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Genetic and metabolic hallmarks of clear cell renal cell carcinoma

Danielle J. Sanchez^{1,2} and M. Celeste Simon^{1,2,*}

¹Abramson Family Cancer Research Institute, 456 BRB II/III, 421 Curie Boulevard, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104-6160, USA

²Department of Cell and Developmental Biology, 456 BRB II/III, 421 Curie Boulevard, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104-6160, USA

Abstract

Clear cell renal cell carcinoma (ccRCC) is a malignancy characterized by deregulated hypoxia-inducible factor signaling, mutation of several key chromatin modifying enzymes, and numerous alterations in cellular metabolism. Pre-clinical studies have historically been limited to cell culture models, however, the identification of critical tumor suppressors and oncogenes from large-scale patient sequencing data has led to several new genetically engineered mouse models with phenotypes reminiscent of ccRCC. In this review, we summarize recent literature on these topics and discuss how they inform targeted therapeutic approaches for the treatment of ccRCC.

Keywords

Cancer; hypoxia-inducible factors; genetics; mouse models; lipid metabolism; therapy

1. Introduction

Kidney cancer is the 8th most prevalent cancer diagnosed each year in the United States, with an estimated 64,000 new cases in 2017 [1]. The median age at diagnosis for both sexes is 64, and total overall incidence rate has been increasing over the past decade. Clear cell renal cell carcinoma (ccRCC) accounts for 70–80% of kidney cancer diagnoses, and represents the most aggressive subtype of this disease [2]. Risk factors for ccRCC include smoking, obesity, von Hippel-Lindau (VHL) disease (defined below), and hypertension. If disease is detected early and still localized within the kidney, surgical resection or nephrectomy is performed with a 5-year survival rate of ~92%. However, if the tumor has spread locally or systemically, 5-year survival rates drop to 67% or 12%, respectively, due to limitations in current standard-of-care therapies for metastatic disease [1].

A defining morphological hallmark of ccRCC is robust lipid and glycogen accumulation in the cytoplasm of tumor cells, giving rise to the name “clear cell”. Widespread metabolic reprogramming in glucose, lipid, and amino acid metabolism broadly contributes to the clear

*To whom correspondence should be addressed: Dr. M. Celeste Simon (celeste2@pennmedicine.upenn.edu, Office: 215-746-5532).

cell phenotype [3], although the advantages conferred unto tumor cells harboring these adaptations are incompletely understood. Compared with other solid tumors driven by loss of classical tumor suppressors or activation of oncogenes (e.g., those encoding p53 and K-Ras), ccRCC has been labeled as a “metabolic disease” supported by alterations in myriad bioenergetic pathways [4], [5]. Genetic hallmarks include biallelic inactivation of the *VHL* tumor suppressor gene, the negative regulator of hypoxia-inducible factor (HIF) proteins, as well as copy number alterations of chromosome 3p, 5q, and 14q genes, and high frequency of mutation in chromatin modifying enzymes (e.g., *PBRM1*, *SETD2*, and *BAP1*). Here, we discuss recent advances in ccRCC research related to elucidating the underlying genetic drivers of human disease, progress on obtaining an autochthonous, genetically engineered mouse model (GEMM) for pre-clinical studies, metabolic reprogramming, and therapeutic strategies moving forward.

2. Genetics of ccRCC

2.1 VHL loss and HIF- α stabilization

The most common genetic event of ccRCC is copy number deletion, inactivating mutation, and/or epigenetic silencing of *VHL* (encoding pVHL), the recognition component of an E3-ubiquitin ligase complex responsible for targeting HIF-1 α and HIF-2 α for proteasomal degradation under normoxic conditions (Figure 1). VHL disease is a familial disorder marked by increased propensity for ccRCC, retinal and cerebellar hemangioblastoma, and pheochromocytoma development. In hereditary disease, patients inherit only one functional copy of the *VHL* gene, and subsequent loss of heterozygosity (LOH) results in disease [6]. pVHL loss results in constitutive stabilization of HIF- α subunits even under oxygen-replete conditions, which translocate to the nucleus and heterodimerize with HIF-1 β (ARNT) [7]. HIFs transcriptionally activate numerous genes involved in cellular processes including glycolysis, angiogenesis, and metastasis of cancer cells [8]. Notably, while *VHL* inactivation occurs in upwards of 90% of all patients, its loss alone is insufficient to generate ccRCC tumors [9], [10]. Characterization of HIF- α protein accumulation in ccRCC has revealed distinct patterns; ~10% retain wildtype *VHL* and express neither HIF-1 α nor HIF-2 α , ~60% of tumors express both HIF-1 α and HIF-2 α , and ~30% express HIF-2 α alone [11]. This can occur as a result of loss of chromosome 14q as disease advances, on which the *HIF1A* gene resides [12], [13]. Indeed, HIF-1 α has been linked with tumor suppressive functions in ccRCC [13], [14], while HIF-2 α has been established as a dominant oncogenic driver of disease progression [15], [16], [17], [18]. These observations have supported efforts to develop small molecule antagonists of HIF-2 α , which are currently being tested in clinical trials for the treatment of metastatic ccRCC [19], and will be discussed later.

2.2 Copy number and single nucleotide variation

Recent sequencing studies involving large cohorts of ccRCC patients have revealed signatures of copy number amplification and deletion across the tumor genome [20], [21]. A 43 megabase region of chromosome 3p contains multiple *bona fide* or putative tumor suppressor genes including *VHL*, *PBRM1*, *BAP1*, and *SETD2* (discussed below) [22]. Mechanistically, this gene inactivation occurs on one allele through intergenic point mutation, and on the second allele through LOH [23], [24]. On a genome-wide scale, copy

number variation of the following regions are most abundant in ccRCC tumors: chromosome 3p loss (91%), 5q gain (67%), and 14q loss (49%) [25].

While chromosome 3p and 14q genes are generally associated with having tumor suppressive functions in ccRCC, copy number amplification of a ~60 gene region of chromosome 5q35 harbors candidate oncogenes [21], [25]. *SQSTM1*, which encodes the p62 protein involved in activation of NRF2 and resistance to oxidative stress, was shown to be a chromosome 5q gene frequently amplified in ccRCC patient samples and cell lines [26]. Suppression of p62 decreased resistance to redox stress and soft agar colony formation *in vitro* and xenograft tumor growth *in vivo*. Additionally, siRNA-mediated suppression of *SQSTM1* reduced mechanistic target of rapamycin (mTOR) signaling, a key nutrient sensing pathway involved in the regulation of cell proliferation, protein synthesis, and autophagy, suggesting multiple mechanisms by which p62 promotes ccRCC progression [27]. Other work has implicated the chromosome 5q genes *EZH2*, *STC2*, and *VCAN*, which were all copy number gained and overexpressed at the mRNA level, as having oncogenic functions in ccRCC [21], [28]. These data suggest that the chromosome 5q hotspot of gene duplication may contain several clinically relevant targets.

Genome-wide association studies (GWAS) have also been conducted in ccRCC patients to illuminate single nucleotide variants predisposing individuals to the disease. A 2017 study identified seven new susceptibility loci, including a single nucleotide polymorphism (SNP) within a predicted intronic enhancer of *DPF3*, a histone acetylation and methylation reader protein of the BRG1-associated factor (BAF) 180 and polybromo-BAF (PBAF) chromatin remodeling complexes [29]. Interestingly, the location of this SNP (14q24) is deleted in one quarter to half of patients [30], and dysregulation of other components of BAF and PBAF complexes are common features of ccRCC. The same group also identified *BHLHE41*, a gene encoding a basic helix-loop-helix protein located in the 12p12.1 susceptibility locus, from GWAS [31]. The ccRCC risk allele associated with this gene increased its expression, and the authors provided functional evidence that ectopic expression of *BHLHE41* increased xenograft tumor growth.

An earlier GWAS identified two SNPs associated with RCC susceptibility within a 4.2 kb region of the first intron of *EPAS1* (encoding HIF-2 α) as well as another at 11q13.3, which is not localized intergenically but flanks *MYEOV* and *CCND1* (encoding cyclin D1) [32]. Additional work demonstrated that the risk SNP at this *CCND1* enhancer promoted HIF-2 α binding, thereby increasing the mRNA expression of this oncogenic cell cycle regulator [33]. The variants within *EPAS1* are also notable as HIF-2 α inhibition has been repeatedly demonstrated to reduce ccRCC growth [17], [18], [34]). However, the functional relationship between intronic *EPAS1* SNPs and its mRNA expression was not determined. A study of familial renal cell carcinoma identified mutations in the *CDKN2B* gene through exon sequencing as predisposing individuals to tumor development [35]. *CDKN2B* encodes the p15^{INK4B} protein, which normally functions as a tumor suppressor by binding and inhibiting cyclin-dependent kinases 4 and 6 to prevent cell cycle progression [36]. These mutations, which germline-inactivated *CDKN2B* in 5% of patients, were predicted to destabilize the interaction between p15^{INK4B} and the CDKs. Furthermore, expression of wildtype p15^{INK4B}

in vitro was shown to suppress colony formation relative to the mutant isoforms, providing support for its ability to act as a tumor suppressor in ccRCC.

Perhaps not unexpectedly, subclonal heterogeneity is a common feature within ccRCC tumors [24]. Such variation manifests as spatially separated mutational patterns and chromosomal imbalances, which create a range of intratumoral phenotypes. For example, gene expression patterns associated with both good and poor prognoses can be found within the same tumor, and roughly 2/3rd of all somatic mutations are not detectable in every sequenced region [23]. The degree to which clonal heterogeneity affects disease progression and informs treatment strategies will be an important consideration for both researchers and clinicians moving forward. Nevertheless, additional studies on copy number and single nucleotide variation may help elucidate the etiology of ccRCC, as well as identify new therapeutic targets of interest.

2.3 Histone and chromatin modifying enzyme mutations

After mutation of *VHL* (53% of patients), *PBRM1*, *SETD2*, and *BAP1* are the most commonly mutated genes in ccRCC at 40%, 13%, and 10% of patients, respectively [37]. *PBRM1* encodes BAF180, which is the defining subunit of the PBAF switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex which uses ATP for nucleosome repositioning [38]. Varela, *et al.* demonstrated that suppression of *PBRM1* through siRNA resulted in enhanced proliferation, colony formation, and migration of several ccRCC cell lines *in vitro*, consistent with a tumor suppressive function in renal carcinoma [38]. Interestingly, Gao, *et al.* did not recapitulate these *in vitro* findings, but found that *PBRM1* suppression by CRISPR did promote subcutaneous tumor growth in an on-target fashion [39]. Inactivating or truncating mutations in *JARID1C* (also known as *KDM5C*), a histone H3 lysine 4 demethylase, and *UTX* (KMD6A) a histone H3 lysine 27 demethylase were identified in another study as positively selected for in ccRCC compared to other cancers [40], [41]. Depletion of *SETD2*, the histone H3 lysine 36 tri-methyltransferase catalyzing methylation at sites of active transcription, was associated with DNA replication stress and reduced loading of nucleosome components onto chromatin [42]. The authors speculated that *SETD2* loss of function during disease progression could be a source of genomic instability and heterogeneity within ccRCC. *BAP1*, a deubiquitinating enzyme involved in transcriptional repression of genes as a polycomb-group protein, exhibits mutations that are largely mutually exclusive with *PBRM1* [43]. How hypermethylation of enhancer and promoter CpG regions affects ccRCC progression is an ongoing area of investigation in the field [44].

3. Genetic mouse models of renal cell carcinoma

3.1 Tumor suppressor deletion

As *VHL* deletion is insufficient to drive ccRCC tumorigenesis [45], conditional knockout mice have been generated in several laboratories combining deletion of candidate tumor suppressor genes based on large-scale patient sequencing data [25], [46]. A recent report combined *Vhl* and *Pbrm1* deletion throughout renal tubules, collecting ducts, and thick ascending limbs using *Ksp-Cre* [47], and found that 67% of these mice first developed

polycystic kidney disease (PKD) within 10–14 months of age [48]. While deletion of either gene alone did not result in PKD or ccRCC, 50% of the double knockout (*Vhl*^{-/-}, *Pbrm1*^{-/-}) mice showed signs of tumor incidence after 10 months of age, with clear cell morphology, activated mTOR signaling, and reduction in oxidative phosphorylation gene signatures reported. Notably, while the primary tumors were orthotopically transplantable into NSG mice, metastatic tumor burden was not detected in *Vhl*^{-/-}, *Pbrm1*^{-/-} mice.

Though *TP53* deletion or mutation is relatively uncommon in human ccRCC tumors, another study examined the effect of conditional *Vhl*, *Trp53*, and *Rb1* loss in the renal epithelium (utilizing *Ksp-Cre*), as changes in regulators of the p53 pathway and the G1/S cell cycle transition are frequently copy number modified according to The Cancer Genome Atlas (TCGA) data [49]. The authors found evidence of tumor onset as early as 7.5 months following triple knockout in a subset of mice, which were characterized by HIF- α and mTORC1 pathway activation by carbonic anhydrase 9 and phospho-4E-BP1 immunostaining, respectively. Further analyses of a larger cohort suggested 82% of *Vhl*^{-/-}, *Trp53*^{-/-}, *Rb1*^{-/-} mice developed tumors within 15 months of gene deletion, the majority of which were classified as grade 3 or 4 tumors growing in acinar or pseudo-papillary patterns. These renal tumors had clear or weakly stained cytoplasm, and displayed gene expression and mutational signatures similar to human ccRCC. While some *Vhl*^{-/-}, *Trp53*^{-/-}, *Rb1*^{-/-} tumors were sensitive to 1st and 2nd line chemotherapies sunitinib and everolimus, nearly all were resistant to 3rd line treatment with acriflavine, the pan-HIF- α inhibitor [50]. These results mimic the variability in drug responsiveness typical of the human disease. Similar to *Vhl* and *Pbrm1* co-deletion, *Vhl*^{-/-}, *Trp53*^{-/-}, *Rb1*^{-/-} mice developed tumors which remained localized within the kidney.

Consistent with chromosome 3p genes displaying tumor suppressive functions in ccRCC, the effect of *Bap1* deletion in combination with either *Vhl* or *Pbrm1* loss has been examined in several publications from the Brugarolas group. Under the control of *Six2-Cre* driver, which is expressed in multipotent nephron progenitor cells of the kidney during development, the authors first generated *Vhl*^{-/-}, *Bap1*^{+/-} mice (deletion of both alleles of *Vhl* and one of *Bap1*) [51]. These mice developed kidneys with atypical cysts and neoplasia resembling those of patients with VHL syndrome. Some of the lesions observed were similar to early-stage ccRCC tumors by cytoplasmic clearing, Ki67 expression, and phospho-S6 positivity as a marker of mTOR activity, yet tumors remained small overall. Again, tumors from this mouse model did not metastasize, and *Vhl*^{-/-}, *Bap1*^{+/-} mice died after 8 months from renal failure, suggesting that additional events are required for ccRCC progression.

A more recent study from this group used additional Cre drivers to modulate *Pbrm1* and *Bap1* levels in different lineages within the kidney. *Pax8-Cre* was used to delete *Vhl* and *Bap1* or *Pbrm1* within the proximal and distal renal tubules, loops of Henle, collecting ducts, and parietal cells of the Bowman capsule [52]. *Sglt2*- and *Villin-Cre* were also used for more specific deletion within proximal tubule epithelial cells. Tumors generated with *Pax8-Cre* were larger than those generated with the *Six2-Cre* model investigated previously. Interestingly, deletion of both alleles of *Vhl* along with a single allele of either *Pbrm1* or *Bap1* using *Sglt2*- and *Villin-Cre* drivers did not result in ccRCC formation. Based on these

data, the authors speculated that ccRCC may be derived from the parietal epithelial cells of the Bowman capsule, rather than proximal tubule cells, as previous histologic and gene expression data would suggest [53], [54]. The authors also found that activation of mTORC1 signaling through inactivation of one allele of *Tsc2* caused the formation of higher grade ccRCC specifically in *Pbrm1*-deficient kidneys. Notably, patients with *PBRM1* and *BAP1* mutations have distinct gene expression patterns and clinical outcomes, and these alterations are largely mutually exclusive [55]. While *BAP1*-deficient tumors are higher in grade and portend worse prognosis than *PBRM1*-deficient tumors, it is believed that *BAP1* mutations are secondary to *PBRM1* loss in the pathogenesis of ccRCC [56], [57].

3.2 Oncogene activation

An early mouse model of ccRCC was generated using a constitutively active mutant of HIF-1 α , termed TRACK (transgenic model of cancer of the kidney) [58]. HIF-1 α protein is found in *VHL*-deficient renal epithelial cells and in early tubular lesions, cysts, and ccRCC, suggesting it is important for carcinogenesis [45]. TRACK mice expressed a triple-mutant HIF-1 α , which was insensitive to hydroxylation and therefore degradation by the PHD/pVHL system, under the control of the γ -glutamyl transferase (*GGT*) proximal tubule-specific promoter. Phenotypes included abnormal vascularization, renal cysts, cleared cells, and high carbonic anhydrase 9 staining. A small percentage of cleared cells accumulated γ H2AX, a marker of DNA double-strand breaks, although the mechanism by which HIF-1 α led to genomic instability was not described. Even though HIF-1 α exerts tumor suppressive functions in established ccRCC, this study demonstrates it is a critical downstream mediator of *VHL* loss early in disease progression. Accordingly, neither late-stage nor metastatic disease was detected in TRACK mice.

Another two studies investigated the role of MYC overexpression in the kidney and its role in promoting renal tumorigenesis. According to TCGA data, genomic amplification of *MYC* only occurs in about 5–10% of patients, however, the MYC/MAX transcriptional network has been predicted to promote glycolysis, de-differentiation, and growth in a majority of ccRCC [59]. MYC pathway activation is also associated with a hereditary RCC characterized by translocation of chromosomes 3 and 8 [60]. Conditional overexpression of *MYC*, but not *KRAS*, under the *GGT* gene promoter gave rise to a highly aggressive RCC that most resembled collecting-duct carcinoma [61]. Combined gene expression and metabolite analysis, along with small molecule inhibition of glutaminase, revealed a dependency on glutamine for sustained growth of these tumors, similar to the human disease.

In another report, the combination of MYC overexpression along with further deletion of *Vhl* and/or *Cdkn2a* (encoding Ink4/Arf) was investigated for the ability to induce papillary or clear cell renal cell carcinoma [62]. This study utilized the *Ksp* promoter to drive expression of a doxycycline-inducible Myc transgene. When MYC was overexpressed without deletion of either *Vhl* or *Cdkn2a*, the mice developed tumors histologically consistent with papillary RCC. However, *Vhl* deletion in combination with MYC overexpression (“VM”) mice generated tumors more similar to ccRCC based on cytoplasmic clearing, necrosis, and hemorrhage. Addition of *Cdkn2a* deletion to this model (“VIM”)

further potentiated ccRCC formation compared with *Vhl* deletion alone, increasing tumor volume and decreasing median survival time. VIM mice had a median survival of 29.5 weeks following gene inactivation/induction, whereas, VM mice had a median survival of 57 weeks. Importantly, 2 out of 6 VIM mice developed metastasis to the liver, and *in vitro* characterization of a cell line derived from this mouse model showed increased expression of epithelial-mesenchymal transition genes as well as increased matrigel invasion relative to a VM line.

ccRCC research is currently limited by the lack of a GEMM that accurately represents the complete spectrum of phenotypes characteristic of human disease. As evidenced by the publications described above, the various methods used to either delete tumor suppressors and/or express oncogenes in distinct kidney lineages result in different propensities for ccRCC development. Furthermore, the long latency of tumor formation in GEMMs currently available, as well as lack of metastatic spread typical of human ccRCC, poses challenges for researchers studying the molecular mechanisms governing these events. In the meantime, identification of subpopulations of established patient-derived cell lines having metastatic capacity [63] and incorporation of patient-derived xenografts will allow the field to investigate these phenomena in greater detail. Additional GEMMs mimicking the initiation and progression of ccRCC will ultimately be beneficial for the pre-clinical study of targeted therapy. A deeper understanding of the genetic, epigenetic, and metabolic changes that synergize with *VHL* loss to generate ccRCC, and the order in which they occur, will ultimately facilitate the development of such tools.

4. Metabolic reprogramming in ccRCC

4.1 Glucose utilization

Aerobic glycolysis, commonly known as the “Warburg effect,” is a hallmark of many cancers regardless of cell-of-origin [64], and this phenomenon is amplified in *VHL*-deficient ccRCC due to HIF activation. A primary consequence of constitutive HIF activation is decreased pyruvate entry into the TCA cycle and increased glycolytic flux towards lactate due to the actions of pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase (LDHA), respectively [3]. It is believed that enhanced glycolysis benefits tumors through several mechanisms, one being the generation of building blocks for nucleotides, proteins, and lipids through pathways branching from glycolytic intermediates [65]. The pentose phosphate pathway, beginning with glucose-6-phosphate and generating NADPH and ribose 5-phosphate, is important for lipid and nucleotide production. Additionally, NADPH produced from these reactions can be used to reduce oxidized glutathione, helping to maintain antioxidant capacity in rapidly proliferating cells. The hexosamine pathway, beginning with fructose 6-phosphate, is important for the generation of precursors used in *N*-glycosylation of proteins. These modifications are important for the proper folding of growth factor and nutrient receptors, which feeds back to regulate cellular metabolism more broadly [66]. Although an inefficient means of ATP generation relative to oxidative phosphorylation, aerobic glycolysis in cancer cells occurs at a rate much higher than in non-proliferative cells [67]. An equivalent rate of ATP synthesis along with the additional

generation of anabolic precursors and reducing equivalents is a proposed explanation for why tumors rewire metabolism to promote the Warburg effect.

In addition to their canonical, catalytic functions, several reports demonstrate that certain glucose metabolic enzymes exhibit dual functions through physical association with HIFs [68], [69]. The HIF-1 α target gene pyruvate kinase (*PKM*) encodes isoforms PKM1 and PKM2, of which PKM2 is a key regulator of glycolytic flux. The Semenza group showed that in addition to its catalytic function, PKM2 can bind to HIF-1 α as a co-activator and enhance p300 recruitment and localization to hypoxia response elements [68]. Additionally, our group demonstrated that the gene encoding the gluconeogenic enzyme fructose-1,6-bisphosphatase 1, FBP1, is uniformly silenced or deleted in ccRCC patients [70]. Loss of FBP1 promotes ccRCC progression by two distinct mechanisms; first, by promoting glycolytic flux in renal epithelial cells, and secondly, by dissociation with the HIF- α inhibitory domain, facilitating constitutive HIF activity at the chromatin-level. Further research exploring the relationship between HIFs and their cell type-specific transcriptional targets will provide insight into the complex regulation of metabolism in ccRCC.

4.2 PI3K-Akt-mTOR pathway

The phosphoinositide 3-kinase (PI3K) signaling pathway is activated by copy number variation or mutation of its positive effectors *PIK3CA*, *MTOR*, and negative regulators *PTEN* and *TSC1/2* in 28% of patients in ccRCC [25], [37]. PI3K activation activates Akt, also known as protein kinase B, by phosphorylation at the cell surface. One of the major downstream targets of activated Akt is mTORC1, which increases anabolic pathways such as protein, lipid, and nucleotide synthesis [71]. Inactivation of the negative regulators of mTORC1, TSC1 and TSC2, has been associated with increased risk of ccRCC development, which presents at an earlier age than sporadic disease [72]. Collectively, these effects render the pathway constitutively active even in the absence of growth factors. The effects of altered glucose and amino acid metabolism on ccRCC pathogenesis was comprehensively reviewed in 2017 [3], as such, we will focus on reprogrammed lipid metabolism in the remainder of this section.

4.3 Lipid metabolism

As renal proximal tubule epithelial cells (RPTEC) transition into ccRCC, they undergo dramatic rewiring of various metabolic pathways including lipid utilization, synthesis, and storage (Figure 2). Healthy RPTEC are characterized by high rates of beta-oxidation to fuel their primary role in ion exchange and secretion [73]. Beta-oxidation is the process by which lipids are broken down into acetyl-CoA, generating the reducing equivalents NADH and FADH₂ to produce ATP through oxidative phosphorylation. Compared to RPTEC, ccRCC have reduced mitochondrial content and defective mitochondrial structure and activity [74], [75]. At the gene expression level, transcriptional regulators of mitochondrial homeostasis and oxidative phosphorylation are uniformly suppressed in ccRCC relative to adjacent healthy kidney tissue [76]. These adaptations are largely due to constitutive HIF signaling, although additional factors regulating these pathways remain to be explored. One study examined the relationship between HIF- α and PGC-1 α , a peroxisome proliferator-activated receptor gamma (PPAR γ) transcriptional co-activator involved in the process of

mitochondrial biogenesis and respiration [77]. *VHL*-deficient ccRCC cells exhibit elevated levels of Dec1, a transcriptional repressor and HIF- α target gene, which blocks expression of PGC-1 α [78]. Ectopic expression of PGC-1 α increased oxidative stress and slowed tumor growth in a subcutaneous xenograft model. Furthermore, lower levels of *PPARGC1A* (PGC-1 α) mRNA were correlated with worse patient survival, and total mitochondrial content was correlated inversely with tumor grade [79], consistent with its role as a tumor suppressor in ccRCC.

Transcriptional repression of carnitine palmitoyltransferase (*CPT1A*), the rate limiting enzyme involved in fatty acid transport into the mitochondria for beta-oxidation, also occurs in ccRCC via a HIF- α dependent mechanism [80]. A recent report found that pVHL reconstitution in several ccRCC cell lines reduced lipid droplet (LD) accumulation in a CPT1A dependent manner (i.e., knockdown of *CPT1A* in pVHL-reconstituted cells partially increased lipid accumulation). Interestingly, pVHL status had no effect on lipid uptake rates, as measured through the uptake of BODIPY, an unsaturated, fluorescently labeled fatty acid molecule. Ectopic expression of CPT1A increased oxygen consumption rate, decreased LD abundance, and decreased xenograft tumor growth. Clinically, CPT1A levels and activity are reduced in ccRCC compared with healthy kidney tissue, and patients with reduced *CPT1A* expression have worse prognosis.

Concomitant with decreased beta-oxidation in ccRCC tumors is increased lipid synthesis and storage. Fatty acid synthesis occurs when cytosolic citrate is carboxylated by the enzyme ATP citrate lyase, or ACLY, to produce acetyl-CoA. Carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC) generates the building blocks for long-chain fatty acid formation which can go on to be desaturated and further modified to produce more complex lipid species. Two key enzymes in this process are fatty acid synthase (FASN), which produces palmitic acid (C16:0), and stearoyl-CoA desaturase 1 (SCD1), which primarily produces oleic acid (C18:1) from the desaturation of stearic acid. In ccRCC, metabolism of glutamine-derived α -ketoglutarate to citrate by reductive carboxylation fuels lipid synthesis rather than glucose-derived citrate generated by oxidation [81], [82]. In addition to increasing anabolic lipid metabolism for the production of membranes needed during rapid cell proliferation, new evidence suggests additional roles for *de novo* lipogenesis (DNL) and lipid uptake in carcinogenesis. FASN levels correlate positively with tumor aggressiveness and poor survival in ccRCC [83] [84], and genetic and pharmacologic inhibition of SCD1 induces apoptosis in ccRCC cell lines both *in vitro* and *in vivo* [85]. Treatment with ACC inhibitor reduces total phospholipid and triglyceride content, as predicted, and also increases sensitivity to oxidative stress-induced cell death [86]. Other work has demonstrated that lipid uptake protects against endoplasmic reticulum (ER) stress in ccRCC and other cancers, particularly under hypoxia when cells cannot desaturate fatty acids due to the inactivation of SCD1 [87], [88].

The protective effect of LD accumulation in cancer cells is incompletely understood, although recent studies suggest several mechanisms by which LDs can enhance tumorigenesis and cell viability. LDs consist of a core of neutral lipids including triglyceride and cholesterol esters surrounded by a monolayer of membrane from the endoplasmic reticulum and coat proteins including the perilipin family [89]. Inhibition of the LD coat

protein perilipin 2 in ccRCC, a highly-expressed HIF-2 α target gene, reduced neutral lipid accumulation, tumor growth, and increased ER stress both *in vitro* and *in vivo* [34]. In glioblastoma and breast cancer, it was recently demonstrated that lipid accumulation following hypoxia-reoxygenation protects cells from reactive oxygen species (ROS)-induced cytotoxicity [90]. In another hypoxic microenvironment, the *Drosophila* neural stem cell niche, LDs that form during oxidative stress reduce ROS levels and inhibit the oxidation of polyunsaturated fatty acids (PUFA) [91]. When PUFA are stored in LDs rather than in membranes, they are less vulnerable to peroxidation, which inhibits proliferation through damaging macromolecules. In theory, these responses are even more critical to maintain in ccRCC, where lipid accumulation is a defining molecular and morphological hallmark of disease. Taken together, these results suggest pathways involving anabolic lipid metabolism could one day be targeted for therapeutic benefit in ccRCC.

In a recently published lipidomic analysis of 49 patients [92], elevated levels of ether-type phospholipids, cholesterol esters, and triacylglycerols were reported in ccRCC, consistent with previously published metabolomic analyses [93], [94], [95]. These metabolic changes were also correlated with gene expression levels of lipogenic genes. While it is clear that suppression of either HIF-1 α or HIF-2 α markedly reduces LD formation in ccRCC [96], [34], [80], the target genes and mechanisms responsible for the clear cell phenotype have not been completely defined. Additionally, the role of other lipogenic transcription factors such as sterol regulatory element-binding protein 1 or 2 (SREBP1/2), PPAR γ , and carbohydrate-responsive element-binding protein (ChREBP) remain to be fully elucidated in ccRCC.

5. Therapeutic strategies for ccRCC

5.1 Anti-angiogenics and mTOR inhibitors

Anti-angiogenic therapy targeting receptor tyrosine kinases (RTKs) such as vascular endothelial growth factor receptor (sunitinib, sorafenib, etc) or VEGF ligand by monoclonal antibody (bevacizumab) has traditionally been a first-line standard-of-care for ccRCC patients [97] (a graphical representation of the therapies reviewed in this section is presented in Figure 3). Although these agents are being investigated for several malignancies including colorectal and lung cancers, anti-angiogenics are theoretically well-suited for the treatment of ccRCC due to constitutive HIF activity and exceedingly high VEGF accumulation. However, clinical trials indicate that at least as single-therapy, anti-angiogenic drugs are only modestly effective for the treatment of metastatic ccRCC. In a randomized, double-blind trial of bevacizumab, the probability of being progression-free after eight months was significantly extended by 30% (high-dose), 14% (low-dose), and 5% (placebo) [98]. However, there was no significant difference in overall survival between placebo, low, or high-dose bevacizumab. A phase III study of sunitinib in patients with high risk of tumor recurrence after nephrectomy revealed that 64.9% of patients were disease-free at three years after adjuvant treatment with sunitinib compared with 59.5% in the placebo group [99]. At five years, the proportions fell to 59.3% (sunitinib) and 51.3% (placebo). Side effects from sunitinib treatment included diarrhea, hypertension, fatigue, and nausea.

Rapamycin analogs, or rapalogs (everolimus, temsirolimus), are used for the treatment of advanced ccRCC after patients have been treated with a RTK inhibitor. A phase III study in

patients whose disease progressed after receiving either RTK- or VEGF-targeted therapy found that everolimus was effective in prolonging progression-free survival compared with placebo control [100]. Side effects from everolimus included hyperglycemia, hyperlipidemia, and hypercholesterolemia due to effects on mTOR-regulated glucose and lipid metabolism, as well as diarrhea and rash. Yet overall, the risk of severe effects was low and no detrimental effects on health-related quality-of-life was observed in everolimus treated individuals compared with placebo. The clinical trials reported above led to FDA approval for angiogenesis and mTOR inhibitors nearly a decade ago, and current investigation has now shifted toward targeted therapy of HIF-2 α as well as immune checkpoint blockade.

5.2 Targeting HIF-2 α

HIF-2 α is the primary oncogenic driver of ccRCC progression downstream of *VHL* loss. Pre-clinical work from several labs has examined the efficacy of a HIF-2 α inhibitors utilizing a panel of ccRCC cell lines in orthotopic xenograft assays as well as patient-derived xenografts grown subcutaneously [101], [102], [103]. PT2399 blocks the interaction between HIF-2 α and HIF-1 β /ARNT by binding to a pocket within the PAS-B domain of HIF-2 α , thereby eliminating DNA binding. Importantly, PT2399 reduces HIF-2 α target gene expression without affecting HIF-1 α targets [102]. When tested alongside sunitinib, PT2399 decreased tumor growth by 60% across all samples tested compared with 40% for sunitinib treatment, without causing a reduction in body weight as seen with the latter drug [103]. Resistance mechanisms were observed in tumors initially sensitive to PT2399, which were characterized by increased tumor vascularity and VEGF production despite dissociation between HIF-2 α and ARNT. However, they took nearly double the amount of time to develop as tumors treated with sunitinib. Based on these results, a derivative of PT2399, PT2385, is currently being tested in clinical trials for ccRCC [104].

5.3 Targeting glutaminase

Since being reviewed by Wettersten, *et al.* [3], two phase II clinical trials have been initiated to examine the effect of CB-839, a small molecule inhibitor of glutaminase, along with either cabozantinib, everolimus, or placebo, on patients with advanced or metastatic RCC [105]. Additionally, a pre-clinical report on the role of glutamine addiction in ccRCC further supports the possibility of therapeutically targeting this axis in kidney cancer [106]. Aboud, *et al.* demonstrate that ccRCC tumors grown orthotopically show increased uptake of ¹⁸F-(2*S*,4*R*)4-fluoroglutamine relative to adjacent healthy kidney tissue and are sensitive to glutaminase inhibition by CB-839. These results suggest that PET imaging could be useful to identify ccRCC patients likely to respond to glutaminase inhibition clinically.

5.4 Immunotherapy

Immune checkpoint blockade represents a promising strategy for the treatment of many cancer subtypes including ccRCC. Such therapies remove the “brakes” from a patient’s immune system, allowing T-cells to recognize and destroy cancer cells. While ccRCC does not harbor as many somatic mutations per megabase as melanoma or non-small cell lung cancer, which creates a higher neo-antigen load and is thought to predict response to immunotherapy, positive immune responses can be achieved in a subset of patients [107],

[108]. One method of achieving this effect is to antagonize programmed cell death-1 receptor (PD-1), which is found on activated T-cells and interacts with PD-L1 or PD-L2 on tumor cells. This interaction normally allows tumor cells to evade the immune response, but when inhibited restores antitumor immunity [109]. Clinical work comparing nivolumab, a monoclonal antibody targeting PD-1, to everolimus, found that nivolumab extended median survival to 25 months compared with 19.6 months for everolimus treatment in patients with advanced ccRCC [110]. However, durable effects were only seen in 20–25% of patients [108]. Current investigations across cancer subtypes are geared toward defining mechanisms that underlie positive and lasting responses to immune checkpoint blockade. A recent study identified signatures of response to checkpoint therapies in ccRCC [111]. Through whole exome sequencing of 35 tumors of patients with metastatic ccRCC, it was determined that biallelic *PBRM1* loss was associated with significantly longer overall survival than patients with *PBRM1* intact. It was hypothesized that *PBRM1* loss-of-function and subsequent alterations in SWI/SNF signaling networks may underlie this effect. It is the hope that through these analyses, specific patient populations can be identified which would respond best to immunotherapy based on the genetic and mutational landscape of their tumors.

6. Conclusion

In this review, we have discussed recent developments in the generation of a genetically engineered mouse model for ccRCC, how reprogramming of glucose and lipid metabolic pathways facilitate tumor progression, and novel therapeutic strategies informed by these pre-clinical studies. Future research investigating additional epigenetic and metabolic hallmarks of ccRCC will likely identify druggable pathways shared across a large percentage of patients. When inhibited or activated through combinatorial therapy, manipulation of these targets will hopefully be effective in prolonging survival in patients with metastatic ccRCC, which is largely resistant to current standard-of-care therapeutics.

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Figure 1. Regulation of hypoxia-inducible factor (HIF) signaling by the von Hippel-Lindau (VHL) tumor suppressor.

Under oxygen-replete conditions, HIF- α subunits are hydroxylated by prolyl hydroxylases (PHDs) and then ubiquitinated by an E3-ubiquitin ligase complex containing pVHL, tagging them for proteasomal degradation. In hypoxia, or when *VHL* is inactivated (such as in ccRCC), HIF- α subunits escape degradation, translocate to the nucleus, and heterodimerize with HIF-1 β (ARNT). HIFs generally promote a transcriptional program favoring increased angiogenesis, glycolysis, and metastatic capabilities of ccRCC tumors. HRE = hypoxia response element.

Figure 2. Comparison of the metabolic state of renal epithelium and clear cell renal cell carcinoma (ccRCC).

Renal epithelial cells (of the proximal tubule, a proposed cell-of-origin of ccRCC) are characterized by high levels of glucose and fatty acid oxidation to serve their energetic needs. As these cells transition into ccRCC, they begin to store triglyceride and cholesterol ester in cytoplasmic lipid droplets. Furthermore, ccRCC have low levels of fatty acid oxidation and compromised mitochondrial architecture relative to healthy renal epithelium. Nuc = nucleus. Mito = mitochondria. LD = lipid droplet.

Figure 3. Select pharmacological approaches for the treatment of metastatic ccRCC.

Current and future therapeutic strategies for ccRCC include inhibition (denoted by red boxes) of mTOR signaling, angiogenesis, HIF-2 α signaling, and immune checkpoint blockade. RTKi = receptor tyrosine kinase inhibitors. Rapalogs = rapamycin analogs. PD-1i = PD-1 inhibitor. PD-L1i = PD-L1 inhibitor.