The role of the Von Hippel-Lindau tumor suppressor protein in clear cell renal carcinoma

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Abstract

Up to 88% of patients who suffer from clear cell renal carcinoma (ccRCC) have inactivating mutations in the Von Hippel-Lindau tumor suppressor gene. Also patients who have a certain type of the familial Von Hippel-Lindau syndrome, harboring a inactivating germline mutation in the *VHL* gene have a 60% chance of developing ccRCC. The VHL protein, pVHL, functions as an E3-ubiquitin ligase for Hypoxia Induced Factor α (HIFα) causing proteasomal mediated degradation of HIFα under normoxic conditions. In recent years other, non-HIFα related functions of pVHL, have been discovered. pVHL is also involved in *cJun*-NH_2-kinase (JNK) regulation by acting as a scaffold for CK2, and regulation of the NFκB pathway by ubiquitinating atypical PKCs. pVHL also plays a role in the maintenance of the primary cilium. Biallelic loss of *VHL* causes aberrant transcription of HIFα target genes, increased proliferative potential through JNK signaling and nuclear accumulation of NFκB resulting in antiapoptotic factors. The loss of the primary cilium is thought to lead to the formation of renal cysts, which can result in ccRCC depending on other mutations accumulating in the cells. The complexity of ccRCC has made finding a suitable animal model for the disease very difficult. Several attempts have been made in mice so far but the most promising results to date have been attained in zebrafish.

Introduction

Von Hippel-Lindau disease is an autosomal dominant disorder that is associated with a variety of neoplasms, most notably in the retina and the central nervous system (CNS). Eugen von Hippel first described angiomas, benign neoplasms of endothelial origins, in the retina in 1904. In a 1926 paper Arvind Lindau described angiomas of the cerebellum and spine. In the 1960s the term Von Hippel-Lindau disease became commonplace amongst physicians as case reports started to appear in the medical literature, mainly focusing on the effects of the disease on the retina and CNS.

Based on the observed phenotype, VHL disease has been divided into two main subtypes, type 1 which does not include pheochromocytomas and type 2 which does. Type 2 is further divided into 2A, consisting of pheochromocytomas without renal cell carcinoma, type 2B with the full spectrum of VHL disease associated symptoms and type 2C which consists of only pheochromocytomas (Nordstrom-O'Brien *et al.*, 2010).

In the early 1990s researchers had found out that the gene involved in the disease was located on 3p25 - 26, but the identification of the Von Hippel-Lindau gene did not occur until 1993 when Latif *et al*. analyzed mutations in both VHL-disease patients and patients with sporadic renal cell carcinomas. The protein that arises from the *VHL* gene, VHL protein or pVHL, occurs in two isoforms. Firstly there is full length VHL30, which is 213 aa long and 30 kDa in size, and shortened VHL19 which lacks the first 53 residues at the N-terminus. Both isoforms of VHL function as the substrate recognition subunit (E3) of an ubiquitin ligase complex consisting of elongin B, elongin C, cullin 2 and Rbx1 (Lai *et al*., 2011) , which bears similarity to the SCF ubiquitin ligase complex.

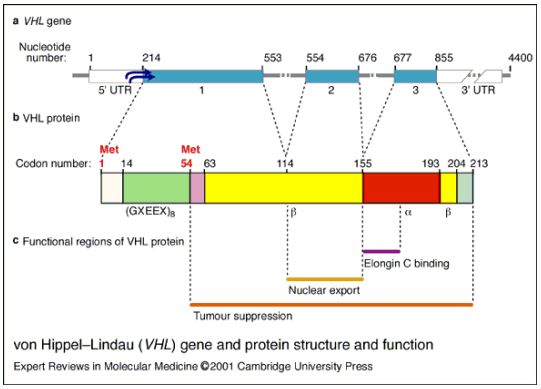

Figure 1: Domain structure of VHL (Richards, 2001)

One the main functions of the VHL E3 ligase complex is the polyubiquitination of Hypoxia Induced Factor α (HIFα). HIFs are a set of transcription factors that regulate the cellular response to hypoxia. Under normoxic conditions HIFα is hydroxylated on two proline residues (402 and 564) (Huang *et al*., 1998) which facilitates the binding to VHL and subsequent proteasomal degradation of HIFα. When cells are in a hypoxic state, HIFα is stabilized and translocates to the nucleus where it, in a heterodimer with a HIFβ family member, upregulates genes that help the cell counter the effects of a lack of oxygen, such as glucose transporter GLUT-1 and vascular endothelial growth factor VEGF. Since neovascularization and increased glucose uptake are also hallmarks of tumorigenesis, HIFα can be regarded as an oncogene, and *VHL*, due to its regulation of HIFα and because it conforms to Knudsons two hit model, it can regarded as classical tumor suppressor.

In recent years HIFα-independent functions of VHL have been discovered. VHL plays an important role in the maintenance of the primary cilium by influencing microtubule dynamics (Lolkema *et al*., 2004). Since renal epithelium is highly ciliated, loss of pVHL has profound effects on these cells and patients with familial VHL disease often have many renal cysts.

Clear cell renal carcinoma (ccRCC) is a type of cancer arising in the cortex of the kidney. According to the WHO, all clear cell type tumors in the kidney are considered to be malignant regardless of size (Lopez-Beltran *et al*., 2009). The predisposition of VHL-disease patients for ccRCC has prompted researchers to investigate the frequency of VHL mutations occurring in sporadic ccRCC cases and it has been found that up to 88% of sporadic ccRCC tumors have biallelic *VHL* inactivation, compared

to 16% in non-clear cell RCC cases, either through promoter hypermethylation or DNA mutations (Moore *et al*., 2011). This review will focus on the molecular characteristics of ccRCC, the role of VHL in the disease and the search for a viable animal model to study ccRCC and VHL disease.

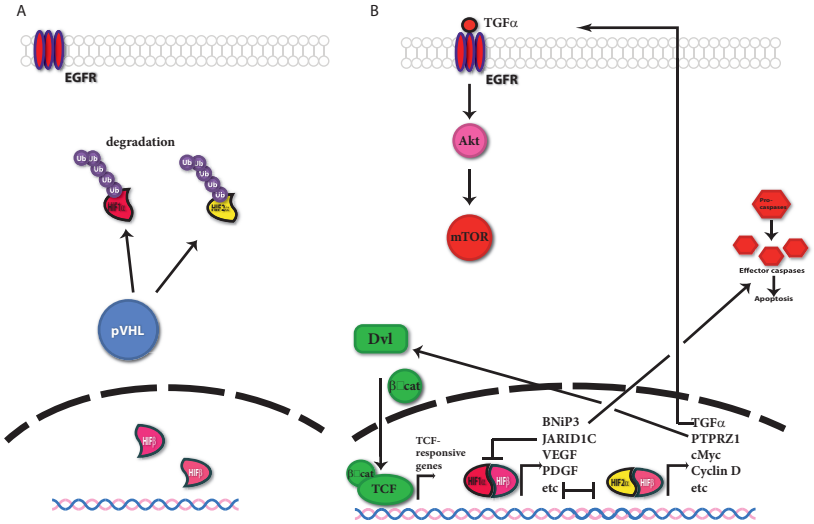

Figure 3: HIFα-dependent processes under normoxic conditions (A) or after pVHL loss (B).

HIFα dependent processes in the formation of ccRCC tumors

HIFs are the main actors in the cellular response to hypoxia. These proteins are basic helix-loop-helix transcription factors that bind to hypoxia responsive elements (HRE) of gene promoters in response to ischaemia. The two HIF isoforms that are best studied are HIF1α and HIF2α, which are both targets of the VHL E3 ligase complex. Both isoforms are hydroxylated on two proline residues by proline hydroxylase domain (PHD) members of the Fe(II) and

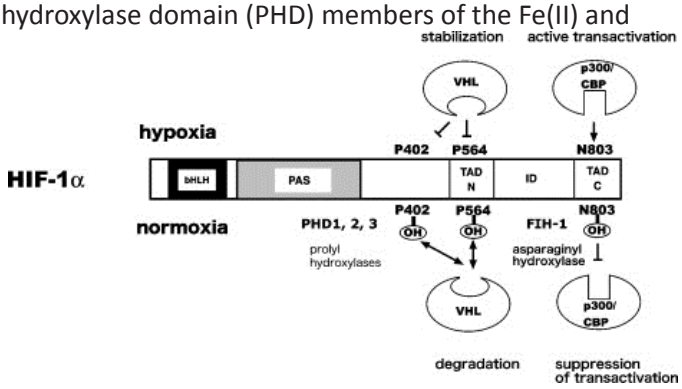

Figure 2: Domain structure of HIF1α. (Hirota & Semenza, 2005)

2-oxoglutarate-dependent oxygenase superfamily, which depend for their activity on the presence of oxygen. Under normoxic conditions, these PHDs are active, HIF1α and HIF2α are hydroxylated and subsequently ubiquitinated by the VHL ubiquitin ligase complex and degraded by the proteasome. In ischaemic conditions, such as myocardial infarction or the inner mass of solid tumors, the PHDs do not hydroxylate HIF and HIF can activate its target genes (Kaelin *et al*., 2008).
VHL deficient tumors have a profound preference for HIF2α over HIF1α expression (Maxwell *et al*., 1999). In normal distal renal tubule tissue HIF1α is mainly expressed, but this changes with tumor progression. Despite their structural similarity, there seem to be a distinct difference between these two HIF isoforms. In neuroblastomas there is a profound difference in HIF isoform expression between well-vascularized areas of the tumor and the area that lacks adequate vasculature (Holmquist-Mengelbier *et al*., 2006). The initial response to hypoxia would seem to be an upregulation of HIF1α and only after prolonged (72 hrs) hypoxia HIF1α is replaced a rising level of HIF2α. This phenomenon is also observed in ccRCC, where Raval *et al*. showed in 2005 that expression of HIF1α negatively regulates HIF2α and vise versa. What they found was that, like in neuroblastoma, higher HIF2α expression strongly correlates with poorer prognosis. Further analysis of the RCC4 (*VHL*-deficient cell line) cells showed that there is reciprocity between HIF1α and HIF2α, with the upregulation of one causing downregulation of the other. Based on their results, it would seem that HIF1α is much less involved in tumorigenesis compared to HIF2α. They studied 5 HIFα target genes that might play a role in cancer: BNip3 (proapoptotic member of the Bcl2 family), CAIX, GLUT-1, VEGF and transforming growth factor α (TGFα). By either inducing or knocking down one of the HIF isoforms they found out that BNip3 and CIAX were induced by HIF1α, but that HIF2α both reduced levels of the proapoptotic BNip3 while simultaneously inducing expression of VEGF, GLUT1 and TGFα, which are associated with tumor progression.
Building on these findings several other groups have studied HIFα. Firstly Zhang *et al*. found in 2007 that HIF1α is mainly involved in reprogramming the cell to cope with hypoxic stress. HIF1α inhibits the oncogene c-Myc, causing a decrease in mitochondria due to a decrease in c-Myc target gene PGC-1β. Since mitochondria are the main reason cells need oxygen and also a part of the apoptotic pathway through Cytochrome c, this decrease in mitochondria simultaneously protects the tumor cells against hypoxia as well as against apoptosis.

Apart from reducing the number of mitochondria HIF1α causes upregulation of pyruvate dehydrogenase kinase 1 which causes pyruvate to be metabolized into lactate instead of acetyl CoA, strongly reducing the need for oxygen in the cell. However this does not provide these cells with any competitive advantage under normoxic conditions. Expressed separately HIF1α and HIF2α seem to have opposing effects on cell cycle progression (Gordon *et al*., 2007). While HIF1α inhibits c-Myc activity resulting in decreased inhibition of p21 and p27 by c-Myc, HIF2α

actively promotes c-Myc activity. This results in repression of p21 and p27, coupled with enhanced Cyclin D2 expression which then leads to increased G1 - S phase progression and subsequent cell division.

Recently another target gene of HIFα was found to correlate with aberrant gene expression and carcinogenesis in ccRCC cell lines. The histone demethylation enzyme JARID1C, was reported to be upregulated in a HIFα-dependent fashion. JARID1C demethylates trimethylated histone 3 lysine residue 4 (H3K4Me3), which alters gene expression in an epigenetic fashion. However this upregulation of JARID1C had a tumorsuppressive effect and malignant ccRCC cell lines had a deactivating mutation in JARID1C. In normal conditions JARID1C could be part of a negative feedback loop for HIFα; upon HIFα activation JARID1C could be produced which then demethylates the promoters of HIFα-responsive genes, resulting in reduced activity. Knocking down JARID1C in 786-O Vhl deficient cell lines caused an increase in tumor size, but the bio logicalsimplications still have to be assessed properly (Niu *et al*., 2012).

Though the sheer number of HIFα targets makes obtaining a complete overview of the biological implications of *VHL* loss practically impossible, it is clear that the importance of HIFα deregulation, especially HIF2α can hardly be understated. By reducing the need for oxygen by increasing glucose uptake and simultaneously reducing the amount of mitochondria, deregulated HIF2α can protect the tumor from hypoxia that arises during rapid growth.

HIFα independent processes in ccRCC

TGF-β target transforming growth factor beta induced (TGFBI) has also been implicated in HIFα-independent regulation by pVHL. Shang *et al* reported in 2012 that cells had a different sensitivity to TGF-β, specifically the induction of TGFBI by TGF-β, depending on *VHL* status. Although they did report increase in adhesion, migration and invasion of RCC cells in culture in response to elevated TGFBI levels in the cells, the exact mechanism of this effect was beyond the scope of their study. Other types of cancer also might have TGFBI involvement. Some groups report a tumor suppressor function, yet in ovarian cancer high TGFBI expression correlates with an agressive tumor phenotype(Ween *et al*., 2013). Interestingly though TGFBI has recently been shown to be essential for blastopore formation in Xenopus laevis. A 2012 study (Wang *et al*., 2012) found that the Xenopus homolog XTGFBI plays an essential role in the induction of Wnt signaling in the developing embryo. The mechanism they proposed was that TGFBI phosphorylates GSK3β, leading to inactivation. This then causes β-catenin to translocate to the nucleus and activate the Wnt-signaling pathway. The Wnt pathway is involved in many types of cancer, and in ccRCC high Wnt signaling correlates with increased tumor diameter, vascular invasion and node involvement compared to patients with low Wnt/β-catenin activity (Kruck *et al.,* 2013).

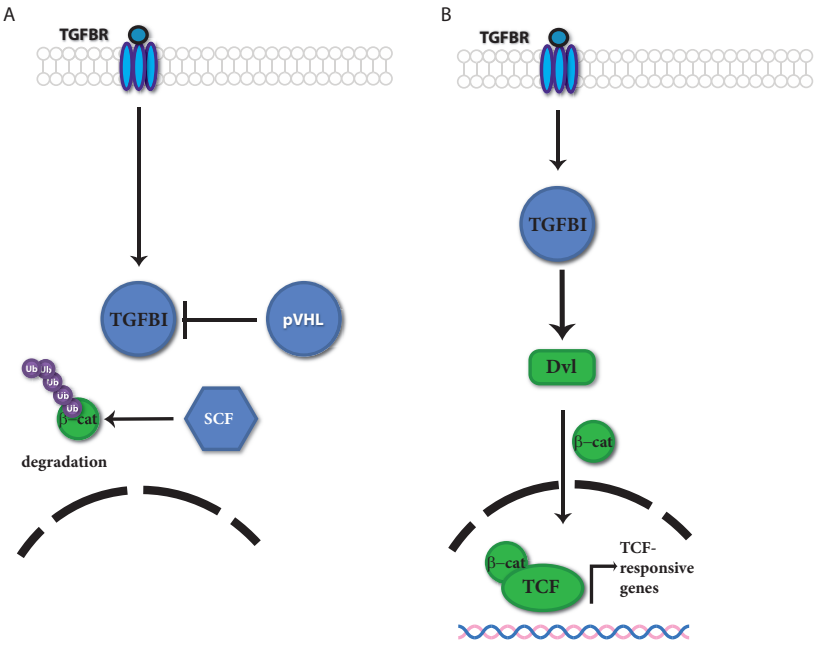

Figure 4 TGFBI signaling in the presence of pVHL (A) and in the absence of pVHL (B)

Whether increased TGFBI activity following pVHL loss could have a similar effect on the Wnt signaling pathway is plausible yet still speculative. Another link to the Wnt/β-catenin pathway and *VHL* loss was provided by Shang *et al*. in 2013. They published that HIF2α causes upregulation of protein tyrosine phosphataseζ (PTPRZ1), which in turn increases the amount of nuclear β-catenin, resulting in increased proliferative potential for these cells.

Yet another novel function for pVHL appears to be in ribosome biogenesis (Zhang *et al*., 2013). They observed with mass-spec that pVHL binds to the 40S ribosomal subunit protein S3 (RPS3). This interaction induces the nuclear retention of the 40S subunits, resulting in lowered overall protein production in 786-O with reconstituted *VHL*. Since ribosome biogenesis is vitally important for rapidly dividing cells, loss of *VHL* could contribute to the increased proliferative potential of ccRCC tumor cells. However the data presented by this group is not very convincing. The decrease in polysomes they proposed is not as dramatic as is seen with essential components of the ribosomal biogenesis machinery, and they concluding argument that pVHL loss decreases protein production can also be explained by the fact that HIFα is degraded in the presence of pVHL. Secondly, preliminary data from the group of Dr. R. Giles (personal correspondence) shows no significant effect of pVHL loss on ribosomal biogenesis. In this case a different tag was used, which might explain the differences in the observed effects.

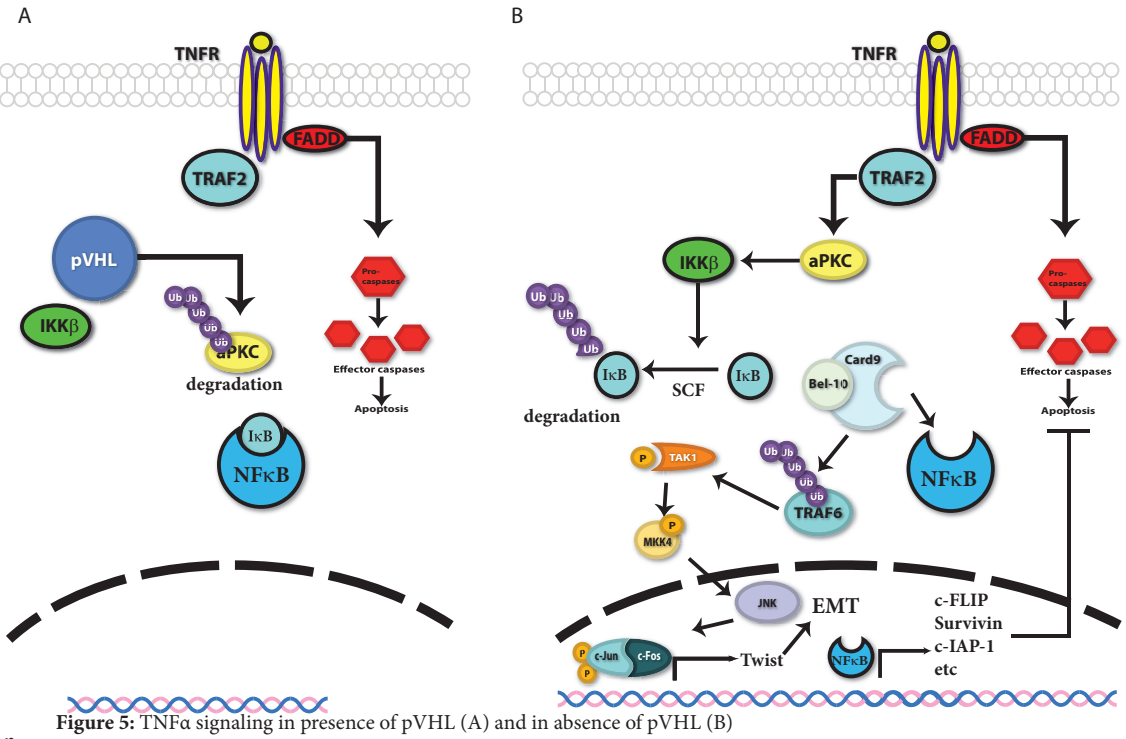

Figure 5: TNFα signaling in presence of pVHL (A) and in absence of pVHL (B)

Another well characterized function of VHL is its involvement in cilia regulation and maintenance (Lolkema *et al*., 2004) This is most likely due to VHL stabilizing microtubules at the cell periphery. A lack of pVHL decreases tubulin turnover in the cilium. ccRCC tumors contain very little cilia, contrary to normal renal tissue. Basten *et al*. found in 2013 that this is not due to increased proliferation, as during normal mitosis the cilium is degraded, but that this is due to loss of *VHL*. These cilia are involved in non-canonical Wnt signaling, resulting in planar cell polarity, but also in the inhibition of canonical Wnt signaling. The protein nephrocystin 2 localizes to cilia where it inhibits the actions of Disheveled upon extracellular Wnt signaling, strongly reducing the response and accumulation of nuclear β-catenin (Gerdes *et al*., 2007). Loss of the cilium in renal cells upon loss of *VHL* could then drive the formation of renal cysts. These cysts are then thought to evolve into ccRCC.

Chemoresistance in ccRCC

ccRCC tumors are notoriously resistant to chemotherapy. Most chemotherapeutic agents rely on activating the apoptotic pathways in the cell, and since p53 is one of the master regulators of the apoptotic pathway p53 is one of the most common mutated genes in tumors (Johnstone *et al*., 2002). Roe *et al*. reported in 2006 that pVHL directly interacts with p53 resulting in its stabilization and activation. The interaction they reported would prevent Mdm2-induced ubiquitination of p53 and subsequent nuclear export and degradation. A loss of *VHL* would then directly result in decreased p53 activity and subsequent chemoresistance.

However this assertion falls apart the moment non-degradable HIF2α is expressed in ccRCC cell lines with reconstituted *VHL* expression (Roberts *et al*., 2009). They reported that this non-degradable HIF2α made cells resistant to Fas mediated apoptosis in a p53 dependent fashion, as resistance was similar in isogenic cells with an inactivating mutation in p53. HIF2α causes an upregulation of TGFα which is a ligand for the epidermal growth factor receptor (EGFR) (Gunaratnam *et al*., 2003). The EGFR then phosphorylates Akt, which in turn phosphorylates Hdm2, causing it to suppresses p53 activity, as well as translocating to the nucleus resulting in suppression of nuclear p53 activity. More importantly they found that inhibiting HIF2α resensitizes ccRCC cells to chemotherapy such as doxorubicin.

Another cause of chemoresistance in ccRCC is the dysregulation of the tumor necrosis factor α (TNFα) pathway. TNFα is a proinflammatory cytokine that is mainly secreted by macrophages. As it binds to the TNF receptor (TNFR) there can be two possible outcomes: apoptosis and survival. After Caldwell *et al*. discovered in 2002 that *VHL* deficient cells were much more resistant to TNFα mediated cytotoxicity, Qi and Ohh found in 2003 that this is due to the actions of pVHL on the nuclear factor k B (NFkB) pathway. The model they proposed (figure 1) did not show the exact mechanism which causes pVHL to inhibit the NFkB pathway, but they did manage to prove the effects of *VHL* loss on aberrant NFkB activation and subsequent cellular resistance to apoptosis in ccRCC. Okuda *et al*., 2001 have proposed that pVHL directly ubiquitinates atypical PKC (aPKC) family members, which would mean that upon *VHL* loss aPKC would be free to activate IKKβ. IKKβ then in turn targets NFkB inhibitor IkB for proteasomal degradation, ultimately resulting in nuclear accumulation of NFkB and subsequent upregulation of NFkB-associated inhibitors of apoptosis. However Qi and Ohh did not find conclusive evidence suggesting this is the mechanism causing chemoresistance in ccRCC. Nevertheless they managed to prove conclusively that presence of pVHL is paramount for sensitivity to TNFα-induced apoptosis by measuring caspase activity in RCC cells with reconstituted *VHL*. Jang *et al*. discovered in 2010 that NFkB accumulation in the nucleus is caused by transglutaminase 2 (TG2), which reduces the levels of IkB in cancer cells (Kim *et al*., 2006). And since the

gene contains six HREs it is considered a direct target of HIFα. Apart from the NFkB pathway they also showed a more direct action of TG2 in survival, namely that it crosslinked the terminal effector caspase 3, creating inactive aggregates of caspase 3. There is however one issue with their study because they only looked at HIF1α and not HIF2α despite the fact that Holmquist-Mengelbier *et al*. showed in 2006 that prolonged hypoxia (i.e.: 72 hrs) leads to accumulation of HIF2α and not HIF1α. Whether the main driving force of NFkB accumulation in the nucleus is TG2 or aPKC remains to be seen, however these proteins could be potential drug targets.

Other studies suggest a more direct involvement of pVHL in NFkB regulation. Considering the fact that amongst different types of familial VHL disease there are patients who have type 2C VHL disease. These patients do not have a full deletion of the *VHL* gene but rather a mutation in one allele that does not affect HIFα regulation. Despite having showing symptoms that correlate with HIFα dysregulation, such as polycythemia caused by upregulated erythropoietin production, they do not develop ccRCC or hemangioblastoma. Evidence for HIFα independent NFkB regulation by pVHL surfaced in 2007, when Yang *et al*. found that pVHL directly binds to caspase recruitment domain-containing protein 9 (CARD9), a known NFkB agonist. They published that pVHL does not ubiquitinate CARD9 but instead acts as a scaffold for casein kinase 2 (CK2) which then phosphorylates CARD9 on the C-terminus. This C-terminal phosphorylation inhibits NFkB activation by CARD9. Conversely mutating the phosphorylation sites on CARD9 causes hyperactivation of NFkB regardless of pVHL status. Furthermore elimination of CARD9 in cells lacking pVHL resensitized these cells to proapoptotic signaling.
Building on these findings it was discovered very recently that pVHL also plays a role in inhibiting c-Jun¬-NH2-kinase (JNK) in a HIFα-independent manner (An *et al*., 2013). JNK is a mitogen activated protein kinase (MAPK), required for Ras-induced transformation and in many model systems linked to tumorigenesis. Their study showed that if CARD9 is not inhibited by pVHL and CK2, it will form a complex together with Bcl10 and TNF-receptor associated factor 6 (TRAF6). This causes transautoubiquitination of TRAF6 on K63, which contrary to K48 ubiquitination, causes activation and not proteasomal degradation. Once this has happened TRAF6 dissociates and activates TGF-β activating kinase 1 (TAK1), which acts as a MAPKKK in the JNK pathway. TAK1 subsequently phosphorylates MKK4, a MAPKK and this leads to the activation of JNK. This activated JNK subsequently phosphorylates c-Jun, allowing c-Jun to bind to c-Fos and together form an active transcription factor complex that activates Twist transcription. Twist protein expression seems to give rise to the epithelial to mesenchymal transformation (EMT) phenotype seen in ccRCC, including a cadherin switch from E-cadherin to N-cadherin and increased invasiveness. Inhibiting JNK in pVHL deficient cells lowered Twist expression and strongly reduced invasiveness. Ectopic expression of Twist however restored the EMT phenotype upon JNK inhibition. If this is truly the case, than that means that the NFkB pathway driven by CARD9 is a cause of both chemoresistance and invasiveness.

The search for adequate model organisms

The search for an adequate animal model that shares the same phenotype as seen in VHL disease patients has been long and arduous. The first attempts consisted of complete knockouts in mice of the *VHL* gene and these attempts resulted in embryonic lethality between 10.5 and 12.5 days post gestation. These mice died *in utero* because embryonic vasculogenesis in the placenta failed to occur in *Vhl* -/- mice resulting in hemorrhagic lesions (Gnarra *et al*., 1997). The discovery of conditional knockouts proved to be a way to circumvent this problem, but so far knocking out *Vhl* in murine systems has not yielded a phenotype that resembles ccRCC.
One group has managed to induce the formation of epididymal tumors found in VHL patients in mice by knocking out both *Vhl* and phosphatase and tensin homologue (Pten). Pten is a tumor supressor, like *VHL*, which dephosphorylates the product of phosphatidylinositol 3-kinases (PI3K), phosphatidylinositol 3,4,5-triphosphates (PI3P). Loss of *Pten* results in aberrant PI3P signaling, of which downstream targets are mammalian target of rapamycin (mTOR), S6kinase (S6K) and JNK. Deleting both of these tumor suppresor genes does cause tumorigenic transformation of the genital tract in these mice, it does however not correspond to the ccRCC phenotype (Frew *et al*., 2008).

Another noteworthy attempt at a mouse model for ccRCC was made in 2011 (Fu *et al*., 2011). The so-called TRACK-mouse was produced by knocking in constitutively active HIF1α. Since HIF1α is known to occur in early lesions leading up to kidney cysts (Mandriota *et al*., 2002), they chose this approach to make a model. The mice do indeed show formation of kidney cysts, but problems arise when they performed molecular analysis, because the only molecular characterization of the model consists of direct HIF1α target genes. One would only expect genes such as CAIX to be upregulated when HIFα is constitutively active, as well as pathological signs such as extensive vascularization due to aberrant VEGF production and subsequent effects. Nothing was mentioned on chemoresistance, leaving little support for their bold conclusion that these TRACK-mice can serve as an actual model for ccRCC.

The model that is the closest resemblance to actual ccRCC in a murine model came in 2013. Albers *et al*. made a mouse model that has a conditional knockout for both *Vhl*

and *Trp53*. First they assessed whether this dual knockout would promote aneuploidy and immortalization of mouse embryonic fibroblasts (MEF) in vitro. The MEFs were immortalized and displayed a marked increase in aneuploidy, however this did not hold true for renal epithelial cells. Deletion of *Trp53* did rescue an apparent inhibition of proliferation in these cells upon deletion of *Vhl*, but single cell seeding on agar plates yielded no colonies implying a lack of immortalization. The *Vhl*-/-;*Trp53*-/- mice that were subsequently produced proved to be subviable, with 25% of the mice dying within the first 3 months with no apparent tumors being found. However the mice that did make it to older age showed great promise as a model for VHL disease. By 5 months of age small clusters of disorganized cells appeared in the kidneys of the mice, contrary to either single *Trp53* or *Vhl* knockout mice. At 11 - 13 months the double knockout mice displayed multiple hyperproliferative lesions in the kidneys and multiple cysts. 16 neoplastic cysts were found in 24 kidneys of these mice, the tumor cells of which showed weak cytoplasmic eosin staining, although to a lesser extent than human ccRCC tumor cells. All cysts showed a marked increase in the amount of both HIFα isoforms, very much like ccRCC. *c-Myc* was also found to be upregulated in almost all neoplastic lesions. The primary cilium was also absent in the majority of cells in the cysts, independent of proliferation. The main difference however is that these neoplastic lesions still expressed E-cadherin, indicating that EMT had not taken place which is a clear sign of ccRCC. Interestingly though, the *Vhl;Trp53* double knockout displayed a similar phenotype in epididymal tumors as the *Vhl;Pten* double knockout when it came to epididymal dysplasias.

Nevertheless in only 14% of the COSMIC-database samples both *VHL* and *TP53* are mutated. Albers *et al*. found in their samples only 9% of ccRCC tumors with simultaneous *VHL* and *TP53* mutations. It raises the question whether a system that models 9% - 14% of patient cases can be used to adequately study ccRCC. Lastly, the finding that 9% - 14% of ccRCC patients harbor *TP53* mutations does not implicate a direct causative effect in the rise of ccRCC, it could just as well be an extra added advantage in a later stage of tumor progression. Furthermore the lack of EMT and the high mortality of the double knockout mice produced presents a problem for further study. Late stage ccRCC cannot be studied if the vast majority of the mice die before reaching 13 months of age. Though it would be interesting to study what kind of secondary mutations the double knockout mice accrue over time as their cystic lesions develop into tumors.

A quick look at the struggle to find adequate murine models for another unrelated kidney pathology, diabetic nephropathy (DN), reveals that it might be an inherent problem with mouse models (Soler *et al.,* 2012). C57BL mice are notoriously absent of the characteristic features of diabetic nephropathy seen in human patients. Since 1966 researchers have been trying to model it in mice, but even the most recent attempts do not mirror the human disease phenotype. Although most recent mouse models do show albuminuria, processes such as glomerular hyalinosis do not seem to occur in the mice, and central processes of DN such as podocyte loss, do not occur in all models. Though the cause of this lack of disease in murine kidneys is a mystery, it does raise an important question: if more 50 years of intensive research has not produced any sort of suitable murine model for DN, would it not be better to abandon the mouse as a model for kidney diseases?

In recent years different organisms have been studied as a model for ccRCC and VHL disease, particularly zebrafish (Danio rerio). Genomic comparison revealed an ortholog for *VHL* in zebrafish, which lacks the first 53 amino acids of VHL, corresponding to human pVHL19 (van Rooijen *et al*., 2009). Another possible ortholog with high transcript sequence similarity was found during this comparison, dubbed *vhl-like*(*vhll*), but the first zebrafish knockout models they made did not have a *vhl/vhll* double knockout. Like the murine models, if *vhl* is knocked out the fish are embryonic lethal and they die at the larval stage (8 - 11 days post fertilization). However, unlike murine models, zebrafish embryos can be studied during their development because their eggs are transparent. During development they display signs of systemic hypoxia. Upon development of the mouth, at 4 to 5 days dpf, they start to hyperventilate and heart rate was significantly elevated. HIFα target genes are strongly upregulated, with increased amounts of vegfa, glut-1 and epo mRNA levels being present at 7.5 dpf. At 8 dpf polycythemia is observable due to accumulation of blood in the yolk sac. Chemical induction of HIFα mimicked the observed phenotype, and interestingly human VHLp30 mRNA rescued this phenotype, indicating that the sequence homology between human *VHL* and the zebrafish ortholog is high enough to restore its function upon endogenous *vhl* loss. There were also some renal abnormalities arising in the fish, possibly paving the way for use in ccRCC studies. A 2010 follow up on this study, using the same strains of zebrafish mutants, showed that the fish develop angiogenesis-related eye problems similar to those seen in human VHL disease patients (van Rooijen *et al*., 2010). Although it is a very promising model for VHL disease, they did not make a tissue specific knockout or study ccRCC in this model.

An affiliated group (Santhakumar *et al*., 2012) have devised a way to circumvent the two main problems in the search for animal models for VHL related cancers, being the developmental problems with a systemic double *vhl* knockout and that a heterozygous loss of *vhl* does not predispose mice nor zebrafish to cancer. Because PHD3, which under normal conditions targets HIFα for pVHL-

mediated ubiquitination, is also a HIFα target it normally forms a negative feedback loop. Upon biallelic *vhl* loss this negative feedback loop is broken. The model they made consisted of zebrafish with one functional and one inactivated *vhl* allele combined with EGFP with a PHD3 promoter. Under normal conditions these fish are not predisposed to tumors of any kind, so they treated the animals with a known zebrafish carcinogen called dimethylbenzanthracene (DMBA). The beauty of this system is that due to the HIFα-dependent expression of GFP, the moment the fish lose their last working *vhl* allele the cells in which this happens express EGFP. It is then relatively easy to study the effects of *vhl* heterozygosity and DMBA treatment on carcinogenesis. It turned out that the heterozygous fish were much more prone to develop neoplasia upon DMBA treatment than the zebrafish with two working *vhl* alleles. As early as two months after DMBA treatment EGFP+ cells were visible in the treated batch of fish, of which 89% was heterozygous. After 14 months the fish were culled and stained for a proliferative marker showing strong correlation between EGFP and neoplasia. Interestingly there was also strong proliferation in the epithelium of the renal ducts, which indicates that this zebrafish model might be used to study ccRCC in vivo.

Conclusions

Clear cell renal carcinoma is a very complicated disease for which a cure has eluded us so far. The role of pVHL in cellular processes is vast and still not fully understood. However it is abundantly clear that the loss of pVHL can confer an immense advantage to developing tumors, both by HIFα-dependent resistance to hypoxia and increased angiogenesis as well as HIFα-dependent and independent resistance to chemotherapy and apoptosis. Secondly the observation that loss of pVHL alone does not give rise to tumors, meaning other mutations need to be present for the onset of ccRCC, adds another layer of complexity. Depending on the other mutations present the growing tumor will most likely respond differently to different treatments.

Nevertheless the advancements made in recent years show an emerging path towards the discovery of a cure. First a group of common secondary mutations was identified in 2011 (Varela *et al*., 2011) pointing at a role for chromatin remodeling in the onset of ccRCC. Secondly the role of HIFα-independent processes has been further elucidated in recent years, for instance CARD9 induced NFkB activation and JNK-mediated proliferation and EMT. It would be very interesting to study whether CARD9 inhibition would resensitize these tumors to caspase-mediated apoptosis. Also the establishment of the zebrafish as a viable model for VHL disease, especially if a kidney-specific inducible knockout can be made to study sporadic ccRCC. Another model system might come from the field of stem cell research. Future advances in stem cell technology might create the possibility to create stem cell lines directly from ccRCC patients, which could ease targeted drug development. Mouse xenograft studies could then be used to test any drugs that show effectiveness in vitro.

In the near future the targeting of CARD9 and JNK, combined with current angiogenesis inhibitors, could stabilize and possibly reduce the tumor load on patients.

The fact that loss of *VHL* alone does not initiate the formation of a tumor creates immense heterogeneity in the patient population. Although the end result may be similar, it is likely that the difference in mutations that different patients harbor might require differential treatment. Although this does hamper the search for a clue, building a library of ccRCC tumors could greatly advance our understanding of the underlying causes of the chemoresistance and agressive phenotype it displays. This could then ultimately lead to the discovery of ways to overcome chemoresistance, not just in ccRCC, but in other kinds of tumors as well.

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