Modeling Clear Cell Renal Cell Carcinoma and Therapeutic Implications

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Abstract

Renal cell carcinoma (RCC) comprises a diverse group of malignancies arising from the nephron. The most prevalent type, clear cell renal cell carcinoma (ccRCC), is characterized by genetic mutations in factors governing the hypoxia signaling pathway, resulting in metabolic dysregulation, heightened angiogenesis, intratumoral heterogeneity, and deleterious tumor microenvironmental (TME) crosstalk. Identification of specific genetic variances has led to therapeutic innovation and improved survival for patients with ccRCC. Current barriers to effective long-term therapeutic success highlight the need for continued drug development using improved modeling systems. ccRCC preclinical models can be grouped into three broad categories: cell line, mouse, and 3D models. Yet, the breadth of important unanswered questions in ccRCC research far exceeds the accessibility of model systems capable of carrying them out. Accordingly, we review the strengths, weaknesses, and therapeutic implications of each model system that are relied upon today.

Keywords

Renal cell carcinoma; Clear cell RCC; Cell lines; Genetically engineered mouse models; 3D Organoids; Tumor microenvironment; Angiogenesis

Introduction

Biological models enable researchers to mimic discrete tumor attributes and have paved the way for therapeutic innovations in cancer. The hallmarks of cancer, outlined by Hanahan & Weinberg, describe a dynamic set of characteristics meant to simplify the complexities of tumor progression and set the stage for more in-depth understanding of narrow research questions¹. Tumor-specific characteristics, including mutational burden, tissue of derivation, intratumoral heterogeneity, and microenvironmental interactions, are just a few variables that make a catch-all platform for basic cancer research and therapeutic exploration impractical²,³.
Renal Cell Carcinoma (RCC), cancer arising from nephric epithelial cells, constitutes a group of diseases characterized on the basis of anatomical origin, histological features, molecular hallmarks, and therapeutic outcomes\(^4,5\). The majority of RCC falls into three major histologically defined subsets including: clear cell RCC (ccRCC), papillary RCC (pRCC), and chromophobe RCC (chRCC); however, several other rare subtypes are also recognized\(^6–8\). Cell lines, 3D organoid systems, and both xenograft and genetically engineered mouse models (GEMMs), have all been exploited in RCC research (Figure 1). Here, we outline current and developing model systems, and describe the implications and opportunities for each.

**Defining the Genetic and Histological Landscape of ccRCC**

ccRCC is the most common of the RCC subsets, accounting for ~80% of adult clinical cases\(^9\). Due to the high incidence of ccRCC, we focus our attention to model systems attempting to mimic the ccRCC phenotype, noting that some highly cited RCC cell lines are of pRCC origin\(^10\). ccRCC is characterized by loss or mutation of the von Hippel Lindau (\(VHL\)) gene, which is responsible for encoding the oxygen sensing mediator protein pVHL\(^11,12\). pVHL is a crucial component of the E3 ubiquitin ligase complex, necessary for exposing the family of hypoxia-inducible transcription factors (HIFs) for proteasomal degradation in the presence of oxygen\(^8,12,13\). Without pVHL, HIF-1\(\alpha\) and HIF-2\(\alpha\) accumulation promotes gene expression programs that ultimately dysregulate glycolysis, angiogenesis, and lipolysis regardless of oxygen status\(^11,14–16\). Greater than 90% of human ccRCC types occur following \(VHL\) loss of heterozygosity (LOH) in a 2-hit phenomenon\(^14,17\). Thus, models of ccRCC are typically defined by the biallelic loss of \(VHL\) (Figure 2). Recently, multi-regional sampling and whole genome sequencing (WGS) enabled researchers to track landmark mutagenic events in ccRCC\(^18\). Results indicated that deletion of chromosome 3p typically occurs decades before diagnosis, usually during childhood or adolescence; this event is the projected ‘first-hit’ to \(VHL\) and the initial driver of sporadic ccRCC types\(^18\). A subsequent ‘second-hit’ occurs as the result of a somatic intragenic mutation or gene silencing event, in the remaining \(VHL\) allele\(^19,20\).

Intracellular HIF stabilization promotes over-expression of HIF-related pro-angiogenic and glycolytic target genes that lead to nutrient and oxygen imbalances in the tumor microenvironment (TME)\(^21,22\). A classic example is the accumulation of the angiogenic growth factor, VEGF-A, which promotes activation of its cognate receptor, VEGFR, on endothelial cells to induce a pro-angiogenic switch\(^21\). VEGF-A and VEGFR have been successfully exploited as therapeutic targets for angiogenesis in the treatment of ccRCC\(^23\). Hypoxia-driven dysregulation of glycolytic modulators, including glucose uptake transporter-1 (GLUT1) and pyruvate kinase-M2 (PKM2), have also been explored as therapeutic targets in ccRCC\(^24,25\).

In 2013, The Cancer Genome Atlas (TCGA) put forward a comprehensive analysis of over 400 RCC specimens, alongside matching normal kidney tissue, to reveal a recurrent pattern of metabolic reprogramming that correlated with disease severity\(^26\). Integrative unbiased pathway analysis disclosed that aggressive tumors resembled a metabolic shift similar to the Warburg phenotype, as determined by downregulation of TCA cycle intermediates and
upregulation of glycolysis and the pentose phosphate pathway. In addition, decreased PTEN and AMPK protein activity and increased Acetyl-CoA carboxylase correlated with aggressive tumor phenotypes.

cCRCC biology can be subdivided based on recurrent mutations in the chromatin modifying co-driver genes (PBRM1, SETD2, BAP1, KDM5C, KDM6A, and MLL2) believed to contribute to intratumoral heterogeneity in cCRCC and serve as a potential etiology of drug resistance. At the genomic level, intratumoral heterogeneity (ITH) is defined as the outgrowth of genetically different clonal subpopulations within the same tumor. Phylogenetic analyses of multiple unique regions within the same cCRCC tumor determined that only VHL mutations were similar and abundant across all regions and additional mutations in co-driver genes varied across these tumor regions. A comprehensive study using TRAcking cancer evolution Rx (TRACERx) and multi-regional WGS in cCRCC exposed clonal and sub-clonal drivers that contribute to the evolution of intratumoral heterogeneity and genomic instability. Seven evolutionary subtypes were classified according to their metastatic phenotype. Primary tumors characterized as BAP1 driven, VHL wild type, or having multiple clonal drivers were shown to exhibit low ITH and rapid progression to lungs, liver, adrenal glands, and lymph nodes. Tumors classified as PBRM1 plus SETD2, PBRM1 plus PI3K, and PBRM1 plus somatic copy number alteration (SCNA) displayed high ITH and attenuated metastatic progression to the bones and lungs. Primary tumors classified as VHL monodriver did not exhibit metastasis. Together, TRACERx Renal and TCGA studies highlight factors that have the potential to predict a myriad of disease phenotypes and associated level of aggression.

**Biomarker Development and Treatment in Advanced cCRCC**

To date, no genetic biomarker has been implemented as a reliable predictor of cCRCC prognostic or therapeutic outcome. Prediction of cCRCC tumor recurrence, using the multigene assays, ClearCode34 and the 16-gene recurrence score (RS) assay, have demonstrated success in aiding clinicians in judging risk of recurrence beyond existing pathological parameters. ClearCode34 is a 34 gene expression signature originally designed to define molecular variation but has demonstrated the capacity to predict tumor recurrence in primary RCC tumors. Based on expression of the 34 gene signature, ClearCode34 classifies tumors as either indolent (ccA) or aggressive (ccB). The predictive value of ClearCode34 has also been validated clinically in connecting primary tumor biology with distant, metastatic sites in cCRCC. A 16-gene recurrence score (RS) assay has also been used in predicting tumor recurrence and projecting patient benefit to adjuvant therapies. The 16-gene assay includes cCRCC related genes involved in vascular, immune, cell-cycle division, and growth pathways. ClearCode34 and the 16-gene RS assay are both valuable tools for research purposes and could be considered for personalized therapeutic selection based on molecular tumor characteristics.

ccRCC is typically non-responsive to traditional cytotoxic chemotherapeutic drugs. Prior to the introduction of small molecule targeted therapies and immune checkpoint inhibitors, cytokine therapy (IFN-α and IL-2) was the standard of care for patients with metastatic RCC (mRCC); however, response rates remained low. A mainstay of therapy, since their...
introduction in 2005, are the anti-angiogenic small molecule tyrosine kinase inhibitors (TKI) primarily targeting the VEGF signaling pathway\textsuperscript{39,40}. The following TKI small molecule inhibitor drugs are approved for clinical use: axitinib, cabozantinib, lenvatinib, pazopanib, sorafenib, and sunitinib\textsuperscript{40}. Additionally, mTOR signaling is tightly linked to HIF activation and increased metabolic demands, which makes mTOR inhibition a viable therapeutic target for some RCC patients\textsuperscript{41}. Everolimus and temsirolimus are both approved for use in advanced RCC\textsuperscript{42}, and more recently, everolimus has been applied in combination with VEGF inhibition\textsuperscript{43}.

Checkpoint inhibitors targeting CTLA-4, PD-1 and PD-L1 work by unleashing the adaptive immune system’s potential to target and kill cancer cells\textsuperscript{44,45}. Ipilimumab blocks CTLA-4 while nivolumab and pembrolizumab block the negative regulator PD-1 on T cells. Avelumab targets the negative regulation of T cells by blocking PD-L1 on cancer and other immune cells\textsuperscript{46,47}. Checkpoint blockade therapies initially showed improvement in patient survival with use of nivolumab in the second line treatment setting\textsuperscript{48}. Shortly after, checkpoint inhibition moved to the first-line standard of care in metastatic ccRCC with a combination of antibodies targeting CTLA-4 and PD-1\textsuperscript{46}, and more recently, finding that combination of checkpoint inhibition and VEGF TKI increased objective response rates and overall survival\textsuperscript{47,49,50}.

Newer generation immunotherapies represent a promising approach to patient care in ccRCC, with the potential to provide a long duration of response\textsuperscript{49}. However, many patients harbor non-responsive tumors and are faced with a poor prognosis. Additionally, for patients who initially respond, many will progress while on treatment as tumor cells develop resistance\textsuperscript{49,51}. Ongoing efforts seek to identify and confirm clinically relevant predictive biomarkers in ccRCC to aid clinicians in immunotherapy selection\textsuperscript{52,53}. As such, model systems capable of recapitulating the TME are crucial to enhance our understanding of drug interactions.

Cell Line Models of Renal Cell Carcinoma

Immortalized tumor cell lines are the most frequently utilized biological system in cancer research, contributing to our current molecular and genetic understanding of RCC biology\textsuperscript{54}. Cost-effectiveness, ease of maintenance and manipulation, and infinite replicative capacity made accessible by immortalized cell lines allow for the three “R’s” in humane research: replacement, reduction, and refinement\textsuperscript{55}. In the 1980s, the National Cancer Institute’s 60 cell line (NCI-60) comprehensive anticancer drug screening study included the following RCC cell lines: 786-O, A-498, ACHN, CAKI-1, RXF 393, SN12C, TK-10, and UO-31\textsuperscript{56}.

The NCI-60 study led to intense characterization and publicly available RCC specific genetic and molecular cell line data\textsuperscript{56}. Consequently, the eight RCC cell lines included were established as first-line research models.

786-O is among the earliest ccRCC cell lines established and remains the most frequently cited today\textsuperscript{57}. The COSMIC Cell Lines Project (CCLP) and Broad Novartis Cell Line Encyclopedia (CCLE) report a p.G104fs VHL frameshift deletion in 786-Os, and consistently, 786-O cells display increased HIF-2α and VEGF protein expression, making
this cell line a workhorse for ccRCC research. The negative regulatory role of the pVHL-HIF axis was first demonstrated in 786-O cells. When transduced with wild type (WT)-pVHL, in vivo tumor growth was inhibited. 786-Os were also used to show that HIF overexpression alone promotes tumor growth, thus validating that HIF activation occurs downstream from the pVHL interaction. More recently, 786-O cells were employed to decipher the role of hypoxia induced resistance to the VEGF inhibitor, sorafenib, via the HIF-2α-Cox2 pathway, thereby illustrating a potential drug resistance mechanism.

Histologically, 786-O cells do not exhibit clear cytoplasm in mouse xenograft models; rather, tumors are poorly differentiated with spindle shaped sarcomatoid features, a quality indicative of genetic and morphological regression. Stem cell-like side populations (SPs) have been observed in 786-O cells, making this line a potential model for cancer stem cell (CSC) studies in aggressive forms of ccRCC. SPs are understood to be a population of cancer stem cells (CSCs) that, when enriched, confer resistance to chemotherapy drugs and promote tumorigenicity.

The second most highly cited RCC line, A-498, is classified as ccRCC based on VHL mutation status. The CCLP and CCLE report p.G144fs*14 and p.V142fs VHL mutations, respectively. A498 cells exhibit clear cytoplasmic histological signatures when xenografted into nude mice. 786-O and A-498 cell lines were utilized in a landmark study implicating mTOR activation as a driver of tumorigenesis in ccRCC. This finding fueled further investigations into mTOR activity as a prognostic indicator and therapeutic target. A-498 and 786-O cells were also used to illustrate the functional redundancy and dominance of HIF protein subunits in transcribing the angiogenic factor, VEGF. When HIF-1α and HIF-2α are both present and functional, VEGF is preferentially expressed by HIF-1α; however, when HIF-1α is deleted or nonfunctional, HIF-2α is upregulated and VEGF levels remain unchanged.

ACHN, the third most highly cited line, was established from a metastatic pleural effusion and exhibits poorly differentiated sarcomatoid histological features. ACHN cells harbor a pRCC specific mutation in c-MET and do not have classical VHL mutations. Though not representative of the ccRCC genetic phenotype, ACHN cells were exploited to demonstrate VHL deficient sensitization to mTOR inhibition by shRNA knockdown. Increased uptake of the glucose tracer fluorodeoxyglucose (FDG) was decreased in VHL knockdown tumors treated with the mTOR inhibitor, CCI-779, compared to the vehicle treated control tumors by positron emission tomography (PET). This study provided support for clinical use of mTOR dependent inhibition in the treatment of ccRCC. These features make ACHN cells an attractive model for studying aggressive tumor types but impractical for studies aimed at gaining a broad understanding of ccRCC diseases.

While the top three cited cells lines in RCC literature are reviewed above, many other useful cell line models exist and should be considered and applied depending on the biologic question. We have summarized the major classifications and characteristics of established cell lines used in RCC research today (Table 1). In summary, thoughtful consideration regarding experimental aims and well-established genomic and molecular signatures should be employed in the process of choosing an appropriate cell line for each RCC project.
Overview of Preclinical Mouse Models

Mice are utilized extensively as a model system for the study of cancer biology. The mouse genome is experimentally accessible, allowing manipulation of relevant onco- and tumor suppressor genes that promote cancer hallmark characteristics in vivo. Until recently, RCC murine models have been limited to cell-line-derived (CDX), or patient-derived xenografts (PDX). New developments in genetically engineered mouse models (GEMMs) have allowed for kidney-specific conditional knockout of Vhl with at least one other ccRCC associated co-driver gene. VHL bi-allelic inactivation is thought to occur during early stages of ccRCC development in humans with additional co-drivers leading to enhanced tumor progression at a later stage. Most ccRCC GEMMs rely on complete gene deletion during early embryogenesis, thereby failing to model sequential mutagenic events through clonal selection. Ultimately, the complexity involved with studying RCC cell mechanisms and therapeutic responses in mice is far too immense for one mouse model to encapsulate. Cell-line derived xenograft (CDX), Patient-derived xenograft (PDX), and Genetically engineered mouse models (GEMM) each provide unique opportunities to interrogate various questions in ccRCC.

Xenograft Models

CDX and PDX RCC models can be established by orthotopic placement in the subcapsular region of the kidney or by heterotopic transplantation: e.g. subcutaneous (s.c.), intraperitoneal (i.p.), or intramuscular (i.m.). To prevent rejection of the tumor cells from successful engraftment into the host animal, xenograft models using human cell lines or tissues rely on non-obese diabetic (NOD) severe combined immunodeficient (scid) mice, or equivalent severely immunocompromised models. Xenograft models have been shown to maintain patient tumor histology, gene expression, DNA copy number alterations, mutagenic profiles, and general drug responsiveness. Successful xenograft transplantation of established RCC cell lines or primary patient-derived tissues has led to the development and current mechanistic understanding of the anti-angiogenic drugs heavily utilized to treat metastatic RCC (mRCC). Xenograft models have been shown to recapitulate important aspects of the TME, specifically the dense vascular bundling that is characteristic of ccRCC. Rojas and colleagues were able to detect responses to sunitinib treatment and deduce tumor responsiveness one week prior to changes in tumor volume using ultrasound-derived microvascular density imaging. In another study, CDX models using 786-O, A498 and CAKI-1 cell lines demonstrated changes in tumor blood flow rates correlating with anti-angiogenic resistance of sorafenib treatment. In this model, arterial spin labeling (ASL) magnetic resonance (MR) imaging was used to measure changes in blood flow signal intensity and spatial patterning during blood vessel formation. In 786-O xenografts, blood vessel growth in anti-angiogenic resistant tumors primarily occurred at the periphery of the tumor, whereas endothelial formation in A498 xenografts occurred closer to the tumor’s center. ASL-MR imaging was also useful in identifying tumors with initial low blood flow intensity, as observed in CAKI-1 xenografts, a characteristic that could aid in treatment selection and prediction. Overall, changes in blood flow intensity provided valuable diagnostic and mechanistic information at earlier
timepoints than previously assessed, thereby highlighting the potential for measuring functional tumor activity, in addition to gross anatomical quantification.

PDX models are created by direct transplantation and subsequent passaging of surgically resected RCC tissues into immunocompromised mice. PDX models provide a closer to human model of tumor growth, but incur important selective pressures which likely skew the distribution of tumor phenotypes. PDX modeling has demonstrated a propensity to select for the growth of aggressive forms of cancers, a phenomenon that allowed for the discovery of BAP1 as a tumor suppressor gene in ccRCC. Patient tumors harboring BAP1 mutations have since been shown to associate with worse cancer specific survival and exhibit aggressive tumor features.

Immune competent mice can be used to study ccRCC in syngeneic CDX models. Murine renal adenocarcinoma (Renca) is a cell line that arose spontaneously from Balb/c lineage and exhibits progressive growth and metastasis to lymph nodes, liver, and lungs in transplantable subcapsular orthotopic models. Though Renca cells do not fully mimic the genetic profile observed in human ccRCC, Vhl deletion in Renca cells leads to HIF-1α stabilization and epithelial-mesenchymal transition (EMT), both of which classically define human ccRCC. The human kidney tumor-specific antigen, carbonic anhydrase-IX (CA-IX), has also been stably expressed on Renca cells, and has provided an ideal platform for metastatic targeting and tracking studies in ccRCC. Similar to human RCC tumors, Renca xenograft models demonstrate high immune-infiltrating cell populations of both lymphoid and myeloid lineage. In an orthotopic CDX model, Renca tumors were used to identify non-canonical expression of the T-regulatory-specific transcription factor, Foxp3, in tumor associated macrophages (TAMs) of the TME. In addition, initial studies aimed at understanding functional mechanisms in which chemokine networks can promote tumor progression have identified chemokine receptor 4 (CCR4) as a potential target in RCC.

Ultimately, xenograft models retain histology and genetic signatures of the original cell line or tissue type, thereby implementing an appropriate system for elucidating tumor mechanisms of response and resistance to candidate drugs. However, the requirement of immune suppression in non-syngeneic models limits accurate representation of the TME and ITH in xenograft models.

**Genetically Engineered Mouse Models (GEMMs)**

Reproducing common mutational events in genetically engineered mouse models (GEMMs) under kidney epithelial specific Cre drivers of deletion has proven challenging in ccRCC for several reasons (Figure 3). Contrary to human disease, Vhl heterozygosity has not been shown to predispose mice to ccRCC tumor development. This dilemma is fueled by inter-species locational variances of commonly mutated ccRCC genes. In humans, an initial ‘first-hit’ VHL mutagenic event is thought to occur when chromosome arm 3p is lost in near entirety; a subsequent ‘second-hit’ occurs when the remaining VHL allele is mutated, resulting in loss of function of VHL. The ccRCC associated chromatin modifying genes PBRM1, BAP1, and SETD2 are all present on chromosome arm 3p in the human genome; thus, ‘first-hit’ and ‘second-hit’ mutagenic events described for VHL also apply to...
these secondary ccRCC related genes. In the mouse genome, however, Vhl is located on chromosome 6, Seta2 on chromosome 9, and Pbrm1 and Bap1 reside on chromosome 14 (Figure 2). Additionally, models that alter genes atypically associated with classic human ccRCC have been shown to give rise to robust tumors resembling the disease in mice. Thus, differences observed in mouse versus human RCC tumor development highlight the need for consideration regarding basic species-specific kidney function.

Despite present challenges, GEMMs are poised to impact our current therapeutic and basic biologic understanding of tumor development in a number of ways. Considering the inevitability of drug resistance conferred by mono-therapeutic anti-cancer agents in ccRCC, immunocompetent GEMMs also offer an appropriate platform to address combination therapy. Importantly, GEM models mimicking ccRCC can develop de novo sporadic tumors, thereby providing an in vivo platform for thorough investigations of the complex interactions between tumor cells and the TME during tumorigenesis and progression. In this discussion, we highlight five of the most relevant GEM models currently available in ccRCC research (Figure 4).

**Vhl−/− Pbrm1−/−**

Two independent groups have published their attempts to recapitulate human ccRCC in mice by deletion of both Vhl and Pbrm1, the 1st and 2nd most commonly altered genes observed in ccRCC, respectively. PBRM1, a tumor suppressor subunit of the SWI/SNF chromatin remodeling complex, located on chromosome 3p, is mutated in 45% of human ccRCC cases. Nargund and colleagues utilized the Ksp-Cre system to conditionally delete Vhl and Pbrm1 in renal epithelial cells. With this system, mice developed preneoplastic polycystic kidney disease (PKD) by 6 months of age and showed a 50% tumor incidence by 10 months, assessed by histological examination. Tumors exhibited classic ccRCC characteristics, including clear cytoplasm, hyperactive mTORC1 signaling, downregulation of critical oxidative phosphorylation genes, and HIF-1α accumulation (Figure 2).

Gu and colleagues developed a combined Vhl and Pbrm1 conditional knockout model using paired box gene 8 (Pax8) as the Cre driver of gene deletion. This approach gave rise to bilateral, homogenous tumors by 9 months of age in 85% of mice. The author’s described the Vhl, Pbrm1 deleted tumors as low-grade, based on aggression and histologic analysis; a result consistent with loss of PBRM1 in human ccRCC. The nephron is made up of a complex capsular and tubular system rich in capillary beds and collecting ducts, specially designed to filter blood. Based on immunohistochemical (IHC) staining and gene expression analysis of specialized nephric regions, the proximal convoluted tubule (PCT), consisting of at least 13 distinct epithelial cell types, has been recognized as the most likely site of origin of human ccRCC mutations. However, Gu and colleagues found that Vhl and Pbrm1 deletion in a murine system using a Pax8 Cre promoted tumorigenesis, where the Cre driver expression was limited to the parietal cells of Bowman’s capsule. In addition, the PCT-specific Cre drivers, Sglt2 and Villin, failed to promote ccRCC tumor growth. IHC staining led the authors to propose that the parietal epithelial cells of Bowman’s capsule may be the location of tumor origin in their model. Therefore, future studies should seek to validate ccRCC cell specific nephric lineage signatures.
and determine locational differences based on co-driver gene mutations. Importantly, both investigations targeting Vhl and Pbrm1 for deletion confirm the tumor suppressive role of Pbrm1 in vivo and will be valuable tools moving forward in ccRCC tumor therapeutic investigations.

**Vhl−/− Bap1+/−**

The histone deubiquitinase, BRCA1-associated protein-1 (BAP1), is a tumor suppressor that functions in DNA repair and chromatin remodeling in the nucleus. BAP1 deletion or mutation occurs in 10–15% of ccRCC tumors. BAP1 mutations were identified through next generation sequencing as strongly associated with high grade tumors and increased risk of tumor recurrence following surgical resection. Combined targeting of Bap1 and Vhl, using the nephron progenitor transcription regulator, Six2, as a Cre driver, resulted in ccRCC development; however, pups only survived up to one month. When the same group utilized the nephric specific transcription factor, Pax8, as the Cre driver of complete Vhl deletion and heterozygous manipulation of Bap1, features reminiscent of ccRCC were observed. Vhl and Bap1 deficiency gave rise to high grade tumors with clear cell histology and positive carbonic anhydrase 9 (CAIX) staining. Future studies should aim to validate tumor responsiveness to drug therapies in this model.

**Vhl−/− Cdkn2a−/− and c-Myc activation**

MYC activation has been recognized as a common genetic anomaly in both pRCC and ccRCC. One study revealed a 3-fold increase in c-MYC expression when compared to matched healthy kidney tissue. CDKN2A (INK4A/ARF), a tumor suppressor gene, encodes a protein that functions to promote cellular senescence. Consequently, CDKN2A loss-of-function promotes tumor growth and metastasis in many cancers. Bailey and colleagues exploited a previously described doxycycline inducible c-Myc transgenic mouse to explore Myc activation alone, and with Ksp-Cre promotor driven Vhl and Cdkn2a deletion in renal tubular cells, thus generating three useful models. Based on histological features and RNA-seq, c-Myc overexpression alone gave rise to renal tumors most representative of type 2 pRCC, assessed by pathologic staining. c-Myc overexpression plus Vhl deletion resulted in tumors demonstrating some ccRCC pathological features, such as cytoplasmic clearing, necrosis, and intratumor hemorrhaging. Deletion of Cdkn2a, in addition to Vhl deletion and c-Myc overexpression, gave rise to an aggressive ccRCC phenotype, exhibiting liver metastasis in 30% of mice. Though the authors demonstrated successful recapitulation of metastatic tumor sites resembling ccRCC, the genetic drivers in this model only occurs in 6% of human RCC cases. This model is representative of a small aggressive ccRCC subset and is unique in its ability to metastasize.

**Vhl−/−, Trp53−/−, and Rb1−/−**

Single nucleotide alterations affecting the function of tumor suppressor genes, RB and TP53, during the G1-S cell cycle transition, are relatively infrequent in ccRCC. However, copy number gains or losses in RB and TP53 are common, and TCGA patient data demonstrates independent evolution in these regulators from those occurring in VHL, PBRM1, BAP1, and SETD2. Hence, the conditional triple-mutant GEMM model, inactivating Vhl, Trp53, and Rbl, was predicted to confer the evolution of genetically distinct tumors overtime. This
model utilized kidney specific cadherin (Ksp-cadherin), expressed on tubular epithelial cells of the kidney and genitourinary (GU) tract, as a Cre driver of deletion\textsuperscript{110}. Sporadic tumors, formed in the mouse kidney, displayed classic ccRCC pathological features, including necrosis, tumor hemorrhaging, and nuclear accumulation of HIF-1\(\alpha\) and HIF-2\(\alpha\).\textsuperscript{128} Notably, a pathological phenotype reminiscent of ccRCC did not result from a \textit{Trp53, Rb1} double knockout model, thus confirming the role of \textit{Vhl} loss as necessary for tumor progression\textsuperscript{128}. Greater than 80\% of the triple-mutant mice developed tumors within 25–61 weeks, with each mouse averaging five tumors\textsuperscript{128}. Male mice developed more tumors than female mice in this model, mirroring the gender bias observed in human ccRCC\textsuperscript{128}. Similar to human ccRCC responses to current therapies, Harlander et al. described heterogeneity in therapeutic responses to a panel of relevant drugs in the triple-mutant model\textsuperscript{128}. Notably, VEGFR and mTOR treatment demonstrated a moderate decrease in tumor growth with development of drug resistance in some tumors\textsuperscript{128}. These results implicate this model as a viable platform for investigating drug resistant mechanisms in an immunocompetent \textit{in vivo} model\textsuperscript{128}. Although the specific mutagenic profile driving tumor progression in this triple knockout is rarely seen in human ccRCC, this system does promote evolution of genetically distinct tumors with variable drug responses and classic pathological ccRCC features.

**GEMM Limitations and Conclusions**

GEMM models described here are unable to fully represent the genetic and pathological phenotypes identified in human disease. In addition, \textit{Setd2} GEM models have yet to be developed, despite the urgent need for qualitative understanding of \textit{SETD2} mutations in an \textit{in vivo} setting. Most current RCC GEMMs rely on simultaneous deletion of target genes during early embryogenesis, rather than promoting sequential loss of function. Thus, future GEMMs in ccRCC should attempt to alter or delete genes of interest sequentially in an attempt to mimic clonal selection. This approach has already been utilized to dissect pancreatic ductal adenocarcinoma (PDAC) using CRISPR/Cas9 technology\textsuperscript{106}.

For many genes residing on human 3p, notably \textit{SETD2}, the ‘first-hit’ mutation results in a haploinsufficiency phenotype that is not yet technically feasible to recapitulate in a murine setting. Indeed, heterozygosity phenotypes for \textit{SETD2} have been implicated in tumorigenic processes as an early driver of genomic and microtubule instability\textsuperscript{129}. Additionally, complete gene deletion, as opposed to mutation, limits investigations aimed at examining adverse interactions conferred by truncated or nonfunctional proteins, which may contribute to tumor progression in human ccRCC.

Comprehensive evaluation of the TME and ITH, in the above described GEMMs, have yet to be fully evaluated; however, researchers are actively exploiting the presence of a competent immune system to evaluate these features and associate the results with human disease. Specifically, future work should aim to determine if the tumor mutation status correlates with immune and non-immune cancer-associated cellular components in mice. Additionally, multi-regional sampling, whole exome sequencing, and single cell RNA sequencing, may uncover translatable tumor vulnerabilities that have not yet been identified in human tumors.
Ultimately, the complexity involved with studying ccRCC cell mechanisms and therapeutics in vivo suggests the need for current and continued development of xenograft and genetically modified mouse models, as each provide unique opportunities to interrogate various questions about the biology and treatment of RCC.

**Patient-Derived Organoid (PDO) Model**

Recently developed organoid technology promotes an in vitro structural organization that is comparable to an in vivo tumor architecture, while also maintaining the vascular-stromal-inflammatory milieu that makes up the TME\(^{130}\). Several studies have successfully created tumor organoid models using primary patient specimens from colorectal\(^{131}\), pancreatic\(^{132}\), bladder\(^{133}\), breast\(^{134}\), and lung cancers\(^{135}\). Grassi and colleagues became the first group to successfully establish and characterize organoid cultures from both normal and matched ccRCC adult-patient-derived-pluripotent stem cells of the kidney\(^{136}\). Differential organization was observed between ccRCC organoids, compared to their normal kidney counterparts, by immunofluorescence (IF)\(^{136}\). In addition, nephron region-specificity of the PCT and DCT, Bowman’s capsule, loop of Henle, thick ascending limb, and collecting duct tissues were all confirmed by cell type specific IF in both tumor and normal organoid cultures\(^{136}\). Successful transplant of RCC PDOs into immunocompromised mice, by orthotopic injection, demonstrated a 75% engraftment rate\(^{136}\). Furthermore, transplantation showed an increased capacity to invade kidney parenchyma and disorganized tissue structure, as compared to normal tissue PDO, thereby achieving tumor-propagating properties\(^{136}\). The authors contribution of organoids derived from normal adult kidney tissue is crucial, not only as a matched control for ccRCC, but also for the provision of a physiological relevant platform for basic kidney research\(^{136}\). Normal kidney tissue derived organoids also provide a foundation for future targeted gene editing in 3D culture utilizing CRISPR-Cas9 technology, an approach that has been established in organoids originating from intestinal and pancreatic tissue\(^{137,138}\). Initial selection of undifferentiated renal adult stem cells and rejection of hematopoietic derived cells limit this model’s ability to fully encompass the native TME in ccRCC.

Other groups, such as Neal and colleagues, developed an air liquid interface (ALI) method to cultivate patient derived organoids (PDOs) from surgically resected ccRCC biopsied human specimens. In a collagen-based matrix, the authors effectively retained primary tumor epithelium alongside immune and transformed non-immune TME components\(^{139}\). In addition, immune checkpoint blockade therapy with PD1 and PD-L1 propagated expansion of TILs and production of granzyme b cytotoxic CD8 T cells, thereby killing tumor cells and recapitulating immune checkpoint functionality in a 3D system\(^{139}\). Continued development of 3D organoid culture will likely provide opportunities to assess the efficacy of other immune- and metabolic-based therapies.

**Challenges to Overcome in RCC PDOs**

Technical challenges involved in RCC organoid development are not trivial, and standardization of optimized techniques to support experimental reproducibility have yet to be established. For unknown reasons, studies have observed a growth advantage of non-
cancerous epithelial cells residing in the TME when exposed to supplemented cell culture conditions\textsuperscript{140}. Modifications, including initial starvation of growth factors, followed by robust nutrient supplementation, have been shown to reduce contaminated normal epithelial cells in some tumor types\textsuperscript{139}. This phenomenon is indicative of cell responses to \textit{in vitro} culture conditions that may be different from those occurring in an \textit{in vivo} tumor environment. In-depth analyses of the nutrients available in the native TME of human RCC tumors may be key to improving organoid development.

ITH is a challenging feature to capture in PDO models that rely on a small fraction of the resected tumor tissue. Given that ITH is a hallmark of ccRCC, methods aimed at sampling multiple regions of the tumor will likely provide a more representative view of any given tumor. Features common to RCC tumors, including necrosis, intratumoral hemorrhaging, inflammation, and hypoxia, make simultaneous retrieval and maintenance of viable tumor epithelial and TME cell populations challenging to obtain\textsuperscript{7,141}. Despite current limitations, however, continued research development of PDOs will provide a reliable framework for investigations of RCC biology, therapeutic development, and evaluation of native TME/tumor crosstalk and organization.

**Concluding remarks**

Each model system in RCC research offers a unique set of advantages and disadvantages that must be considered by investigators. Well established RCC cell lines have propelled our molecular and genetic understanding of renal cancers, both in cell line models and murine CDX models, thus providing a system for high-throughput drug screening. Results obtained from historical research utilizing cell lines, CDX, and PDX models identified angiogenic and mTOR signaling as candidate therapeutic targets that ultimately translated into significant improvements in patient care and prognosis. The relative ease of manipulation allowed by cell line and xenograft models are important for enhancing our understanding of established and newly identified RCC subtypes. Despite advancements permitted by cell line and xenograft models, unpredictable tumor recurrence and therapeutic resistance in mRCC remain challenging obstacles in the field. Therefore, sophisticated models are particularly critical as the TME emerges as a major factor in cancer control mechanisms. Complex crosstalk occurring within the TME, has prompted a shift toward immunotherapy-based treatment as the standard of care. Consequently, there is a need for improved immunocompetent \textit{in vivo} models and PDO \textit{in vitro} 3D systems to allow for accurate recapitulation of the TME.

GEMMs have demonstrated successful development of \textit{de novo} sporadic ccRCC tumors, providing an \textit{in vivo} platform for thorough investigations of tumorigenesis and progression. Continued development of GEMM models will result in an increased capacity to mimic the clonal evolution believed to occur in human ccRCC tumors and allow the underlying mechanisms that contribute to tumorigenesis and progression to be fully appreciated by researchers. ccRCC organoid systems are poised to uncover tumor specific vulnerabilities in the context of an intricate stromal-vascular-inflammatory interaction. As rapid scientific advancement continues to push the technical boundaries of cancer research toward the
development of precision medicine and immune therapy-based treatment, so, too, should disease-specific model systems evolve.

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Figure 1.
Advantages and disadvantages of model system platforms in RCC. Advantages and ideal applications, and disadvantages of traditional cell line, organoid, xenograft, and GEM models in RCC. As physiological relevance increases, ease of manipulation decreases.
Figure 2.
Location of ccRCC Associated Genes Human and Mouse Genome

*VHL* and additional driver genes (*SETD2*, *PBRM1*, and *BAP1*) are located on chromosome 3p in the human genome. WGS identified two key events in the evolutionary progression of ccRCC: loss of one 3p arm during childhood or adolescents, followed an additional mutation in the remaining *VHL* allele\textsuperscript{18}. Corresponding genes in the mouse are located on chromosomes 6, 9, and 14.
**Figure 3.**
Cre drivers in kidney epithelial cell deletion.
Cre drivers in kidney epithelial cell gene specific deletion and tissues affected are outlined.
Figure 4.
Genetically engineered RCC mouse models.
Strengths and limitations of relevant RCC GEM models, and the pathology recapitulated by each are outlined.
Table 1.

Top cited RCC cell lines.
Histologic and genetic characteristics, including ccA and ccB sub-classification, and 3p deletion status as defined by Sinha et al., are outlined. \textit{VHL} and other known significantly mutated genes, determined by TCGA, are defined by CCLE and CCLP databases.

| Cell line | Classification | Sub classification \textsuperscript{10} | Histological features | 3p status \textsuperscript{10} | VHL status \textsuperscript{56,67} | CCLE mutations \textsuperscript{66,67} |
|-----------|----------------|---------------------------------|----------------------|----------------|----------------|-----------------|
| 786-O     | ccRCC         | ccB                             | Poor differentiation with sarcomatoid features \textsuperscript{10} | Deleted        | Null           | \textit{PTEN}, \textit{TP53}, \textit{TSC2}, \textit{MLL3} |
|           |                |                                 | (Sinha et al., 2017) |                |                |                 |
| A-498     | ccRCC         | ccB                             | Compact nests of tumor cells with clear cytoplasm \textsuperscript{10} | Deleted        | Null           | \textit{MLL3}, \textit{SETD2} |
|           |                |                                 | (Sinha et al., 2017) |                |                |                 |
| ACHN      | pRCC          | Unknown                         | Poor differentiation with sarcomatoid features \textsuperscript{10} | Deleted        | Wildtype       | \textit{PBRM1}, \textit{NF2} |
|           |                |                                 | (Sinha et al., 2017) |                |                |                 |
| CAKI-1    | ccRCC         | ccB                             | Poor differentiation\textsuperscript{71} | Not Deleted    | Wildtype       | \textit{MET}, \textit{MLL3}, \textit{SETD2} |
|           |                |                                 | (Korhonen et al., 1994) |                |                |                 |
| RCC-4     | ccRCC         | Unknown                         | Unknown              | Deleted        | Null           | \textit{NA}     |
|           |                |                                 |                     |                |                |                 |
| A-704     | ccRCC         | ccA                             | Not tumorigenic in immunocompromised mice\textsuperscript{73} | Deleted        | Null           | \textit{MLL3}, \textit{TP53}, \textit{PBRM1}, \textit{SETD2} |
|           |                |                                 | (Giard et al., 1973) |                |                |                 |
| SN12C     | Unknown        | Unknown                         | Clear cell combined with granular morphology\textsuperscript{74} | Not Deleted    | Wildtype       | \textit{TP53}, \textit{BAP1}, \textit{NF2} |
|           |                |                                 | (Sanchez et al., 1994) |                |                |                 |
| CAKI-2    | ccRCC         | ccB                             | Well differentiated clear cell\textsuperscript{71} | Deleted        | Wildtype       | \textit{PBRM1} |
|           |                |                                 | (Korhonen et al., 1994) |                |                |                 |
| 769-P     | ccRCC         | ccA                             | Unknown              | Deleted        | Null           | \textit{BAP1}, \textit{TSC2} |
| TK-10     | Unknown        | Unknown                         | Unknown              | Deleted        | Wildtype       | \textit{PIK3CA}, \textit{TP53} |
| UMRC-2    | ccRCC         | ccA                             | Unknown              | Deleted        | Null           | \textit{NA}     |
| OS-RCC-2  | ccRCC         | ccB                             | Unknown              | Deleted        | Null           | \textit{PTEN}, \textit{TP53}, \textit{PBRM1} |
| CAL-54    | pRCC          | Unknown                         | Well differentiated with papillary structures\textsuperscript{75} | Deleted        | Wildtype       | \textit{FH}, \textit{MLL3} |
|           |                |                                 | (Gioanni et al., 1996) |                |                |                 |