxia blocks HIF expression in a TSC1/2 and REDD dependent pathway [155]. HIF1α appears to be both TORC1 and TORC2 dependent, whereas HIF2α is only TORC2 dependent [275]. Signaling via TORC2 appears to upregulate HIF2α in an AKT dependent manner [69].

Figure 6. Different subtypes of clear cell RCC can be defined by HIF patterns as well as by transcriptomic expression as defined by ccA and ccB subtypes. Papillary RCC also demonstrates distinct histological subtypes. A recently described variant denoted as clear cell papillary RCC is VHL wildtype (VHL WT), while other clear cell tumors are characterized by VHL mutation, loss, or inactivation (VHL MT).

Figure 7. Immune regulation of renal tumor cells. A: When an antigen presenting cell (APC) engages a T-cell via a cognate T-cell receptor (TCR) and CD28, T-cell cell activation occurs. B: Early and late T-cell inhibitory signals are mediated via CTLA-4 and PD-1 receptors, and this occurs via engagement of the APC via B7 and PD-L1, respectively. C: Inhibitory antibodies against CTLA-4 and PD-1 can overcome T-cell downregulation and once again allow cytokine production.
REFERENCES


89. Ding, Z., et al. High throughput screening to identify molecules that alter VHL stability


231. Escudier, B.J., et al., Update on AVOREN trial in metastatic renal cell carcinoma (mRCC): efficacy and safety in subgroups of patients (pts) and pharmacokinetic (PK) analysis. J Clin Oncol, 2008 (suppl; abstr 5025). 26: p. 15S.


Figure 1. Overview of Histone 3 modifications implicated in RCC genetics.
Figure 2. pVHL mutants are categorized as Class A, B and C depending on the affected step in pVHL protein quality control

Class A: mutations in pVHL box 1 or 2 required for TriC binding (G114R, A149P)

Class B: mutations that inhibit the interaction between pVHL and Hsp90/Hsp82, Sti1/HOP, Sse1/Hsp110 (L158P, C162S)

Class C: mutations that inhibit the interaction between pVHL and HIF-1α (Y98H, Y112H)

SCF ubiquitin ligase complex

E2 ligase pairs ubc4/5, ubc6/7

26S proteasome

Loss of pVHL

(VBC complex=pVHL, elongins B and C)

Hsp90/Hsp82, Sti1/HOP, Sse1/Hsp110

TriC/CCT

26S proteasome

Loss of pVHL

(Adapted from Feldman et al. Molecular Cell. 2003.)
Figure 3. Immunofluorescent images of primary cilia in VHL+ and VHL- cells
Figure 4. Regulation of Prolyl Hydroxylases and Keap1 by Krebs cycle.
Figure 5. Regulation of mTORC1.
Figure 6. Subtypes of clear cell and papillary RCC, and a new subtype, clear cell papillary.

- Clear Cell RCC
  - VHL MT
    - HIF1/HIF2
      - ccA
      - ccB
    - HIF2
  - VHL WT

- Clear Cell Papillary RCC

- Papillary RCC
  - Papillary Type I
  - Papillary Type II
Figure 7. T cell regulation in RCC

A. Cytokines produced

B. Early Inhibitory Signals

C. Antibodies to block inhibitory signals: e.g. anti-CTLA-4, anti-PD-1
State-of-the-science: An update on renal cell carcinoma

Eric Jonasch, Andrew Futreal, Ian Davis, et al.

Mol Cancer Res  Published OnlineFirst May 25, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-12-0117

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.