Chapter 8
Research Translation and Personalized Medicine

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8.1 Case Studies in Personalized Cancer Medicine

A better understanding of the molecular genetics of tumors, together with the availability of molecularly targeted therapies, sets the foundation for "personalized cancer therapy," tailoring treatment to the particular genetic alterations of a tumor in an individual. The opportunities and challenges from such an approach are illustrated by the following two cases.

8.1.1 A Young Man with a Recurrent Epithelioid Angiomyolipoma

A 24-year-old man presented with a recurrent epithelioid angiomyolipoma (EAML) [1]. Five months earlier, the patient had undergone a right radical nephrectomy with removal of a 24-cm EAML that ruptured during surgery. At the time of presentation, there was a 20-cm tumor with evidence of intratumoral hemorrhage (Fig. 8.1), and the patient's hemoglobin was 3.8 g/dL. After transfusion, arterial embolization of

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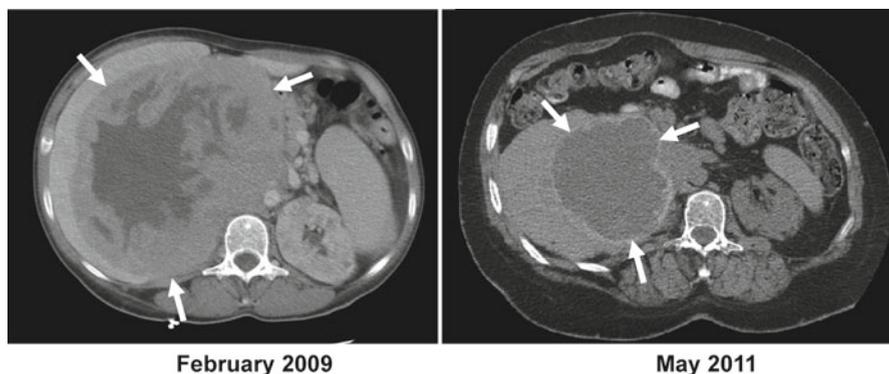e-mail: james.brugarolas@utsouthwestern.edu

Fig. 8.1 EAML in TSC patient. CT scans (with or without contrast) at presentation and following treatment with sirolimus

the tumor was attempted without success, and the patient continued requiring 5–7 units of packed red blood cells weekly. The mass was compressing the small bowel, and total parenteral nutrition (TPN) became necessary. The patient's performance status was very poor and he was deemed not to be a surgical candidate. Unlike angiomyolipomas (AMLs), EAMLs exhibit an aggressive behavior and metastasize, and there is no standard medical therapy [2]. Hospice placement was recommended.

However, the patient had familial tuberous sclerosis complex (TSC). TSC is a syndrome that results from germline mutations in the eponymic genes, *TSC1* and *TSC2* [3]. The *TSC1* and *TSC2* genes encode proteins that form a protein complex TSC1/TSC2 [4, 5] that functions as a negative regulator of mammalian target of rapamycin complex 1 (mTORC1) [6]. The TSC1 protein is necessary for the stabilization of TSC2 [7], and the TSC2 protein functions as a GTPase-activating protein (GAP) toward the small GTPase Ras homologue enriched in brain (Rheb) [8–12]. Inactivation of TSC1/TSC2 results in a Rheb-dependent activation of mammalian target of rapamycin complex 1 (mTORC1) [8–14], and mTORC1 is active in tumors from TSC patients [15].

Because the patient had TSC and since *TSC1* and *TSC2* function as two-hit tumor suppressor genes, it was likely that the event initiating tumor development was the loss of the remaining wild-type allele. This would result in constitutive mTORC1 activation, and high level of mTORC1 activity was observed in the EAML [1]. Thus, we speculated that the patient may benefit from treatment with an mTORC1 inhibitor. In addition, while EAMLs differ from benign AMLs, benign AMLs seem to be responsive to sirolimus, the prototype mTORC1 inhibitor [16]. Sirolimus was started and sirolimus dosing was adjusted so as to maintain levels deemed to be therapeutic [1]. Within 72 h, intratumoral bleeding stopped and no further transfusions were required. The patient had substantial improvement clinically and TPN was discontinued. Two years after the initial presentation, the patient continues on sirolimus, and the tumor remains under control (Fig. 8.1).

8.1.2 A Young Woman with a Papillary Type 2 Renal Cell Carcinoma

A 24-year-old woman presented with a 45-cm left renal mass [17]. The tumor was resected, and this required a partial pancreatectomy, a splenectomy, and a partial colectomy. Histologically, the tumor was a papillary renal cell carcinoma type 2 (pRCC-2). The tumor invaded into the pancreas and there were multiple lymph nodes involved. One month after the surgery, a CT scan showed several nonvisceral metastases that were increasing in size measuring up to 3 cm in diameter. Temsirolimus, which in a phase III trial of histologically unselected renal cell carcinoma (RCC) patients had shown unrestricted activity [18, 19], was begun. Three months later, metastases remained stable.

The presentation with a pRCC-2 at 24 was unusual. The patient did not have a family history of malignancy, but genetic testing was recommended. An increased predisposition to pRCC-2 has been described in the context of hereditary leiomyomatosis and renal cell cancer (HLRCC) [20]. HLRCC patients typically develop leiomyomas in the skin and uterus. There were no cutaneous leiomyomas, but the patient had uterine fibroids. The gene responsible for HLRCC is fumarate hydratase (*FH*) [21, 22]. *FH* sequencing from peripheral blood mononuclear cell DNA revealed a novel mutation. The mutation was a missense mutation resulting in the substitution of an aspartate at position 341 for an asparagine. The aspartate residue was evolutionarily conserved, and the mutation was nonconservative suggesting that it may impair function.

Like the *TSC1* and *TSC2* genes, *FH* functions as a two-hit tumor suppressor gene [20–23], and sequencing studies of the pRCC-2 tumor revealed loss of heterozygosity (LOH) [17]. Furthermore, FH enzymatic activity was absent in the tumor [17]. FH is an essential enzyme of the tricarboxylic acid (TCA) cycle (also referred to as the Krebs cycle), and disruption of FH function truncates the cycle leading to the accumulation of intermediates [24, 25]. The TCA cycle is essential for mitochondrial ATP production, and interruption of the cycle should markedly reduce ATP generation by the mitochondria. To meet the ATP demands of the cell, glucose uptake and glycolysis rates are increased [25–28].

Given these findings, the patient was evaluated with a 2-deoxy-2-(^{18}F)fluoro-D-glucose (^{18}FDG)-PET scan, which is not recommended for routine assessment of RCC. Five months after the initiation of temsirolimus, a PET/CT scan showed diffuse ^{18}FDG uptake throughout the abdomen and pelvis consistent with peritoneal carcinomatosis. The size of the implants was small, and they may have been missed by CT alone. Ominously, a dilated loop of small bowel was observed concerning for an impending small bowel obstruction. Treatment options were reevaluated.

There are no established therapies for pRCC-2 in HLRCC patients. The patient may be treated with small molecule kinase inhibitors such sunitinib or sorafenib, which are approved for advanced RCC, but whether sunitinib and sorafenib are effective against non-clear-cell RCC is undetermined. Furthermore, inasmuch as sporadic

pRCC-2 lack *FH* mutations [29, 30], sporadic and familial pRCC-2 are likely to represent altogether different entities.

We sought to exploit the knowledge we had acquired about the tumor. FH-deficient cells have a high demand for glucose [26–28] and the tumor was intensely FDG avid. pRCC-2-derived FH-deficient tumor cells are unable to grow in 50 mg/dL glucose concentrations, which support the growth of other tumor cell lines [26]. Thus, FH-deficient tumor cells would be expected to be exquisitely sensitive to glycolytic inhibitors, such as 2-deoxy-D-glucose (2DG). 2DG is very similar to FDG and, like FDG, should accumulate in tumor cells, where it would competitively inhibit the glycolytic enzyme, glucose-6-phosphate isomerase.

2DG was obtained for compassionate use, and 2DG dosing was determined based on two phase I clinical trials, which showed overall similar results [31, 32]. While *FH* loss in the tumor may make the tumor exquisitely sensitive to 2DG, given the heterozygous state of the patient, she could be at increased risk for toxicities. Furthermore, as the FH enzyme is a homotetramer [33], the possibility existed that FH activity in normal cells may not be 50% (as would be expected from the loss of one *FH* copy), but much lower. Should the activity of the complex be compromised by the incorporation of a single mutant FH protein into the tetramer, FH activity could be as low as 20%.

To gain insight into the effects of the mutation, we evaluated the role of Asp[341] in a previously reported FH tetramer crystal structure (Protein Data Bank ID 3E04). Asp[341] formed part of the interface between FH monomers and was involved in an intramolecular interaction with Lys[337]. Mutation of Asp[341] to Asn would leave Lys[337] unpaired, which, if anything, should destabilize the complex. To determine experimentally whether the FH[Asp341Asn] protein could be incorporated into complexes, FH-deficient pRCC-2 tumor cells [26] were reconstituted with either wild-type or mutant FH protein. Whereas wild-type FH led to the formation of tetrameric complexes, tetrameric complexes did not form in cells expressing FH[Asp341Asn]. These data were reassuring and suggested that FH[Asp341Asn] did not function as a dominant negative.

Nonetheless, because the patient was heterozygous, 2DG was started at 1/8th of the target dose (8 mg/kg PO q.d.). The patient tolerated the first dose well, and given concerns about an impending small bowel obstruction, 2DG was rapidly escalated, within 8 days, to the target dose (63 mg/kg). There were no toxicities except for grade I electrolyte abnormalities. However, 1 week after the target dose was reached, the small bowel obstruction progressed to a complete obstruction, which was not amenable to surgical intervention. 2DG was stopped and the patient was started on TPN.

We sought to understand the lack of 2DG activity and evaluated the effects of 2DG on FH-deficient pRCC-2 tumor cells in vitro. FH-deficient cells were cultured with near-physiological glucose concentrations (150 mg/dL) and supplemented with 10% 2DG for 4 h a day. Based on PK studies [32], such a regimen should mimic drug exposure in the patient. While 2DG slowed the proliferation of FH-deficient tumor cells, the effect was quite modest [17]. We speculated that a

more sustained exposure may have a greater effect and found that continuous treatment with 10% 2DG abrogated cell proliferation.

After a discussion with the FDA and the IRB, the frequency of 2DG administration was increased, initially to every 8 h and subsequently to every 6 h. To minimize competition, carbohydrates were held from the parenteral nutrition. 2DG was given while the patient was hospitalized and on telemetry. On this regimen, the patient developed symptoms of hypoglycemia (blurred vision, tachycardia, clamminess, etc.), but there were no serious toxicities. After a week on 2DG, and following a period >24 h from the last dose (to avoid competition), a PET/CT was performed. Unfortunately, no evidence of antitumor activity was observed.

To evaluate this further, the effects of 2DG on the metabolism of FH-deficient tumor cells in the laboratory were explored. Whereas at 50% 2DG concentrations, glycolysis was suppressed, glycolysis was largely unaffected by 10% 2DG. However, 10% 2DG had a modest effect on ATP levels. While the drop in ATP was quite small (~10%), this was sufficient to activate the master energy regulator, AMP-activated protein kinase (AMPK). AMPK has been previously shown to be sufficient to inhibit mTORC1 [34], and 10% 2DG led to an AMPK-dependent inhibition of mTORC1 in FH-deficient pRCC-2 tumor cells [17].

These results explained why 10% 2DG inhibited cell proliferation in vitro and provided an explanation for the discrepancy between the effects on FH-deficient cells in culture and those in the patient. The patient had been previously treated with an mTORC1 inhibitor, to which the tumor had become resistant, and thus, should the effect of 2DG be dependent on mTORC1 inhibition, 2DG would be expected to have no effect on the tumor.

In summary, an unusual presentation led to the identification of a novel germline *FH* mutation. Studies in the laboratory determined that the mutation was a loss-of-function mutation and that in the tumor, the wild-type allele was lost and FH activity was absent. This defect was exploited for diagnostic and therapeutic purposes. An inhibitor of glycolysis was evaluated, and dosing was adjusted for the heterozygous state of the patient and the possibility that mutant FH functioned as a dominant negative. The drug was shown to be active against FH-deficient pRCC-2 tumor cells in culture, and the 2DG regimen was optimized based on in vitro studies. Finally, insight was obtained into the mechanism of 2DG action at pharmacologically relevant concentrations, and an explanation was obtained for the discrepancy between the in vitro studies and the results in the patient. More broadly, this represents the first attempt to inhibit glycolysis in a tumor with a genetic defect in an enzyme of the Krebs cycle, and these type of tumors may be most suitable for the evaluation of glycolytic inhibitors in cancer.

These two reports illustrate attempts at personalized cancer therapy. In both instances, the treatment was informed by the molecular characteristics of the tumor. The mutations exploited were germline mutations in two-hit tumor suppressor genes. As the first hit was already present in all cells, the loss of the corresponding wild-type allele is likely to have been the tumor-initiating event, and as a consequence, tumors developed early in life. Tumor-initiating events are likely to engender a greater dependency in tumor cells than subsequent mutations, and this may explain

the sustained benefit of mTORC1 inhibitors in the first patient. While only a small percentage of renal tumors are accounted for by germline mutations, the principles governing the use of molecular genetic information for therapeutic purposes are similar and therefore applicable to sporadic tumors.

8.2 From Genes to Drugs: A Historical Perspective

8.2.1 The von Hippel–Lindau Pathway

Up until 2005, a single drug, interleukin-2, had been approved for RCC by the FDA. From 2005 to 2011, six drugs (or drug combinations) reached marketing approval. These drugs belong to two classes, inhibitors of angiogenesis and mTORC1 inhibitors [35]. The large expansion in the armamentarium against RCC has its roots in a greater understanding of the molecular genetics and the biology of RCC, particularly of clear-cell RCC (ccRCC) [36]. In 1993, the gene von Hippel–Lindau (*VHL*) was identified as the gene responsible for conferring an inherited predisposition to ccRCC development [37]. Subsequently, *VHL* was found to be frequently mutated in sporadic ccRCC [38]. *VHL* is inactivated either genetically or epigenetically [39] in over 90% of sporadic ccRCCs [40].

Over the following decade, the function of the VHL protein (pVHL) was elucidated. pVHL was determined to be an essential component of an oxygen signaling pathway. When pVHL is inactive, genes normally induced under conditions of hypoxia become constitutively expressed [41]. In *VHL*-deficient cells, the hypoxia-inducible factor (HIF) transcription factor, which would normally be active only during hypoxia, was constitutively active [42]. HIF refers to a family of heterodimeric transcription factors (HIF-1, HIF-2, and HIF-3) composed of a labile α subunit and a stable β subunit. pVHL acts as the substrate recognition subunit of an E3 ubiquitin ligase complex that targets HIF-α subunits for degradation [42–46]. In the presence of oxygen, molecular oxygen is used by a family of prolyl hydroxylases to hydroxylate HIF-α at specific prolyl residues [47, 48]. This creates high-affinity binding sites for pVHL leading to HIF-α ubiquitylation and degradation [49–51]. When oxygen levels are low, prolyl residues remain unmodified, and HIF-α subunits escape pVHL recognition, interact with β subunits, and form an active heterodimeric transcription factor [52]. However, when pVHL is inactive, HIF-α subunits accumulate regardless of oxygen levels, leading to increased HIF activity and the expression of, among others, vascular endothelial growth factor A (VEGF-A or VEGF) and platelet-derived growth factor B (PDGF-B) [53], which are implicated in angiogenesis and may explain the vascular nature of ccRCC tumors.

The importance of *VHL* in tumor suppression was further established through reconstitution experiments. Reintroduction of wild-type *VHL* into *VHL*-deficient RCC cells inhibited tumor formation in xenograft assays [54]. Using similar approaches, HIF-2α was shown to be both necessary and sufficient for tumor growth downstream

of pVHL [55–57]. HIF-1α function in ccRCC is less clear [58–60]. Somatic loss-of-function mutations have been found in *HIF-1α* in ccRCC [61, 62], and while they are rare, *HIF-1α* has been recently proposed to act as a tumor suppressor [63]. Further studies are needed to clarify the role of HIF-1.

Overall, these findings led to the notion that interfering with VEGF and PDGF-B signaling downstream of HIF may affect ccRCC development and laid the foundation for the evaluation of bevacizumab, a VEGF neutralizing antibody [64, 65–67]. Similarly, small molecule inhibitors of VEGF receptor-2 (VEGFR2) and PDGF receptor-β (PDGFRβ) including sorafenib, sunitinib, and pazopanib, were evaluated against ccRCC and found to be effective [68–70].

To date, most efforts at targeting the pVHL pathway have focused on the development of drugs inhibiting angiogenesis. However, other pVHL and HIF functions may be important for tumor development [36]. Among the most striking effects of HIF are its effects on metabolism [53]. HIF-1 activation under conditions of hypoxia reroutes ATP production from oxidative phosphorylation (which requires oxygen for electron disposal) to glycolysis, which can occur anaerobically [53]. Interestingly, recent experiments in mice showed that acute *VHL* disruption in hepatocytes, which results in an accumulation of lipid reminiscent of ccRCC [71–75], causes a HIF-dependent inhibition of mitochondrial respiration [75]. *VHL* loss suppresses glucose and ketone production by the liver leading to the death of mice within days [75]. The effects of *VHL* inactivation are abrogated by simultaneous disruption of *HIF-1β*, which is required for both HIF-1 and HIF-2 function [75]. Probably as a result of a blockade in mitochondrial oxygen utilization, partial oxygen pressures in *VHL*-deficient livers are increased [75]. Thus, no other pathways exist in hepatocytes that allow oxygen utilization when HIF is active. The relative contribution of HIF-1α and HIF-2α to this process remains to be determined, but HIF-2 may play an important role [73]. Should a similar inhibition of mitochondrial respiration be found in ccRCC, it would portend a dependency on glycolysis for energy generation which may be amenable to therapeutic exploitation.

Vulnerabilities resulting from *VHL* inactivation in ccRCC have also been explored through more pragmatic approaches. W.G. Kaelin and colleagues conducted a synthetic lethal RNAi screen to identify kinases that, when downregulated, reduced the fitness of *VHL*-deficient cells [76]. shRNAs targeting 15% of the human kinome were evaluated in *VHL*-deficient (and reconstituted) RCC tumor cells. Knockdown of CDK6, cMET, and MEK1 preferentially affected *VHL*-deficient RCC cell lines. Because kinases are amenable targets for drug development, the findings could have therapeutic implications. Furthermore, a small molecule CDK6 inhibitor showed increased activity against *VHL*-deficient cells, and this is important, as interfering with gene expression may have effects beyond enzymatic inhibition.

A.J. Giaccia and colleagues reported a chemical-genetic screen to identify compounds preferentially targeting *VHL*-deficient cells [77]. Among 64,000 compounds screened, several were identified with increased activity (in the low micromolar range) against *VHL*-deficient cells.

8.2.2 *The mTORC1 Pathway*

Research we conducted provided a rationale for targeting mTORC1 in RCC [78]. Similarities between VHL and TSC syndromes (and the corresponding genetically engineered mouse models) led us to hypothesize that a functional overlap existed between pVHL and the TSC1/TSC2 complex [78]. We found that like pVHL loss, disruption of TSC1/TSC2 resulted in HIF-1 activation and increased VEGF production and that the effects were reverted in part by mTORC1 inhibition [78]. Parenthetically, deregulation of HIF and VEGF in response to mTORC1 activation appears to be a common feature of familial hamartoma syndromes [79].

mTORC1 includes mTOR, an atypical protein kinase with a kinase domain with structural similarities to phosphatidylinositol 3-kinase (PI3K), and regulatory-associated protein of mTOR (Raptor), an adaptor protein that plays an important role in determining substrate specificity [80–82]. Mammalian lethal with Sec13 protein 8 (mLST8) also forms part of this complex [82, 83], but mLST8 is dispensable for mTORC1 activity, at least during development [84]. In addition, other regulatory proteins associate with and inhibit mTORC1, proline-rich Akt substrate of 40 kDa (PRAS40) [85, 86], and DEP domain-containing TOR-interacting protein (Deptor) [87].

8.2.2.1   Regulation of Protein Translation by mTORC1

mTORC1 plays a critical role in the regulation of cell growth (cell mass), which at least in part results from increasing protein translation (Fig. 8.2). The best characterized substrates of mTORC1 are implicated in regulating protein synthesis, S6 kinase 1 (S6K1) [88] and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). Phosphorylation of S6K1 by mTORC1 contributes to its activation, and S6K1 in turn phosphorylates the small ribosomal subunit protein S6, eIF4B, programmed cell death 4 (PDCD4), and cap-binding protein 80 (CBP80) [88, 89]. 4E-BP1 phosphorylation by mTORC1 leads to its dissociation from the 5′ cap of mRNAs, which serves as a launching pad for translation initiation [90]. The disengagement of 4E-BP1 allows eIF4G binding and the assembly of a translation preinitiation complex that will scan the mRNA untranslated region until the start codon, at which point protein translation will begin.

8.2.2.2   Transcription Factor Regulation by mTORC1

Besides its role in the regulation of protein translation, mTORC1 has also been implicated in the regulation of gene expression (Fig. 8.2). Several transcription factors are regulated by mTORC1. mTORC1 regulates HIF-1 [78, 91–96], and while the
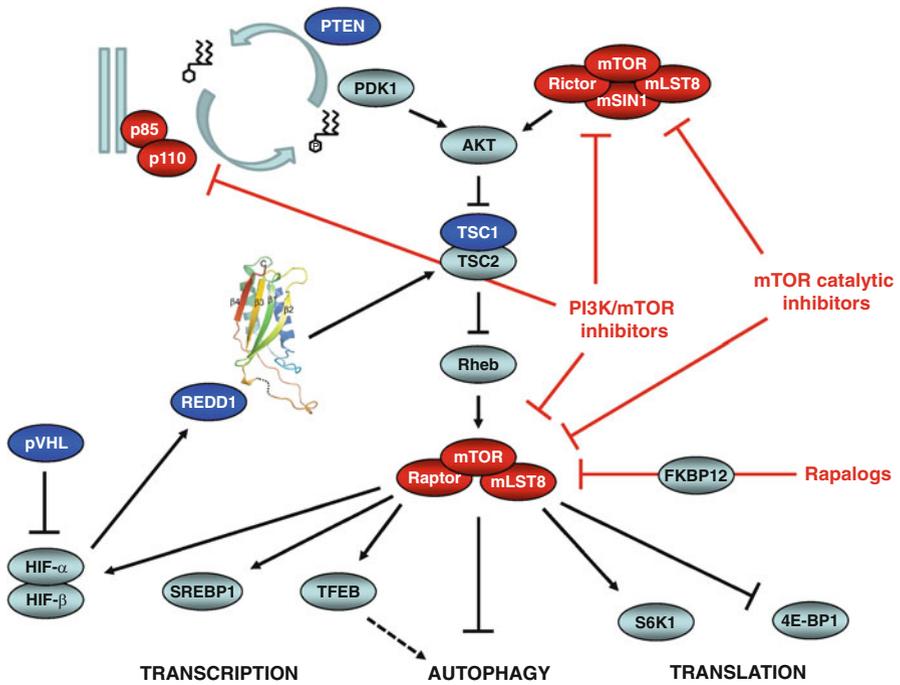
Fig. 8.2 Cross talk between pVHL and mTORC1 pathways in ccRCC. In *red*, mTOR complexes and PI3K; in dark *blue*, tumor suppressor proteins mutated in ccRCC; ribbon diagram, REDD1 structure (reprinted with permission from Vega-Rubin-de-Celis et al. *Biochemistry* 49 (11):2491–2501; Copyright 2010, American Chemical Society)

precise molecular mechanism remains to be fully elucidated [97–99], this provides a link between trophic functions and angiogenesis. In addition, mTORC1 regulates sterol regulatory element-binding protein 1 (SREBP1) [98, 100]. mTORC1 promotes the nuclear localization of the mature form of SREBP1 coupling thereby lipogenesis to protein synthesis and cell growth.

Recently, we discovered another transcription factor regulated by mTORC1, the transcription factor EB (TFEB) [101]. mTORC1 coordinately regulates the phosphorylation and nuclear localization of TFEB. TFEB is a basic helix-loop-helix (bHLH) transcription factor of the Myc family, microphthalmia transcription factor (MITF) subfamily, and a master regulator of lysosomal biogenesis [102]. TFEB has also been recently implicated in autophagy [103]. mTORC1 promotes TFEB nuclear localization, and TFEB is responsible for a large percentage of genes whose expression is induced by mTORC1 [101]. Interestingly, the *TFEB* gene is translocated in an uncommon type of renal tumor that occurs primarily in children and young adults. In these translocation carcinomas, which may also involve the closely related family member *TFE3*, *TFEB* is constitutively activated [104, 105].

Given that TFEB nuclear localization is regulated by mTORC1, should this be the case also in translocation carcinomas, mTORC1 inhibitors may be highly effective.

8.2.3 Interplay Between pVHL and mTORC1 Pathways

Arguably, the two most important pathways in ccRCC pathogenesis are those governed by pVHL and mTORC1. However, little is known about the interplay between these two pathways. We have found that regulated in development and DNA damage response 1 (REDD1), a negative regulator of mTORC1 [106], links pVHL and mTORC1 pathways in ccRCC [107] (Fig. 8.2). REDD1 is physiologically induced by hypoxia [108] and REDD1 is sufficient to inhibit mTORC1 [109]. mTORC1 inhibition under hypoxia allows cells to shift resources from protein translation, an energy-consuming process, to more pressing activities.While REDD1 is broadly implicated in mTORC1 regulation by hypoxia [106, 110–112], in some cell types, hypoxia signals are transduced to mTORC1 independently of REDD1 [112].

REDD1 expression is upregulated in the majority of ccRCC [107]. REDD1 is induced by both HIF-1 [108] and HIF-2 [107], and since REDD1 overexpression is sufficient to inhibit mTORC1 [106, 113], these results present a paradox, particularly since mTORC1 has been reported to be active in a large percentage of ccRCCs [114, 115]. Interestingly, strategies have evolved in tumors to disengage mTORC1 from REDD1 control [107]. In some ccRCCs, *REDD1* is mutated, but the frequency, although comparable to that of *PTEN*, is rather low. *PTEN* loss may also uncouple mTORC1 from REDD1 [107]. Another mechanism involves the inactivation of the TSC1/TSC2 complex, as REDD1-induced mTORC1 inhibition is TSC1/TSC2-dependent [106]. Despite previous reports [116], we recently discovered that *TSC1* is mutated and inactivated in sporadic ccRCC [107]. TSC1 inactivation blocks REDD1 action on mTORC1 [106, 107]. In contrast, we did not find mutations in *TSC2* [107]. Interestingly, mutations have also been reported in *mTOR* [61, 117]. These mutations appear to selectively activate mTORC1 and may similarly uncouple mTORC1 from REDD1 [118]. However, even when taken together, all these mutations still account for a small percentage of ccRCCs, and how the remaining tumors maintain mTORC1 activity despite REDD1 upregulation is unclear.

Light may be shed into this problem by uncovering the molecular mechanism of REDD1 action. REDD1 has been proposed to function by binding to and sequestering 14-3-3 proteins away from TSC2 [119]. However, it is unclear how REDD1 would sequester 14-3-3 proteins, which are very abundant and interact with over 100 proteins in cells [120]. In addition, structural and mutagenesis studies we conducted show that the putative 14-3-3 binding site in REDD1 does not conform to any 14-3-3 binding sites known and that residues typically critical for 14-3-3 binding

are dispensable for REDD1 function [121]. Thus, how REDD1 inhibits mTORC1 remains a mystery. The answer to this question may provide fundamental insights into ccRCC progression.

8.2.4 Targeting mTORC1

TOR was identified on the basis of genetic [122] and biochemical assays [123, 124] using rapamycin (also called sirolimus), a macrolide antibiotic with growth inhibitory properties. Rapamycin functions as an allosteric inhibitor. Rapamycin binds to FKBP12 (FK506-binding protein, MW of 12 kDa) and, as a complex, interacts with a region upstream of the kinase domain of mTOR, referred to as the FKBP12-rapamycin binding (FRB) domain (Fig. 8.2). Interestingly, despite that mTOR is present in a second complex, mTORC2, for reasons that are not completely understood, sirolimus does not bind to mTORC2 [82, 125, 126]. However, prolonged exposure to sirolimus in some cell types results also in mTORC2 inhibition [127, 128], possibly because mTOR becomes sequestered by sirolimus.

Two sirolimus analogues have been approved for treatment of advanced RCC, temsirolimus and everolimus. Both analogues differ from sirolimus by side chain substitutions at a single carbon atom of the macrolactone ring that is not directly involved in FKBP12 or mTOR binding. Thus, while these modifications alter the pharmacokinetic properties of the compound, the inhibition of mTORC1 by all three drugs is likely to be indistinguishable. Furthermore, temsirolimus is largely a sirolimus prodrug. Seventy percent of circulating drug levels following temsirolimus administration are actually sirolimus [129, 130], and we previously reported the treatment of a patient with RCC with sirolimus before temsirolimus became commercially available [131].

Temsirolimus was evaluated in a phase III trial involving patients with previously untreated metastatic RCC of clear-cell and non-clear-cell types that were in a poor prognostic group as defined by modified MSKCC criteria including metastasis in multiple organs [18]. By comparison to interferon-α, temsirolimus improved median overall survival (OS) (10.9 vs. 7.3 months; HR for death, 0.73; 95% CI, 0.58–0.92; $p=0.008$).

Everolimus was evaluated in patients with metastatic ccRCC progressing to antiangiogenic therapy [132]. Most patients had received sunitinib or sorafenib, and approximately 25% had received both. In addition, over 50% of patients had also been treated with immunotherapy. Everolimus resulted in an improvement in median progression-free survival (PFS) of 2.1 months by comparison to placebo (1.9 vs. 4.0 months; HR for progression, 0.30; 95% CI, 0.22–0.40; $p<0.0001$).

Both temsirolimus and everolimus exhibit a similar adverse effect (AE) profile that includes, among the most serious AEs, pneumonitis. Other less serious, but more common AEs include stomatitis, hyperlipidemia, hyperglycemia, thrombocytopenia, and anemia [133]. While little is known about the pathogenesis of anemia, it

tends to be microcytic, and given the role of mTORC1 in protein translation, it may reflect reduced hemoglobin synthesis. Whether this effect could have clinical application, for instance in the treatment of sickle cell anemia, is unknown.

What determines responsiveness to mTORC1 inhibitors is unclear, but presumably only tumors with active mTORC1 would be responsive and this is supported by a small retrospective study [134]. However, at least as determined by phospho-S6$^{S235/236}$ (pS6$^{S235/236}$) levels, most RCCs appear to have increased mTORC1 activity [114, 115]. While pS6$^{S235/236}$ levels may be influenced by other signaling pathways besides mTORC1 [135], pS6$^{S235/236}$ levels tend to correlate well with pS6$^{S240/244}$ in ccRCC [107]. Interestingly, Pantuck et al. observed a correlation between pS6$^{S235/236}$ and Fuhrman grade [114]. This correlation could be explained by the fact that mTORC1 regulates ribosome biogenesis, a process that takes place in the nucleolus, and that nucleolar size is an important determinant of the Fuhrman grading scale. Indeed, nucleolar size is affected by mTORC1 [136, 137]. This has several implications. First, nucleolar size (and perhaps Fuhrman grade) may serve as a pharmacodynamic indicator of mTORC1 activity. Second, the prognostic significance of the Fuhrman grading scale may be due, at least in part, to mTORC1. Finally, since mTORC1 inhibitors reduce nucleolar size [137], the assessment of Fuhrman grade could be affected by prior mTORC1 inhibitor therapy.

8.2.5 Uncovering Mechanisms of Resistance to mTORC1 Inhibitors

To identify mechanisms of resistance to mTORC1 inhibitors, we opened a phase II clinical trial, "Neoadjuvant everolimus for advanced RCC before cytoreductive nephrectomy with correlative tumor studies" (NCT00831480). Patients presenting with metastatic RCC and a primary tumor in place who are eligible to undergo cytoreductive nephrectomy (CRN) will receive everolimus for 3–5 weeks, then undergo CRN, and subsequently receive everolimus until progression. The primary endpoint of the study is PFS at the end of the fourth month of everolimus treatment following CRN, and the trial is designed to provide an exceptional platform to explore how resistance to mTORC1 inhibitors develops (Fig. 8.4). The primary tumor will be biopsied prior to everolimus initiation, tissue will be obtained at nephrectomy, and a metastatic site showing progression will be biopsied at that time. During nephrectomy, biopsies will be performed of the tumor prior to ligation of the renal artery so as to minimize confounding effects from tissue ischemia. In addition, in order to provide an adequate context for the interpretation of pharmacodynamic studies in the tumor tissue, they will be integrated with measurements of circulating drug levels as well as pharmacodynamic analyses on peripheral blood mononuclear cells. Finally, everolimus will be continued for 24 h after the biopsy

of the metastasis. Should mTORC1 be active in the metastasis, we should be able to determine that this occurred despite mTORC1 inhibition in PBMCs and adequate circulating drug levels.

8.2.6   Novel Approaches to Targeting mTOR

Catalytic inhibitors that bind to the kinase domain of mTOR are being evaluated. These inhibitors more potently inhibit mTORC1 phosphorylation of 4E-BP1 [138, 139], and as a consequence may have greater effects on cell proliferation and tumorigenesis [140–144]. Unlike sirolimus analogues, these inhibitors also target mTORC2. mTORC2 shares with mTORC1, mLST8, and DEPTOR, but whereas mLST8 is dispensable for mTORC1 activity, mTORC2 requires mLST8 [84]. In addition, mTORC2 function requires rapamycin-insensitive companion of mTOR (Rictor) [125] and mSIN1 [145].

mTORC2 phosphorylates Akt [146], serum- and glucocorticoid-regulated kinase (SGK) [147], and several protein kinase C (PKC) isoforms [84]. Akt phosphorylation by mTORC2 at S^{473} contributes to its activation, and Akt promotes survival and proliferation. Akt^{S473} phosphorylation is important for the phosphorylation of some substrates including forkhead box protein O1 (FoxO1) and FoxO3 [84, 145]. Phospho-rylation of FoxO transcription factors by Akt sequesters them in the cytosol where they are unable to activate gene expression and induce apoptosis [148].

Several lines of evidence suggest that mTORC2 inhibition in RCC may be beneficial. First, mTORC2 inhibition may downregulate Akt activity, and Akt appears to be phosphorylated in the majority of RCCs [114]. Second, mTORC1 is involved in several negative feedback loops that dampen growth factor receptor signaling when mTORC1 is active, and mTORC1 inhibition increases the levels of receptor tyrosine kinases [149] and adaptor proteins [150–153]. Increased growth factor receptor signaling with mTORC1 inhibitors may be offset, at least partially, by Akt inhibition. Finally, at least in a *TSC1* heterozygous background, experiments in mice suggest that FoxO proteins block renal tumor development [154]. Therefore, by inhibiting Akt, mTORC2 inhibitors may activate FoxO proteins and suppress RCC development.

Akt activation is also regulated by phosphorylation at T^{308}, which is mediated by 3-phosphoinositide-dependent kinase 1 (PDK1). In response to growth factor stimulation, class Ia PI3Ks are recruited to sites of receptor phosphorylation via the p85 regulatory subunit, leading to the activation of the catalytic p110 subunit. p110 is thereby brought into proximity to its lipid substrates at the plasma membrane resulting in the generation of the second messenger phosphatidylinositol-3,4,5-tris-phosphate, which in turn recruits and activates PDK1 [155, 156] (see also Fig. 8.2). Because the kinase domain of mTOR is similar to that of PI3Ks, inhibitors are available that target not only mTOR (mTORC1 and mTORC2) but also PI3Ks, and this

may further reduce Akt activation and increase antitumor activity [157]. One such inhibitor, GSK2126458, which targets mTOR complexes and class I PI3Ks was recently shown in a first-in-human trial that included 25 patients with previously treated RCC to result in two partial responses [158].

Since reactivation of feedback loops following mTORC1 inhibition results not only in the activation of Akt but also of extracellular signal-regulated kinase (ERK) [159], mTORC1 inhibitors are also being studied in combination with mitogen-activated protein kinase/ERK kinase (MEK) inhibitors. Attempts have also been made to combine mTORC1 inhibitors with sorafenib and sunitinib, but these combinations are poorly tolerated at full doses of each agent. In contrast, mTORC1 inhibitors can be combined with bevacizumab at full doses, but there are no conclusive studies presently as to whether the combination is synergistic.

8.3   Uncovering New Targets with Genome Sequencing

By comparing tumor and normal genomes from the same patient, a list of somatically acquired mutations can be compiled among which driver mutations are to be found. However, distinguishing "driver" from "passenger" mutations is challenging. Since the probability that random mutations be activating is very low (the number of possible changes that could be introduced in a protein-coding sequence that would enhance protein function is typically very small), gain-of-function mutations have a higher likelihood of being tumorigenic. However, loss-of-function mutations, which represent the majority of mutations in tumors, are more difficult to interpret. The probability that a loss-of-function mutation is pathogenic may be increased if it is associated with LOH, but this criterion alone is not enough.

Genes that are recurrently mutated in tumors of a specific histology have a higher likelihood of driving tumor formation. Statistical analyses adjusted for multiple comparisons may single them out. In addition, while the mutation frequency of a particular gene in a specific tumor type may not be statistically significant, in the context of other genes acting in the same pathway, the findings may be significant. Assuming that the pathway was linear, mutations at multiple levels would be expected not to confer a selective advantage and should be infrequently observed together.

In keeping with large scale recent studies, in possibly the first whole genome sequence of a ccRCC to be reported [160], approximately 6,500 somatically acquired mutations were identified. There were 63 mutations in protein-coding genes (or splice sites) including a mutation in *VHL*. Interestingly, there was no enrichment for mutations in protein-coding regions (which account for ~1% of the genome) suggesting that other regions in the genome may be similarly important.

A strategy utilized by several groups, including ours, has been the search for recurrently mutated genes in small numbers of tumors. An analysis of seven ccRCCs by the Sanger Institute identified polybromo 1 (*PBRM1*) [117], a gene subsequently found to be mutated in 35% of ccRCCs, which encodes a component of a SWI/SNF

nucleosome remoding complex. The same group previously reported mutations in *SETD2*, *KMD5C* (*JARID1C*), and *KMD6A* (*UTX*) in 1–3% of ccRCCs [61, 161]. Another study of seven ccRCC exome pairs that included follow-up sequencing of ~80 chromatin remodeling genes in 96 samples identified the following genes and mutation frequencies: *SETD2* 12%, *MLL2* 10%, *KDM5C* 6%, *MLL* 4%, *ARID1A* 4%, *ASH1L*, and *KDM6A* 1–2% [162]. Overall these data highlight the importance of chromatin remodeling in ccRCC and may offer opportunities for therapeutic intervention.

The sensitivity of RCC sequencing studies is limited however, by stromal contamination. A potential avenue to increase mutation detection involves the study of human tumors growing in mice (referred herein as tumorgrafts). In tumorgrafts, human stroma is replaced by the host thereby eliminating normal human DNA contamination [163]. However, there are intrinsic challenges associated with genome sequencing efforts of tumorgrafts [164]. Overall, the number of mutations in protein-coding genes in ccRCC may be 40–80, and the number may increase in patients with a history of tobacco use.

Thus far, only two genes have been identified in ccRCC with mutation frequencies greater than 25%, *VHL* and *PBRM1*. This may make the development of molecularly targeted therapies broadly applicable to large number of patients difficult. However, genes may be integrated into pathways, and pathways may emerge that are more broadly deregulated in ccRCC.

8.3.1 *"Actionable" Mutations*

While the number of frequently mutated genes may be low, genome sequencing efforts may uncover rare, but "actionable," mutations. Presently, the main class of actionable mutations is made up of activating mutations in protein kinases. Protein kinases are amenable targets for drug development, and because most inhibitors target the kinase domain, which has shared structural features, kinase inhibitors tend to be active against multiple kinases [165]. Thus, while an inhibitor might have not been developed to target a particular kinase, one may exist that is cross-reactive.

Unfortunately, kinases are infrequently mutated in ccRCC [166]. To date, the only oncogenic kinase found to be mutated and possibly activated in ccRCC is ERBB4 (our unpublished observations). ERBB4 is a receptor tyrosine kinase of the EGFR family, and ERBB4 activating mutations have been previously reported in melanoma [167]. *ERBB4* mutations are rare in ccRCC (1–2%). Nonetheless, should ERBB4 be a driver in ccRCC, inhibitors with cross-reactivity against ERBB4, such as lapatinib [168], may prove efficacious for this subset of patients. In addition, other kinases for which inhibitors are available, such as JAK2, have been found to be amplified in ccRCC [162]. However, regions of amplification in tumors tend to be large [169, 170], and the driving gene(s) may be unclear.

8.3.2   Turning Mutations into Druggable Targets

In contrast to activating mutations in oncogenes, whose protein products may serve as a target for chemical inhibitors, harnessing inactivating mutations in tumor suppressor genes for drug development is more complex. The identification of effector pathways activated downstream such as VEGF/VEGFR2 in *VHL*-deficient tumors may help. This process requires biological insight and an understanding of the relative contribution of the particular effector pathway to tumor development.

Another approach, which may also be applicable to activating oncogenic mutations, involves the identification of dependencies created by the mutation. Mutations may engender a dependency on another pathway for survival, a pathway that is not normally essential for viability. Experiments in yeast suggest that the majority of nonessential genes (~80%) are involved in such synthetic lethal relationships with one or more genes [171]. Should a similar number of genes be involved in synthetic lethal interactions in humans, this could be a fertile ground for drug development. Several screens have been conducted to identify chemicals (or genes) that target pathways synthetic lethal with *VHL* [76, 77]. Given the high frequency of pVHL inactivation in ccRCC, such efforts may result in a drug broadly active against ccRCC. In addition, inasmuch as synthetic lethal approaches target genetic defects specific to tumor cells, the drug should have a suitable therapeutic window. However, there may be tissues in which the gene (i.e., *VHL*) is not normally expressed and this may cause toxicity.

8.4   Improved Model Systems

A bottleneck in oncology drug development results from the lack of preclinical models that faithfully recapitulate human cancer. More than 80% of anticancer drugs in clinical trials fail to reach FDA approval [172, 173]. The rate of failure for anticancer drugs is twice that of drugs in other categories [172]. The annual toll on patient lives and resources is enormous. Thus, current paradigms and preclinical models are clearly inadequate.

Most preclinical studies evaluate drugs on established tumor cell lines generated sometimes decades ago (i.e., NCI-60 panel) [174]. However, the value of the cell lines is compromised by the acquisition of new mutations [175, 176]. Indeed, the number of DNA copy number alterations in RCC cell lines is substantially larger than in patient tumors [169]. Cell lines may be injected into immunocompromised mice but the tumors they form tend to be poorly differentiated and dissimilar from the tumor from which they were originally derived [175–178].

Tumor samples when implanted directly in mice (tumorgrafts) may better preserve the characteristics of patient tumors. The implantation of tumor fragments orthotopically in mice without disaggregation or additives results in tumors that reproduce the histological characteristics (Fig. 8.3) and molecular genetic altera-
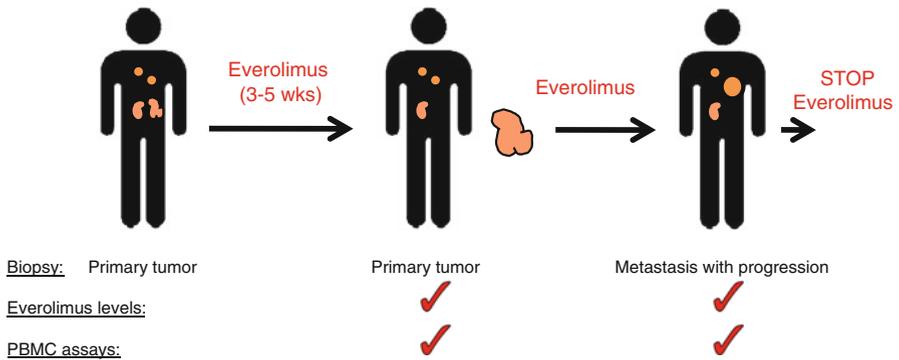
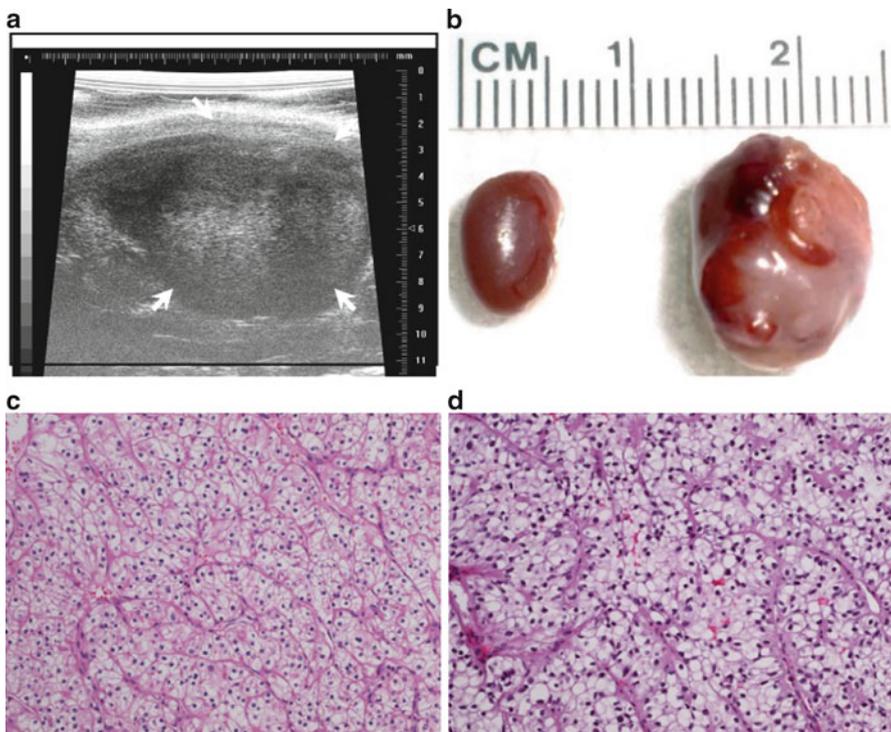**Fig. 8.3** Schema of neoadjuvant everolimus trial in patients with advanced RCC with correlative studies

Fig. 8.4 Tumorgraft model of RCC. (**a**) Ultrasound of a tumorgraft growing orthotopically in a mouse kidney. (**b**) Pictures of a normal kidney in a mouse and the contralateral kidney with a RCC. (**c**, **d**) Histological tissue sections of tumor from the patient (**c**) and corresponding tumorgraft (**d**)

tions (mutations and DNA copy number alterations) of the corresponding tumors in patients [179]. Most importantly, tumorgrafts reproduce the drug sensitivity of RCC in the clinic. After PK studies to identify regimens mimicking human drug exposures, tumorgraft growth was inhibited by drugs active against RCC but not by a control drug [179].

While growth in mice may select for most aggressive tumors (or most aggressive components within a tumor) [180], tumorgrafts represent the most suitable preclinical model available. They reproduce the histology, molecular genetic alterations, and treatment responsiveness of RCC in patients.

Tumorgrafts may be used for the evaluation of novel agents, to explore drugs in different contexts (i.e., frontline vs. second line) and to prioritize drug combinations. When a drug fails, tumorgrafts facilitate determining whether the failure was due to inadequate target inhibition (in which case, the target may still be valid) or whether it occurred despite adequate target inhibition. This determination, which is of utmost importance [181], is not often possible in the clinic. Tumorgrafts can also be used to explore mechanisms of resistance and may be particularly helpful in elucidating resistance to agents that target the tumor microenvironment. In addition, tumorgrafts may be instrumental for the development of pharmacodynamic markers.

Tumorgrafts are helpful for a variety of other applications. An important obstacle to characterizing the genome of solid tumors is that tumors are an admixture of tumor and normal stroma. In contrast, the stroma in tumorgrafts is derived from the host [163], and tumorgrafts represent nearly pure populations of human tumor cells. This is useful to determine whether a mutation is heterozygous or homozygous. In our own experience, tumorgrafts were critical in determining that 20% of the protein-coding gene mutations we identified were associated with loss of the wild-type allele. In addition, tumorgrafts provide a means to study rare forms of cancer and a tumorgraft line has been generated from the pRCC-2 of the HLRCC patient described before. Finally, tumorgrafts may be used for the evaluation of novel imaging modalities. We reported recently that acute *VHL* loss in the liver of the mouse is sufficient to inhibit mitochondrial respiration with a consequent increase in partial oxygen pressures as determined by MRI oximetry [75]. The availability of tumorgrafts would allow us to establish whether *VHL* loss in ccRCC similarly blocked mitochondrial oxygen consumption.

8.5   Genomic Medicine

Tumor genome sequencing may not only present candidates for drug therapy but is likely to pave the way for the identification of prognostic and predictive markers. The discovery of a somatically acquired mutation in *TSC1* in a ccRCC from a patient whose disease progressed on frontline sunitinib after 3 months, but who remained progression-free on everolimus for 13 months [107, 160] led us to hypothesize that TSC1 is a predictor of extraordinary responsiveness to mTORC1
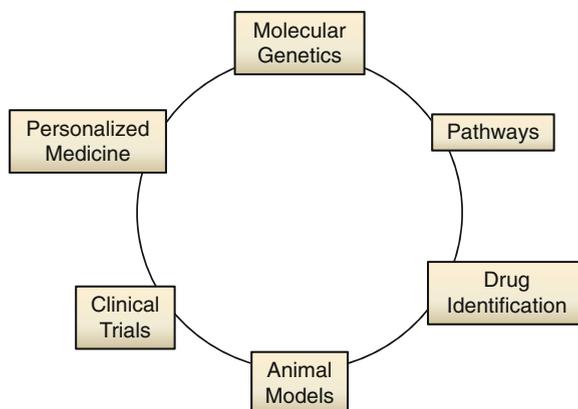
inhibitors clinically. The prolonged tumor control with everolimus contrasted with both the rapid progression on sunitinib and a median progression-free interval on everolimus in the pivotal phase III clinical trial of 4 months [132]. While this represents a single case, there have been other reports of unusual responsiveness to mTORC1 inhibitors [182], and we conjecture that these tumors may similarly have mutations in *TSC1* or other proximal mTORC1 regulators. In addition, sequencing of the normal genome as a reference may provide information with respect to drug metabolism or even about haplotypes associated with drug responsiveness.

Establishing the clinical utility of cancer genome sequencing will be a challenge. One approach may involve clinical trials in which patients are randomized to treatment decisions based on genome sequencing data or decisions made without such information. The outcome of such a trial would depend, however, on the interpretation of the genomic information, and furthermore, unless genome-based decision algorithms were clearly laid out, the approach would not be transferable to other settings. Alternatively, as recently illustrated in a different setting [183], patients may serve as their own control, and the effectiveness of genomic-based treatment decisions may be compared to outcomes of an immediately prior experimental, nongenomic-based regimen. However, this paradigm is subject to some of the same limitations.

8.6   Conclusions

Herein, I have attempted to present a vision for research translation and personalized medicine. As is the case for drugs recently approved, future therapeutic developments are likely to result from a deeper understanding of the molecular genetics of RCC. Very often, the findings from cancer genome sequencing studies will be novel and the significance of the mutations unclear. Their evaluation will require functional studies and an integrated research translation program (see Fig. 8.5). The function of mutated genes can be explored through molecular and cellular biological

approaches and the consequences of gene mutation exploited in synthetic lethal chemical-genetic screens. These screens may result in chemical leads, which after optimization, could be evaluated in suitable animal models and perhaps subsequently in clinical trials.

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