



# The von Hippel-Lindau Tumor Suppressor Gene: Insights into Oxygen Sensing and Cancer

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## INTRODUCTION

As a third-year medical student at Duke University, I worked in Dr. Randy Jirtle's laboratory studying tumor blood flow using radioactive microspheres (Kaelin *et al.*, 1982, Shrivastav *et al.*, 1983, Kaelin *et al.*, 1984). During that year I read about tumor angiogenesis and became intrigued by the idea, then championed by Dr. Judah Folkman, that blocking angiogenesis would be a way to arrest tumor growth (years later I learned that this idea actually can be found in the 19<sup>th</sup> century German medical literature). I also learned that some tumors, such as kidney cancers, are particularly rich in new blood vessels and that highly angiogenic tumors, including kidney cancers and hemangioblastomas, are a feature of a rare hereditary cancer syndrome now referred to as von Hippel-Lindau (VHL) disease.

I did my house staff training in internal medicine at Johns Hopkins. As a house officer I would often memorize the potential causes, or differential diagnosis, for various symptoms and signs encountered in patients. This, and my knowledge of rare eponymous syndromes such as VHL disease, served me well when I was later selected to be assistant chief of service, equivalent to a chief resident, for the Johns Hopkins internal medicine service (also called the Osler medical service). One such differential diagnosis was for polycythemia, or excess red blood cell production. Included among the causes of polycythemia are certain tumors that can secrete EPO or EPO-like substances, including the three hallmark tumors seen in VHL disease: kidney cancer, hemangioblastoma, and pheochromocytoma (more broadly, paraganglioma) (Golde and Hocking, 1981). This struck me as odd, especially as hemangioblastomas and pheochromocytomas are otherwise rare tumors. The fact that VHL-associated tumors could promote angiogenesis and erythropoiesis suggested to me that these tumors must have dysregulated oxygen sensing, because angiogenesis and erythropoiesis are normal responses to hypoxia. These tumors were acting like they had an exaggerated response to hypoxia, as though their response to oxygen was “stuck” in the hypoxic position. My chairman when I was an intern at Johns Hopkins was Dr. Victor McKusick, who taught me the importance of medical history and the power of human genetics. My chairman when I was a resident was Dr. Jack Stobo, who began introducing me to new molecular techniques that were being used to study human diseases.

#### THE RB1 TUMOR SUPPRESSOR GENE

I was a medical oncology fellow at the Dana-Farber Cancer Institute. In 1988, shortly after beginning the second year of my fellowship, I joined Dr. David Livingston's laboratory. The retinoblastoma tumor suppressor gene (*RB1*) was cloned about a year earlier by several groups. The predicted primary sequence of the *RB1* gene product, pRB, was relatively featureless and hence did not provide any real clues regarding pRB's likely biochemical functions. Dr. Ed Harlow had shown, however, that the Adenovirus E1A oncoprotein could bind to pRB and David's group, shortly thereafter, showed that SV40 T antigen, via a short peptidic region that was homologous to E1A, could do the same (Whyte *et al.*, 1988, DeCaprio *et al.*, 1988). My first project was to map the minimal region of pRB that could bind to T and E1A. This region, which David coined “the pocket”, turned out to be a hotspot for RB1 missense mutations (Kaelin *et al.*, 1990). This suggested that both pocket mutations and the viral oncoproteins caused cancer by preventing pRB from binding to one or more cellular proteins.

It fell upon me to find these putative cellular proteins. Our initial strategy was to look for cellular proteins that coimmunoprecipitated with pRB in anti-pRB immunoprecipitates. Unfortunately, there was really only one anti-pRB antibody available at that time and its continued supply from a competitor laboratory was in doubt. We had an emergency laboratory meeting to make our own anti-pRB antibodies. I was charged with producing fragments of pRB in *E.coli* with which to immunize rabbits and mice. My first task was to find a suitable prokaryotic expression vector. Producing human proteins in *E. coli* was still a bit of an art back then, and success or failure could depend on choosing the right vector.

One day a company representative left a brochure on my desk for a new commercial prokaryotic expression plasmid that encoded glutathione S-transferase (GST) fused to the protein (or protein fragment) of interest. The GST moiety often improved protein solubility (a big issue with bacterially produced proteins) and enabled rapid purification using glutathione sepharose capture followed by elution with reduced glutathione. After looking at the plasmid map it dawned on me that I could use it to express GST-fused to the pRB pocket and employ the fusion protein, rather than an anti-pRB antibody, to look for pRB-associated proteins. Specifically, I could capture the GST-pRB protein on glutathione sepharose and, after washing the sepharose, use the immobilized GST-pRB pocket to capture <sup>35</sup>S-labelled cellular proteins. I ultimately identified a series of anonymous <sup>35</sup>S-labelled cellular proteins that had the right properties: they bound to GST fused to the wild-type pRB pocket, but not tumor-derived pRB pocket mutants, and their binding to the pRB pocket was prevented by T/E1A-derived peptides (Kaelin *et al.*, 1991).

In parallel, I worked with another Livingston trainee, Xiao Qin, to show that reintroducing the wild-type pRB pocket, but not tumor-derived pRB pocket mutants, into *RBI*<sup>-/-</sup> tumor cells suppressed their growth (Qin *et al.*, 1992). This strengthened the idea that tumor suppression by pRB was linked to its ability to bind to one or more of my cellular pRB-binding proteins. It also showed me the power of marrying biochemical and functional assays when studying tumor suppressor proteins.

My pulldown experiments did not distinguish direct from indirect binders. Moreover, the identities of my pRB-binding proteins remained unknown, partly because I could not recover them in the amounts required for mass spectrometry sequencing back then. Fortunately, my colleague Myles Brown told me about the work of Michael Blanar, who had radiolabeled recombinant proteins using <sup>32</sup>P-ATP, heart muscle kinase, and genetically encoded phosphoacceptor sites (Blanar and Rutter, 1992). I modified my GST-pRB pocket expression vector so that this phosphoacceptor site was inserted between the GST moiety and pRB moiety. In pilot experiments I could readily label my GST-pRB fusion pro-

teins in this way, but they no longer bound to E1A or T. After some troubleshooting, I realized that the brief exposure to 30°C required for the kinase reaction was the culprit. Fortunately, I discovered that the kinase reaction also proceeded at 4°C, and that the protein labeled at this temperature retained the ability to bind to E1A and T.

I proceeded to do “far-western” blot assays using cell extracts that were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and then incubated with <sup>32</sup>P-labelled GST-pRB pocket in the presence of excess unlabeled GST. After a bit more troubleshooting, I was able to clearly detect direct binding of the GST-pRB pocket to E1A and SV40 T in suitable cell extracts. Notably, I also could see a cellular protein (or proteins) that migrated as a doublet of ~50 kD (Kaelin *et al.*, 1992).

In parallel studies, I worked with another Livingston Laboratory trainee, Thomas Chittenden, who used the immobilized GST-pRB to show that pRB could bind, directly or indirectly, to a DNA-binding activity that Joe Nevins had earlier coined “E2F” (Chittenden *et al.*, 1991). The ability of pRB to bind to this activity was also shown by Drs. Joe Nevins, Nicholas LaThangue, and Pradip Raychaudhuri (Chellappan *et al.*, 1991, Bagchi *et al.*, 1991, Bandara *et al.*, 1991a, Bandara and La Thangue, 1991b).

Encouraged by my far-western blot results, I teamed up with Erik Flemington, who was a postdoctoral fellow in Dr. Sam Speck’s laboratory, to screen phage expression libraries with <sup>32</sup>P-labelled GST-pRB pocket in the presence of excess unlabeled GST. The dominant clone we recovered represented the first member of the E2F family, now called E2F1 (Kaelin *et al.*, 1992).

## THE VON HIPPEL-LINDAU TUMOR SUPPRESSOR GENE

I started my own laboratory at the DFCI in 1992, working down the hallway from David Livingston’s laboratory. I did a few additional experiments related to pRB and E2F1 but was appropriately advised that I should probably start to carve out a line of investigation that was clearly distinct from David’s. I briefly tried to clone the *ATM* gene using a functional complementation strategy, without success. In 1993 I read a paper in *Science*, from a group led by Drs. Bert Zbar, Michael Lerman, and Marston Linehan at the National Cancer Institute (NCI) and Dr. Eamonn Maher at the University of Birmingham, describing the isolation of a partial cDNA for the gene that, when mutated, caused the von Hippel-Lindau hereditary cancer syndrome (henceforth von Hippel-Lindau disease now that the causative gene is known) (Latif *et al.*, 1993). It was clear that the *VHL* gene, like the *RBI* gene, was a “Knudson 2-hit” tumor suppressor gene. Patients with *VHL* disease have typically inherited a defective

maternal or paternal *VHL* allele. Tumor development, such as development of clear cell renal cell carcinoma (ccRCC), is linked to loss of the remaining wild-type allele in a susceptible cell. The authors of the *Science* paper also showed that biallelic *VHL* inactivation was a common feature of sporadic ccRCCs, which is by far the most common form of kidney cancer in the general population (Latif *et al.*, 1993, Gnarr et al., 1994). In this setting both mutational events, or “hits”, occur somatically, in contrast to *VHL* disease, where the first hit is typically present in the germline. As was true for *RBI*, the predicted *VHL* open reading frame (ORF) did not provide immediate clues as to its likely biochemical functions.

I immediately thought I should work on the *VHL* gene for multiple reasons. In the early 1990s it seemed to me that most of the advances regarding our molecular understanding and treatment of cancer involved cancers that were fascinating, but uncommon. It was clear to me that we needed to make progress on the ten most common cancers (many of which are epithelial cancers) if we wanted to make a big dent in overall cancer mortality. I hoped that studying the *VHL* gene would provide insights into the pathogenesis of kidney cancer, which is an epithelial cancer and one of the ten most common cancers in the developed world.

Judah Folkman was beginning to describe the purification of endogenous angiogenesis inhibitors such as angiostatin (O’Reilly *et al.*, 1994, O’Reilly *et al.*, 1997). His group identified angiostatin in a phenotypic screen for angiogenesis inhibitors, but its mechanism of action was unknown. I had been intrigued by the idea of treating cancers with angiogenesis inhibitors dating back to my third year of medical school, but I thought this would require targeted angiogenesis inhibitors with defined mechanisms of action and that this, in turn, would require a much deeper understanding of the molecular circuits that govern new blood vessel formation. The highly vascular nature of *VHL*-associated neoplasms suggested to me that studying the *VHL* gene would provide insights into such a circuit.

As a physician I knew that oxygen (or lack thereof) played a role in many diseases, including anemia, myocardial infarction, and stroke, in addition to cancer. The fact that *VHL*-associated tumors were linked to increased angiogenesis and erythropoiesis suggested to me that studying the *VHL* gene would ultimately provide insights into how human cells and tissues sense and respond to oxygen. This seemed like a great puzzle to work on.

I also had some prosaic reasons for working on the *VHL* gene. There were many great laboratories working on the *RBI* gene and the competition sometimes felt intense. I naively thought it might be more fun to work in relative obscurity on a gene linked to a disease few people had

heard of. In addition, the *VHL* cDNA published by the NCI group was thought to be missing about 2 kB of 5' sequence and its predicted ORF was open at the 5' end (Latif *et al.*, 1993). In particular, the *Science* paper reported a *VHL* mRNA size of 6-6.5 kB, based on a Northern Blot, while the cDNA was closer to 4 kB (Latif *et al.*, 1993). I had become very facile at screening phage cDNA libraries with radioactive cDNA probes in the course of my E2F1 cloning work. I had also made an excellent 293T kidney cell cDNA library. I thought we would quickly isolate the 5' end of the cDNA and then be off to the races.

The next postdoctoral fellow to join my laboratory was Othon Iliopoulos, a medical oncology clinical fellow at the Dana-Farber Cancer Institute. He screened multiple cDNA libraries, including the 293T cDNA library described above, with <sup>32</sup>P-labelled cDNA probes derived from the published *VHL* cDNA clone (kindly provided by Dr. Michael Lerman). Over the ensuing year he screened tens of millions of cDNAs and isolated scores of new *VHL* cDNA clones. The problem, however, was that none of them extended the published *VHL* cDNA sequence in the 5' direction. In fact, none of them even contained as much 5' sequence as the published *VHL* cDNA sequence. Othon also tried 5' RACE (Rapid Amplification of cDNA ends) based on the published *VHL* cDNA sequence, without success.

Fortunately for us, however, we had used the partial *VHL* cDNA to express a GST-VHL fusion protein in *E. coli*, which was then used to immunize rabbits. The affinity purified anti-pVHL (pVHL = protein product of *VHL* gene) we made recognized an ~30 kD protein in 293T cells that was absent in human *VHL*<sup>-/-</sup> ccRCC lines that we had purchased from the ATCC (American Type Culture Collection) (Iliopoulos *et al.*, 1995). We realized that the ORF in the published *VHL* cDNA clone could produce a protein with 213 amino acid residues if a particular upstream methionine was used to initiate translation. I was still working in the laboratory back then and had become fairly proficient at making mammalian expression plasmids and at *in vitro* translation while a postdoctoral fellow in David Livingston's laboratory. I made mammalian expression plasmids that would encode this theoretical protein of 213 amino acids and that could support *in vitro* translation. Othon used one of these plasmids to make the theoretical protein as an <sup>35</sup>S-labelled protein by *in vitro* translation. In parallel, he immunoprecipitated the ~30kD protein with the anti-pVHL antibody using cell extracts from 293T cells labeled with <sup>35</sup>S-labelled methionine. The two proteins comigrated when resolved by SDS-PAGE and their partial proteolytic peptide maps were identical. We (and the NCI group) had the complete *VHL* ORF cDNA after all (Iliopoulos *et al.*, 1995). Othon also showed that pVHL resides largely in the cytoplasm under steady-state conditions (Iliopoulos *et al.*, 1995), as determined by

immunofluorescence and cellular fractionation assays, although Dr. Richard Klausner would later show that pVHL can dynamically shuttle between the nucleus and the cytoplasm (Lee *et al.*, 1996).

When I was a clinician working in the hospital we used to sometimes say “believe nothing, trust no one, and do it yourself”. Knowing that the published “partial” VHL cDNA contained the complete ORF and having been unable to extend the published 5' cDNA sequence, we finally did what we should have done on day 1. Othon did his own Northern Blot and determined that the VHL mRNA is closer to 4.4 kB (Iliopoulos *et al.*, 1995). When I approached the NCI group about their published 5' end that we had never seen in our cDNA clones, they confessed that they were so sure the cDNA was open at the 5' end that they had added some 5' VHL genomic sequence to the VHL cDNA sequence *in silico*.

While this early work was on-going, I travelled to the basement of the Countway Medical Library at Harvard Medical School to find the original papers by Eugen von Hippel and Arvid Lindau (von Hippel, 1904, Lindau, 1927). In the 1970s I was probably among the last students to be told that if you wanted to study medicine, you should first learn German. So, I studied German for 3 years in high school. In medical school I learned the origin of this advice. One of my medical school professors told us that if you thought you had discovered something new in clinical medicine, you should take a sabbatical and read the German medical literature, because there was a good chance your discovery was already reported there. At least one of my medical school professors referred to the late 19<sup>th</sup> century, when the German medical literature was so dominant, as the great descriptive era in medicine.

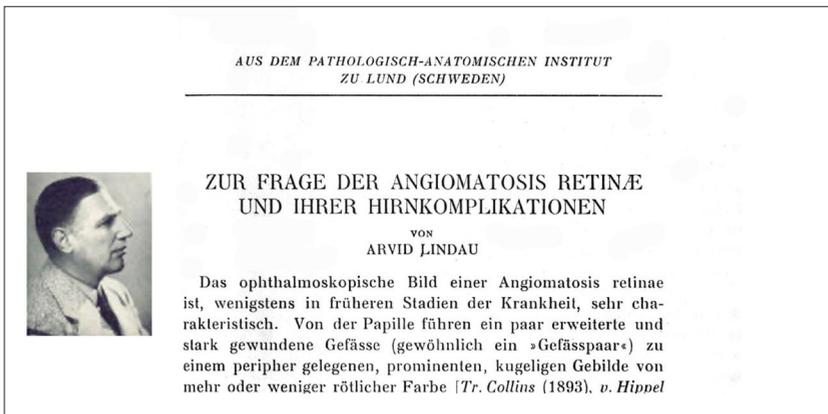


Figure 1. Arvid Lindau's description of von Hippel-Lindau Syndrome. Shown is first page of Arvid Lindau's 1927 paper *Acta Ophthalmologica* (1927) 4: 193–226. Also included is a picture of Arvid Lindau.

Eugen von Hippel was a German ophthalmologist and Arvid Lindau was a Swedish neuropathologist. Despite my high school German, I could not translate Eugen von Hippel's original paper published in 1904 or Arvid Lindau's seminal paper published in 1927 (Figure 1). I came to learn that Eugen von Hippel's paper described the familial occurrence of retinal hemangioblastomas (Figure 2). Years later my former Chairman, Dr. Victor McKusick, informed me that a similar family had been described in 1894 by Treacher Collins (Figure 3) (Collins, 1894). von Hippel's paper was more widely known, however, because it was published in German (the Collins paper was published in English) and because von Hippel's father was himself an academic physician who would disseminate copies of his son's paper at medical congresses. I came to learn that it was really Lindau, however, who noted that these familial retinal hemangioblastomas were a marker for a systemic disease that also affected the brain, spinal cord, and kidney. Now, approximately 100 years after the paper by Treacher Collins, we had the reagents to begin understanding why *VHL* mutations caused cancer.

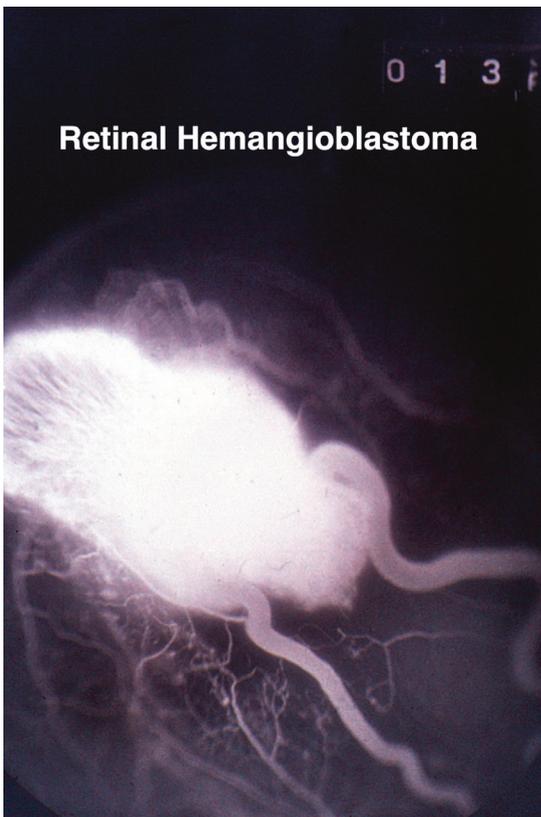


Figure 2. Retinal hemangioblastoma seen on retinal angiography.

My postdoctoral *RBI* studies involved developing functional assays for pRB that could plausibly map to its ability to suppress tumors (e.g. suppression of tumor cell proliferation) and assays for pRB-associated proteins. It seemed reasonable to employ the same strategies here.

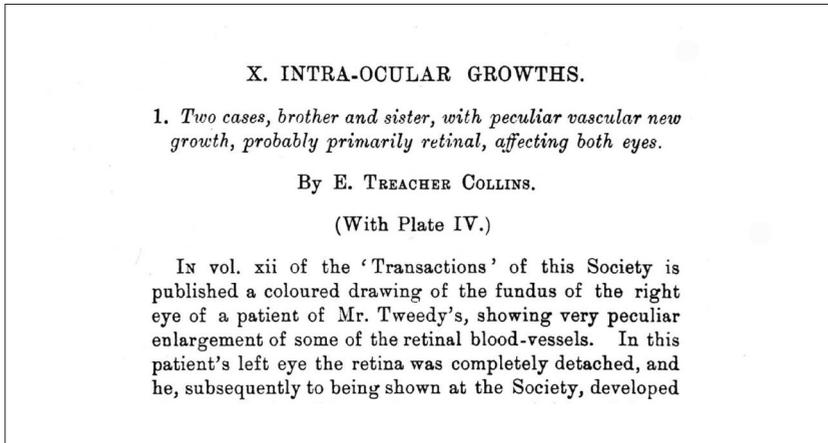


Figure 3. First report of a family with von Hippel-Lindau syndrome. Shown is first page of Treacher Collins' 1894 paper in *Transactions of the Ophthalmological Society* (1894) 14: 141-149.

#### THE *VHL* GENE PRODUCT (pVHL) SUPPRESSES TUMOR GROWTH AS PART OF A MULTIPROTEIN COMPLEX

Othon stably transfected 786-O *VHL* mutant/- (hereafter "-/-" for simplicity) human ccRCC cells to now produce a wild-type version of pVHL ("WT" cells) or a C-terminally truncated version that contained only the first 115 amino acid residues of pVHL ("ARZ" cells). The latter was based on the knowledge that some ccRCC-associated *VHL* mutations caused similar truncations. As an additional control he transfected cells with the empty backbone vector ("pRC" or "EV" cells). He used cloning cylinders to isolate multiple independent clones for each, cognizant that there would likely be some heterogeneity amongst the parental 786-O cells.

The first surprise, which was a bit disappointing, was that it was relatively easy to generate 786-O subclones that now contained wild-type pVHL; they proliferated as well as the control cells under standard cell culture conditions. This was in stark contrast to what would have been seen with other well-studied tumor suppressor proteins, such as pRB and p53.

When Othon implanted the cells subcutaneously in nude mice, however, he saw a dramatic phenotype. The WT cells did not form tumors, in contrast to the ARZ and pRC cells (Iliopoulos *et al.*, 1995). This strength-

ened our suspicion that tumor suppression by pVHL was linked, at least partly, to suppression of angiogenesis.

In parallel, Adam Kibel, a urology resident at the Brigham and Women's Hospital, joined my laboratory and began looking for pVHL-associated proteins. He labelled 786-O cells that stably expressed hemagglutinin (HA)-tagged versions of wild-type pVHL, pVHL (1-115), or a pVHL disease-associated mutant, pVHL (R167W), with  $^{35}\text{S}$  methionine and captured potential pVHL-binding partners in immunoprecipitation assays with either anti-pVHL or anti-HA antibodies. In both cases he saw two proteins with molecular weights of ~18kd (p18) and ~14 kD (p14), respectively (Kibel *et al.*, 1995). The same two proteins coimmunoprecipitated with endogenous pVHL in *VHL*<sup>+/+</sup> ccRCC lines but were not detected in anti-pVHL immunoprecipitates prepared from pVHL-defective 786-O ccRCC cells. Adam then mapped the minimal p18/14 binding region of pVHL to pVHL residues 157–172 using GST pulldown assays and peptide competition assays. In the latter, the binding of p18/14 to pVHL was blocked by a peptide in which residues 157–172 corresponded to wild-type pVHL, but not if the peptide contained the R167W mutation. Gratifyingly, we saw that the 157–172 was clearly a hotspot for mutations in VHL families, arguing that the binding to p18 and/or p14 was linked to pVHL's ability to suppress tumors (Kibel *et al.*, 1995).

We purified p18 and p14 and sent them for mass spectrometry sequencing. The first sequences that came back were from p18 and they did not match any known protein. About this time, I attended an Oncogene Meeting in Frederick, MD and presented a poster on our findings. An adjacent poster from Dr. Richard Klausner described a similar line of investigation. Rick was a legendarily good scientist and decided to work on a human cancer syndrome, namely VHL disease, having recently become the Director of the National Cancer Institute. So much for working in a field without competitors! Over the ensuing years, however, I learned that you actually want competitors, as long as they hold themselves and others to a high scientific standard, and they play fairly. Rick, as well as countless other members of the hypoxia-community, are examples of such investigators.

My heart sank when I learned that Rick's group had identified p18 and p14 as elongin B and elongin C, respectively (I don't remember if this was on his poster or revealed in conversation during the meeting). His group had obtained peptide sequences for both p14 and p18, and p14 matched elongin C. They had then reached out to Drs. Joan and Ron Conaway, who had identified elongin C as part of a transcriptional elongation complex (Elongin or SIII), and learned that p18 was another component of the complex, soon to be deposited in databases as elongin B. Moreover, Rick's group and the Conaway group collaborated to show that pVHL could

inhibit Elongin/SIII by competing with elongin A for elongin B and C. It looked like, for the first time in my young scientific career, I had been scooped!

I spoke to Rick and learned that he had already made a prepublication inquiry to *Science* about his paper. It would have been within his rights to squash me like a bug at this point. Instead, he graciously offered to have us co-submit with him. As is often the case in science, our independent papers were complimentary of one another (for example, our pVHL-binding studies and mapping studies were very strong) and provided simultaneous corroboration of the key findings. As a postdoctoral fellow I had cloned *E2F1*. At that time David and I decided to orchestrate the co-publication of a competitor paper from Ed Harlow's group (Helin *et al.*, 1992). It seemed I was now to be the beneficiary of such generosity.

Rick's paper emphasized a model in which tumor suppression by pVHL was linked to its ability to inhibit the Elongin/SIII complex (Duan *et al.*, 1995). Our data didn't really speak to this. Instead, we made the more conservative conclusion that tumor suppression by pVHL required that it bind to elongin B and elongin C (Kibel *et al.*, 1995). In truth, we were also concerned that the low abundance of pVHL relative to elongin B and elongin C challenged whether pVHL could truly outcompete elongin A for binding to elongin B and elongin C.

#### pVHL REGULATES HYPOXIA-INDUCIBLE mRNAs

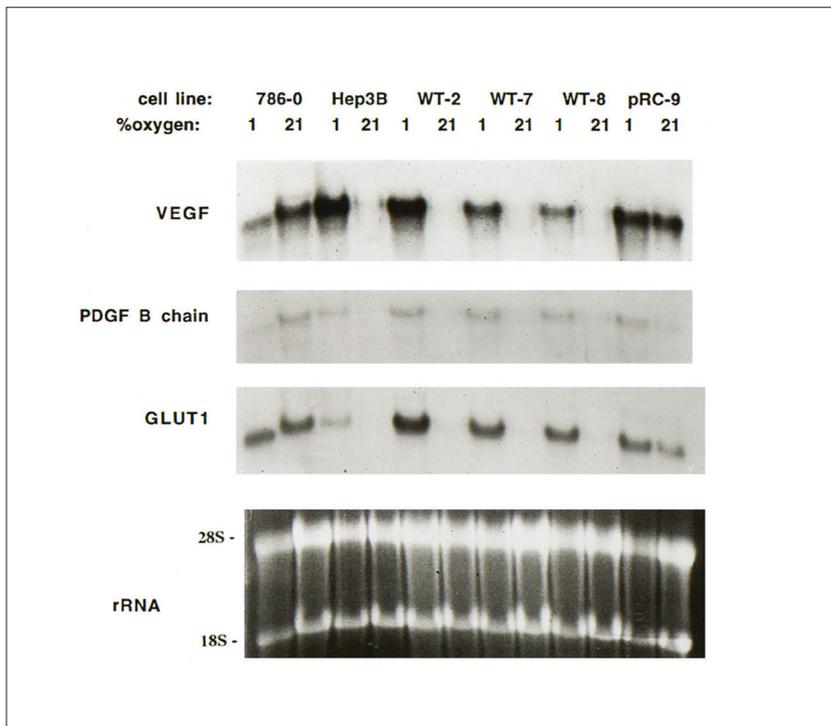
I remained intrigued by the idea that pVHL suppressed angiogenesis, which would explain both the increased angiogenesis of VHL-associated tumors and potentially explain our finding that pVHL suppressed ccRCC cell growth in nude mice, but not on plastic dishes (at least under standard culture conditions). I reached out to Dr. Judah Folkman and provided him with our isogenic WT, ARZ, and pRC 786-O cells. My idea was for him to test the conditioned medias from these cells for their ability to promote angiogenesis in a battery of *ex vivo* bioassays he had developed over the years. To my dismay, his laboratory found no difference (I still cannot explain this).

Partly for this reason I couldn't convince anyone in my laboratory to measure *VEGF* mRNA levels in these cells by Northern blot analysis. One day, however, I went to a lecture by Dr. Hal Dvorak, who showed angiograms of retinal neoangiogenesis driven by VEGF. The pictures reminded me of retinal hemangioblastomas. I remember thinking, "it has to be VEGF!"

I then reached out to Dr. Mark Goldberg, who was then at the Brigham and Women's Hospital. He was experienced measuring hypoxia-inducible mRNAs, including the *VEGF* mRNA, by Northern blot. Better still, he had

an incubator for growing cells at 1% oxygen. He and I designed an experiment to measure hypoxia-inducible mRNAs, including the *VEGF*, *PDGF B*, and *GLUT1* mRNAs, in WT, ARZ, and pRC cells grown at 21% or 1% oxygen. Hep3B *VHL*<sup>+/+</sup> hepatoma cells, a workhorse in the hypoxic gene regulation field, were included as controls. The actual experiment was done by one of his fellows, Andrew Levy.

I will never forget the day they walked into my office with the autoradiogram (Figure 4). As expected, the hypoxia-inducible mRNAs in the Hep3B cells were only detected at high levels if the cells were grown at 1% oxygen. In contrast, the hypoxia-inducible mRNAs were constitutively produced at high levels in the 786-O ccRCC cells, unless they had been engineered to once again produce wild-type pVHL (Iliopoulos *et al.*,



**Figure 4.** Deregulation of hypoxia-inducible mRNAs in cells lacking wild-type pVHL. Northern blots with the indicated probes using RNA from 786-O *VHL*<sup>-/-</sup> clear cell renal cell carcinoma cells, ep3B *VHL*<sup>+/+</sup> hepatoma cells, or independent 786-O subclones stably transfected to produce wild-type pVHL (WT-2, WT-7, and WT-8 subclones) or stably transfected with the backbone vector (pRC-9 subclone). Cells were grown in the presence of 21% oxygen or 1% oxygen, as indicated. Also included is ethidium stained gel prior to transfer to assess loading. From: Iliopoulos, O., *et al.*, Negative Regulation of Hypoxia-Inducible Genes by the von Hippel-Lindau Protein. *Proc. Natl. Acad. Sci.*, 1996. 93: p. 10595–10599. Copyright (1996) National Academy of Sciences, U.S.A.

1996). Mark told me at the time that this was the first example of a mammalian gene that was strictly required for the transcriptional response to hypoxia. Years later I would hear the expression “the first harpoon in the whale”. This was our first real harpoon in the whale. The ability of pVHL to suppress *VEGF* mRNA levels under normoxia was also reported by Marston Linehan’s group in collaboration with Rick Klausner (Gnarra *et al.*, 1996).

#### pVHL FORMS AN E3 UBIQUITIN LIGASE THAT REGULATES THE HIF TRANSCRIPTION FACTOR

Kim Lonergan, a postdoctoral fellow in my laboratory, discovered that Cul2, a member of the cullin family, also bound to pVHL in coimmunoprecipitation assays and did so via elongin C (Lonergan *et al.*, 1998). We collaborated with Joan and Ron Conaway, who showed that Cul2 also copurified with pVHL in native cell extracts. The conclusion that Cul2 bound to pVHL was also reached by the Klausner group working independently (Pause *et al.*, 1997).

Importantly, Kim then tested a series of N-terminal, C-terminal, and missense pVHL mutants for 1) their ability to bind to the elongins and Cul2, and 2) their ability to suppress hypoxia-inducible mRNAs. Binding to the elongins and Cul2 was necessary, but not sufficient, for pVHL to suppress hypoxia-inducible mRNAs (Lonergan *et al.*, 1998). The latter also required sequences N-terminal of the elongin (and hence Cul2)-binding region.

At around this time I had an extremely important conversation with Dr. Steven Elledge, then at Baylor College of Medicine, who was visiting Harvard to give a lecture. He had noticed that the elongin C protein sequence resembled that of the yeast protein Skp1, and that the cullins likewise resembled the yeast protein Cdc53 (Bai *et al.*, 1996). He and others had shown that Skp1 and Cdc53, when bound to a so-called F-box protein, generated an ubiquitin ligase complex in which the F-box protein served as the substrate recognition unit (Bai *et al.*, 1996). He therefore predicted that the pVHL, elongin, Cul2 complex was a ubiquitin ligase.

We collaborated with Nikola Pavletich to solve the three-dimensional structure of pVHL bound to elongin B and elongin C. The 3D structure revealed that pVHL actually had two hotspots for mutations linked to VHL disease (Stebbins *et al.*, 1999). The first, which Nikola termed the alpha domain, gratifyingly corresponded to the elongin C-binding domain we had identified biochemically. The second, which Nikola called the beta domain, had the properties of a potential substrate docking site. Finally, the structure confirmed the potential similarity between elongin C and Skp1, and between pVHL and an F-box protein. Collectively, these find-

ings strengthened the case that the pVHL complex was a ubiquitin ligase complex. In further support of this idea, both the Klausner group and Dr. Willy Krek's group showed that anti-pVHL immunoprecipitates contained ubiquitin ligase activity, as though pVHL was, or was associated with, an ubiquitin ligase (Lisztwan *et al.*, 1999, Iwai *et al.*, 1999). Moreover, our collaborators, the Conaways, detected the ubiquitin-conjugating enzyme (E2) Rbx1 bound to the pVHL, elongin, Cul2 complex (Kamura *et al.*, 1999).

The question then became: is the pVHL complex really a ubiquitin ligase and, if so, what are its substrates? Both our group and the Linehan group observed that the suppression of *VEGF* mRNA levels by pVHL involved a modest effect on *VEGF* mRNA transcription and a much more pronounced effect on *VEGF* mRNA stability (this is still not explained given the findings outlined below) (Iliopoulos *et al.*, 1996, Gnarr *et al.*, 1996). For this reason, we hypothesized that pVHL regulated the stability one or more mRNA-binding proteins. Nonetheless, the heterodimeric HIF (Hypoxia-Inducible Factor) was such an attractive target, given its central role in hypoxia-inducible gene regulation and the oxygen-dependent turnover of its alpha subunit, that we did western blots for HIF1 $\alpha$  using cell extracts from the WT, ARZ, and pRC cells grown at 1% oxygen or 21%, as well as hypoxic control cells. We did not detect HIF1 $\alpha$  in 786-O cells, which furthered our suspicion that pVHL regulated an mRNA-binding protein (and unfortunately quelled any enthusiasm for doing electrophoretic mobility shift assays for HIF, which would have given us the answer).

The VHL Family Alliance is a support network for VHL patients and their family members. They hold annual meetings that bring together scientists, clinicians, and lay stakeholders. The first such meeting I attended, in 1996, was one of the most moving experiences of my life. Up until then, I had never met a patient with VHL disease – they had only existed in textbooks. I became truly inspired by their tenacity, resilience, and courage. In 1998 I attended the VHL Family Alliance Annual Meeting that was held in Paris that year. I met one of Dr. Peter Ratcliffe's postdoctoral fellows, Patrick Maxwell, who kindly told me that he had found that pVHL was strictly required for the degradation of the HIF $\alpha$  subunits in ccRCC under normoxia (Maxwell *et al.*, 1999). The Ratcliffe Laboratory had made a good antibody for HIF2 $\alpha$ . Unfortunately for us, our workhorse cell lines at the time, 786-O and A498, produced HIF2 $\alpha$ , but not HIF1 $\alpha$  (explaining our negative western blot data). In ccRCC cell lines that express both HIF1 $\alpha$  and HIF2 $\alpha$ , both are upregulated by pVHL loss (Maxwell *et al.*, 1999). Patrick and I agreed that if we both discovered the oxygen-dependent signal that regulated the binding of pVHL to HIF $\alpha$ , we would try to coordinate the publications of our papers.

### OXYGEN-DEPENDENT PROLYL HYDROXYLATION OF HIF RECRUITS THE pVHL UBIQUITIN LIGASE

The seminal Maxwell paper appeared in *Nature* (Maxwell *et al.*, 1999), but it did not address whether pVHL directly or indirectly regulated the HIF alpha subunits and it did not describe the mechanism. I had a new post-doctoral fellow in my laboratory, “John”, who had “golden hands” at the bench when it came to biochemical experiments. He quickly showed that pVHL, via its beta domain, bound to a region of HIF1 $\alpha$  (the Oxygen-Dependent Degradation Domain) that was known to be responsible for its oxygen-dependent turnover. Moreover, with the help of Dr. Vincent Chau (then at Millenium), “John” and Michael Ohh showed that the pVHL complex is indeed the E3 ubiquitin ligase for HIF1 $\alpha$  and that this activity is abrogated by tumor-associated *VHL* mutations (Figure 5). We eventually published these findings in *Nature Cell Biology* in 2000 (Ohh *et al.*, 2000). The ability of pVHL to polyubiquitylate HIF1 $\alpha$  was subsequently also reported by Ratcliffe, Conaway, and Dr. Lorenz Poellinger laboratories (Kamura *et al.*, 2000, Cockman *et al.*, 2000, Tanimoto *et al.*, 2000).

While our ubiquitylation paper was out for review, “John” also generated data suggesting that HIF $\alpha$  underwent an oxygen- and iron-dependent posttranslational modification that determined whether it could bind to pVHL. In the summer of 1999, it seemed like we were learning a new

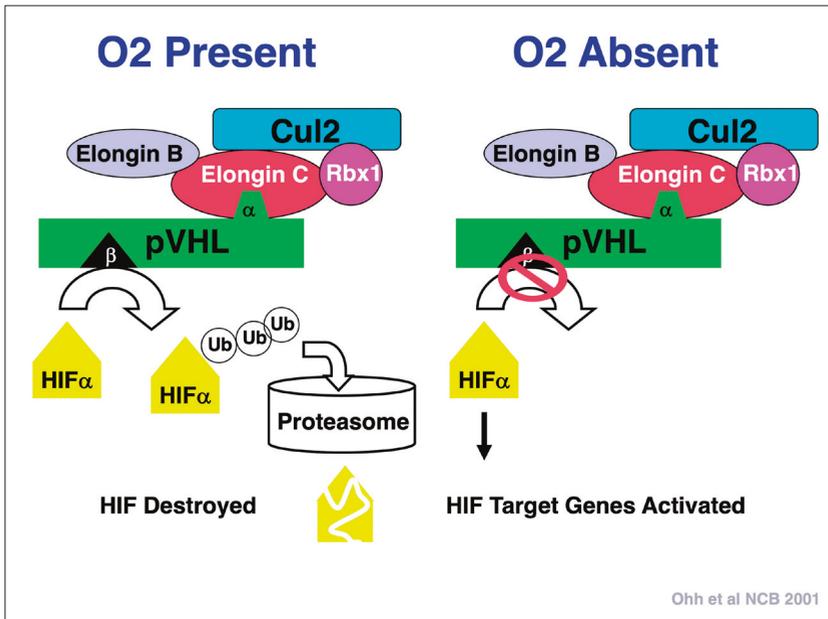


Figure 5. pVHL is the substrate recognition component of a ubiquitin ligase that regulates HIF.

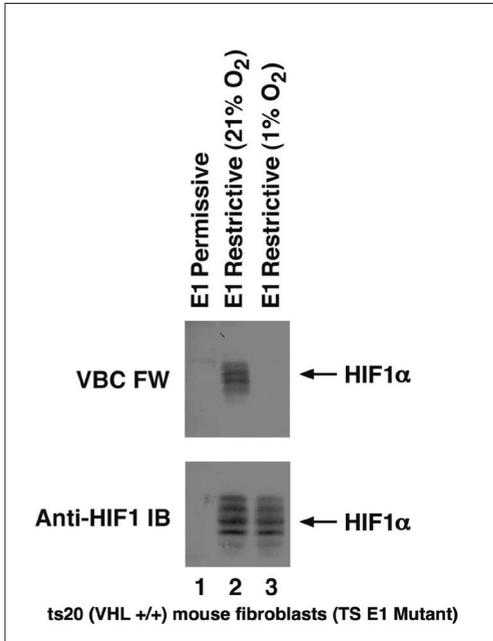
piece of the puzzle almost every week. “John” then told me that he wanted to go to his native country for one week to deal with family matters. One week became two, which became four, which became 7. No word. In the meantime, another postdoctoral fellow in my laboratory confided that “John” had gone home to apply to medical school and to discuss a possible marriage, and that there was a chance he wasn’t coming back. He did come back the day I was to leave for my July vacation. I told him that I was disappointed that he had not been more honest with me and that I would have tried to help him with his medical school application if that was what he really wanted. I then left for my vacation. While I was gone, he came to the laboratory one night, took his notebooks, data, and reagents, and returned to his native country.

I returned to the laboratory a week later and was naturally devastated. Some of the most exciting preliminary data I had ever seen had now vanished. I had an emergency meeting of the laboratory and my postdoctoral fellows Michael Ohh and Mircea Ivan stepped in to remake the key reagents and redo the key experiments. Michael did the additional experiments that allowed us to publish the *Nature Cell Biology* paper showing that pVHL was the ubiquitin ligase for pVHL, while Mircea, with help from two other fellows in the laboratory, Haifeng Yang and Billy Kim, pursued the oxygen-dependent posttranslational modification of HIF $\alpha$ . We were still at the leading edge of the field, but this debacle cost us months.

Mircea did pVHL far-western blot assays modeled on my pRB work. Here, however, he used the recombinant pVHL/elongin B/elongin C complex (leftover from the crystallography studies) and detected the bound pVHL with an anti-pVHL antibody (rather than  $^{32}\text{P}$ ). This taught us that the pVHL bound directly to HIF1 $\alpha$  and HIF2 $\alpha$ , which was not known previously (Ohh *et al.*, 2000) (Ivan *et al.*, 2001).

Haifeng Yang did a powerful experiment in which TS20 *VHL*<sup>+/+</sup> cells, which have a temperature-sensitive mutation in the E1 ubiquitin activating enzyme, were cultured at the restrictive temperature in 1% oxygen or 21% oxygen. As expected, HIF1 $\alpha$  accumulated at the restrictive temperature compared to the permissive temperature, as determined by anti-HIF1 $\alpha$  immunoblot analysis, consistent with loss of ubiquitylation. In far-western assays, however, pVHL, could only bind to the HIF1 $\alpha$  that had accumulated under normoxic conditions (Figure 6) (Ivan *et al.*, 2001).

It was known for years that treating cells with iron chelators or iron antagonists could induce a hypoxic response. Later this was shown to be due to HIF induction. Gratifyingly, Mircea discovered that pVHL could not bind to HIF1 $\alpha$  that had been induced by iron chelators or iron antagonists (Ivan *et al.*, 2001). This strongly suggested that HIF1 $\alpha$  was indeed undergoing an oxygen- and iron-dependent posttranslational modification that was required for its recognition by pVHL.



*Figure 6.* An oxygen-dependent modification of HIF $\alpha$  allows it to bind directly to pVHL. Far-western (FW) Blot with recombinant pVHL/elongin B/elongin C complex and anti-HIF $\alpha$  immunoblot (IB) of TS20 VHL+/+ cells grown at the restrictive or permissive temperature. At the restrictive temperature the cells were grown in 21% or 1% oxygen, as indicated. TS20 cells have a temperature-sensitive E1 mutation and do not support ubiquitylation at the restrictive temperature. From: Ivan, M., *et al.*, HIF $\alpha$  targeted for VHL-mediated destruction by proline hydroxylation: implications for O $_2$  sensing. *Science*, 2001. 292: p. 464–8.

Mircea discovered that recombinant HIF1 $\alpha$  fragments produced in *E.coli* (or as synthetic peptides) could only bind to pVHL if first preincubated with a mammalian cell extract (such as unprogrammed rabbit reticulocyte lysate) under conditions permissive for enzymatic activity (presumably to provide the modifying activity) (Ivan *et al.*, 2001). He also found that HIF1 $\alpha$  *in vitro* translated in rabbit reticulocyte lysate contained an electrophoretically distinct band that 1) was absent when HIF $\alpha$  was translated using wheat germ extracts and 2) specifically bound to pVHL (Ivan *et al.*, 2001). The assumption was that this band reflected HIF $\alpha$  with the modification we were seeking.

To identify this modification, Mircea mapped the minimal pVHL-binding fragment of HIF1 $\alpha$  to a 20mer (corresponding to HIF1 $\alpha$  residues 556–575), which contained at its core the sequence MLAPYIPM (Ivan *et al.*, 2001). This 20mer synthetic peptide, immobilized on streptavidin beads via an N-terminal biotin, could bind to 35S-labelled pVHL (made by *in vitro* translation), but again only if the peptide was preincubated with unprogrammed rabbit reticulocyte lysate at 30°C (Figure 7) (Ivan *et al.*, 2001). Fortuitously, Dr. Jaime Caro at Thomas Jefferson had previously done linker scanner mutagenesis of HIF1 $\alpha$  and shown that replacing the MLAPYIPM sequence with 8 consecutive alanines rendered HIF1 $\alpha$  constitutively stable (Srinivas *et al.*, 1999). Mircea then repeated his binding assays using biotinylated HIF1 $\alpha$  peptides in which the entire MLAPYIPM

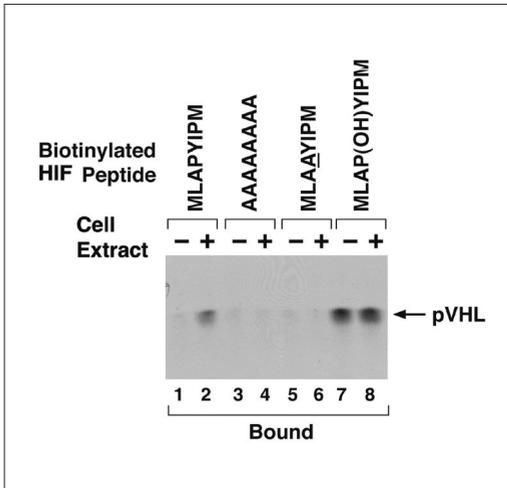


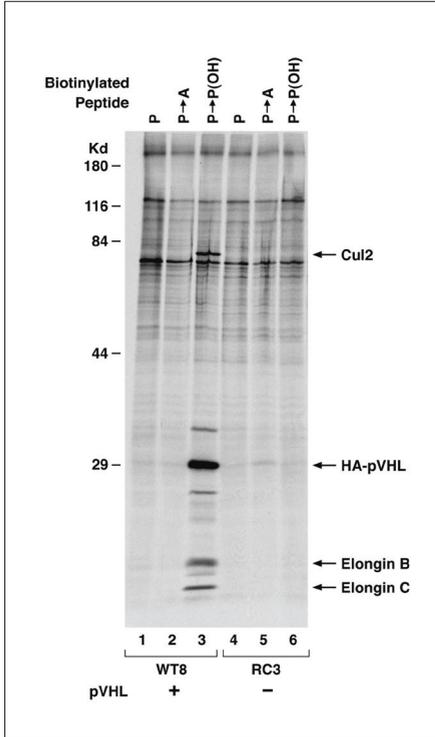
Figure 7. pVHL binds to prolyl hydroxylated HIF $\alpha$ . Binding of  $^{35}\text{S}$ -labeled pVHL to biotinylated HIF1 $\alpha$  (556–575) peptides with the indicated mutations of the core MLAPYIPM sequence. Peptide was preincubated at 30°C with an unprogrammed reticulocyte lysate where indicated with the “+”. From: Ivan, M., *et al.*, HIF $\alpha$  targeted for VHL-mediated destruction by proline hydroxylation: implications for O<sub>2</sub> sensing. *Science*, 2001. 292: p. 464–8.

sequence was replaced with 8 alanines or in which individual residues within the MLAPYIPM were replaced with alanine one at a time (i.e. he did an alanine scan). The two critical residues were the leucine residue and the first proline residue (Figure 7) (Ivan *et al.*, 2001).

I then ran to the computer to look for posttranslational modifications of proline and leucine that might be oxygen- and iron-dependent. Prolyl hydroxylation fit the bill (in fact, I encountered hundreds of papers about collagen prolyl hydroxylation). Mircea also suspected the modification was prolyl hydroxylation, having remembered this posttranslational modification from his college biochemistry course in Romania. He then synthesized his biotinylated HIF1 $\alpha$  peptide so that the prolyl residue was already hydroxylated. This was one of those rare “eureka” experiments. The hydroxylated peptide bound to pVHL, and no longer required the reticulocyte lysate preincubation step (presumably because the relevant modification was already present) (Figure 7) (Ivan *et al.*, 2001).

I always tell trainees to think of the least interesting interpretations of their data before jumping to the rosier interpretations. It was formally possible that the hydroxylated HIF1 $\alpha$  peptide was very “sticky” and would have bound non-specifically to any  $^{35}\text{S}$ -labelled protein (we just happened to look at pVHL). Billy Kim addressed that by doing peptide pulldown experiments with  $^{35}\text{S}$ -labelled cell extracts rather than  $^{35}\text{S}$ -labelled pVHL. Here you could see that the binding was exquisitely specific (Figure 8) (Ivan *et al.*, 2001).

Another concern was that the peptide pulldown experiments implied that prolyl hydroxylation was the signal *or* that prolyl hydroxylation mimicked the authentic signal used by cells. The methionines in our 20mer



*Figure 8.* Binding of the pVHL complex to prolyl hydroxylated HIF $\alpha$  is highly specific. 786-O *VHL*<sup>-/-</sup> cells that were stably transfected to produce wild-type pVHL (WT8) or with the empty vector (RC3) were metabolically labeled with <sup>35</sup>S-methionine, lysed, and incubated with biotinylated HIF1 $\alpha$  (556–575) peptides with the indicated proline 564 substitutions. Bound proteins after washing were detected by autoradiography. From: Ivan, M., *et al.*, HIF $\alpha$  targeted for VHL-mediated destruction by proline hydroxylation: implications for O<sub>2</sub> sensing. *Science*, 2001. **292**: p. 464–8.

peptide were not required for binding to pVHL. This was fortunate, because methionines are prone to spontaneous oxidation, which could have complicated our subsequent mass spectrometry analyses. Mass spectrometry of the wild-type HIF1 $\alpha$  peptide, but not the HIF1 $\alpha$  peptides in which the critical leucine and proline residues were converted to alanine, after incubation with reticulocyte lysate clearly demonstrated prolyl hydroxylation at proline 564 (Ivan *et al.*, 2001). Mircea also showed that HIF1 $\alpha$  *in vitro* translate made with rabbit reticulocyte lysate, but not wheat germ extract, in the presence of radioactive proline contained hydroxyproline, as determined by amino acid hydrolysis and thin layer chromatography (Ivan *et al.*, 2001). Finally, Mircea showed that Pro564A mutation stabilized full-length HIF1 $\alpha$  in cells (Ivan *et al.*, 2001).

As promised, I reached out to Patrick Maxwell before submitting our findings for publication. He appropriately referred me to his mentor, Peter Ratcliffe. I had never met Peter before and was now doing so by phone. I didn't want to deprive him of the joy of discovering the oxygen sensing mechanism on his own nor, I suppose, was I interested in co-publishing with him if he was hopelessly behind us. We therefore proceeded to speak a bit in code, turning over one card at time so to speak, until it was clear

that we had independently arrived at the same conclusion (Ivan *et al.*, 2001, Jaakkola *et al.*, 2001).

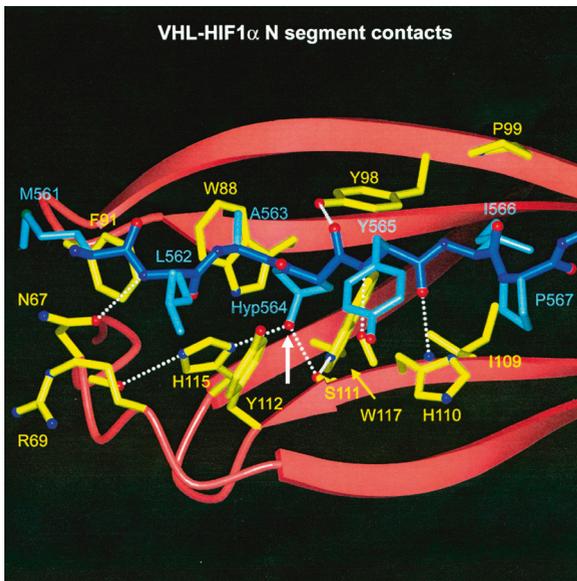
Years earlier, when I was just getting started as an independent investigator, an editor at *Science* named Paula Kiberstis met with me at a Keystone Symposium to find out what I was doing in my laboratory. I felt like I had arrived (recently she confessed to me that she thought the work I described that day was pretty boring). I suggested to Peter that we submit our papers to *Science* and that Paula handle the papers. Peter and I agree that this was a great decision. Firstly, at least in my case, she improved my writing considerably (I still use some of her conventions). More importantly, the reviews were not all positive. One reviewer said we had to be wrong because prolyl hydroxylation only took place in the endoplasmic reticulum. Paula realized this reviewer had tunnel vision based on their own work (presumably on collagen). Another more serious debate amongst the reviewers and editorial board was whether identification of the relevant enzyme(s) should be required for publication. Paula intervened, arguing that our paper was already a major step forward and would open the search for the enzyme(s) to others. Sadly, such editorial interventions seem less common today.

I have learned there are the competitors you know about and the competitors you don't know about. A few months after our two *Science* papers appeared, Frank Lee also reported that HIF $\alpha$  was prolyl hydroxylated (Yu *et al.*, 2001). The Ratcliffe Laboratory would also later report that both HIF1 $\alpha$  and HIF2 $\alpha$  contained second potential prolyl hydroxylation sites (Masson *et al.*, 2001).

#### DEVELOPMENT OF PROLYL HYDROXYLASE INHIBITORS FOR THE TREATMENT OF ANEMIA AND ISCHEMIA

Once we knew that HIF $\alpha$  stability was controlled by prolyl hydroxylation, I became intrigued with the idea of developing prolyl hydroxylase inhibitors as a way of treating diseases where HIF might be beneficial, such as anemia, myocardial infarction, and stroke (Ivan *et al.*, 2001). By now I was familiar with the collagen prolyl hydroxylases and I wondered if anyone had developed small molecule inhibitors against them. An internet search led me to a small company in South San Francisco called Fibrogen, Inc., which was making collagen prolyl hydroxylase inhibitors as antifibrotic agents. My hope was that some of their chemical inhibitors might fortuitously also inhibit the prolyl hydroxylation of HIF $\alpha$  and, if so, be good starting points for making more specific inhibitors of the (as yet unidentified) HIF prolyl hydroxylases.

I called Fibrogen and spoke to a scientist named “Max” (I believe this was in early 2001, before the publication of our *Science* paper). He listened very politely as I told him about our findings. He then proceeded to tell me that I had to be wrong because prolyl hydroxylation takes place in the endoplasmic reticulum, whereas HIF is nuclear. He closed the call by saying we must be studying an artefact. Fifteen minutes later he called me back and said, “Please start again from the beginning”. Years later I would learn that the second phone call was due to Tom Neff, the late CEO of Fibrogen. Tom had passed “Max” in the hallway immediately after the first call and, seeing that “Max” was amused, asked him what was so funny. “Max” proceeded to tell him about the crazy scientist from Boston who thought HIF was prolyl hydroxylated. Tom stopped in his tracks and said, “Don’t you remember that some of our early prolyl hydroxylase inhibitors caused polycythemia in rabbits. This scientist is trying to tell you why. Call him back!”. My longstanding collaboration with Fibrogen began with that second phone call. Shortly thereafter I had dinner with Tom in Boston. He said that a new druggable class of enzymes only comes along every 30 or 40 years. He also laid out his vision for developing a new class of anemia drugs and he predicted that I would one day win the Nobel Prize. I told him that if I was ever so lucky it would probably be because Fibrogen eventually succeeded and that, as a result, our legacies were now joined. Sadly, Tom died suddenly shortly before the Nobel announcement.



**Figure 9.** Structure of pVHL bound to prolyl hydroxylated HIF $\alpha$  peptide. The backbone of pVHL is shown in red and its side chains in yellow. The HIF $\alpha$  backbone is in medium blue and its side chains in light blue. Hydrogen bonds are shown with dotted lines. Note hydrogen bonds between His115 and hydroxylated Pro564 and Ser111 and hydroxylated Pro564. From: Min, J.H., *et al.*, Structure of an HIF-1 $\alpha$ -pVHL complex: hydroxyproline recognition in signaling. *Science*, 2002. **296** (5574): p. 1886–9.

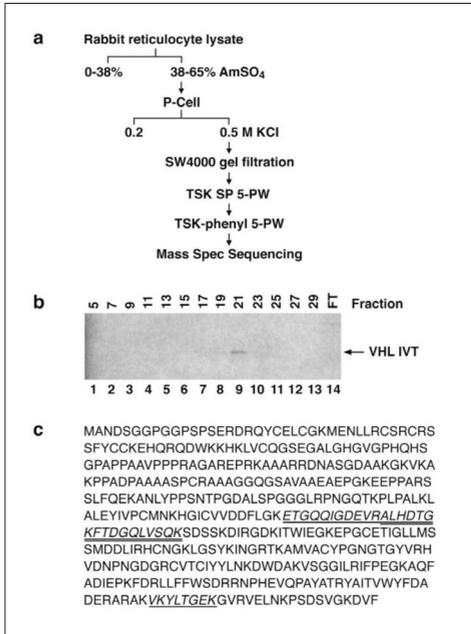
To understand how pVHL discriminated between hydroxylated and non-hydroxylated HIF1 $\alpha$ , we again collaborated with Nikola Pavletich, who then solved the structure of the pVHL/elongin B/elongin C complex bound to our prolyl hydroxylated HIF1 $\alpha$  peptide (Figure 9) (Min *et al.*, 2002). It confirmed that the hydroxylated HIF1 $\alpha$  peptide bound to the pVHL beta domain. The HIF1 $\alpha$  binding site on pVHL consisted largely of hydrophobic residues except for two hydrophilic residues, S112 and H115, that normally interact with water molecules. When the HIF1 $\alpha$  peptide interacts with pVHL, this water is displaced, which is energetically unfavorable. However, if the HIF1 $\alpha$  is hydroxylated on proline 564, the hydroxyl group can hydrogen bond with S112 and H115, thus compensating for the displaced water molecules. As Nikola would later tell me, if you were trying to design a protein that was specific for hydroxyproline, you couldn't do much better than this. A similar paper appeared from the Ratcliffe group (Hon *et al.*, 2002).

At this point we still didn't know the identity of the enzyme (or enzymes) that hydroxylated HIF $\alpha$ . The collagen prolyl hydroxylases were unlikely candidates because of their subcellular localizations and high oxygen affinities (making them relatively insensitive to oxygen). Moreover, the sequences surrounding the collagen and HIF $\alpha$  prolyl hydroxylation sites were highly dissimilar.

I made two assumptions that proved to be correct. The first was that we could biochemically purify the HIF $\alpha$  prolyl hydroxylase by classical biochemical fractionation, monitoring the ability of fractions to hydroxylate our synthetic, biotinylated, HIF1 $\alpha$  peptide (as determined by capture of <sup>35</sup>S-labelled pVHL). That way at least the field would have the answer and could move forward. The second assumption, however, was that someone might get their first using a clever genetic approach.

We again collaborated with Joan and Ron Conaway, who had and have far more biochemical expertise than me. They worked closely with my postdoctoral fellow, Mircea Ivan, who spent time in their laboratory. We ultimately identified a biochemical fraction of rabbit reticulocyte lysate that could hydroxylate HIF1 $\alpha$  despite containing fewer than 10 proteins (Figure 10). One of those proteins was EglN1 (Figure 10) (Ivan *et al.*, 2002). This was an excellent candidate, as a bioinformatic paper by Drs. Aravind and Koonin at the NCI predicted that EglN1 was actually a 2-oxoglutarate-dependent dioxygenase (Aravind and Koonin, 2001). We then confirmed that recombinant EglN1 could hydroxylate HIF1 $\alpha$  (Ivan *et al.*, 2002).

While this work was being completed, I visited UCLA to give a lecture. While there I got a message to call Mircea right away. He informed me that a paper had just appeared from the Ratcliffe group reporting that the three mammalian EglN family members (originally called EglN1, EglN2, and

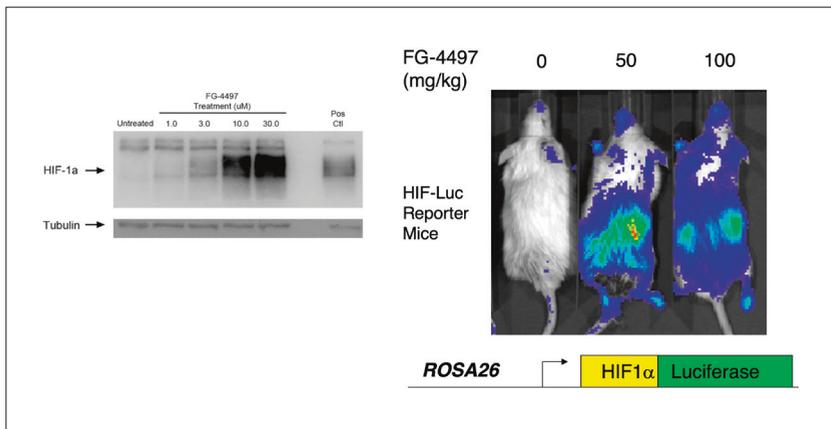


**Figure 10.** Purification of EglN1 as HIF $\alpha$  prolyl hydroxylase. Purification of EglN1. (a) purification schema. (b) Binding of  $^{35}\text{S}$ -labeled pVHL to biotinylated a HIF $\alpha$  (556–55) peptide. Peptide was preincubated at 30 oC with indicated column fractions after TSK SP 5-PW chromatography. FT = flow through. (c) Conceptual open reading frame of EglN1. Peptides identified by mass spectrometry of partially purified HIF prolyl hydroxylase are shown with single or double underline. From: 1. Ivan, M., *et al.*, Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. *Proc Natl Acad Sci U S A*, 2002. **99** (21): p. 13459–64. Copyright (2001) National Academy of Sciences, U.S.A.

EglN3, which they renamed PHD2, PHD1, and PHD3, respectively) were the HIF $\alpha$  prolyl hydroxylases (Epstein *et al.*, 2001). Peter, I would later learn, had a “secret weapon” in his Oxford colleague Chris Schofield, who was an expert regarding 2-oxoglutarate-dependent dioxygenases. He, like Aravind and Koonin, saw that the EglNs were likely to be 2-oxoglutarate-dependent dioxygenases and hence candidate prolyl hydroxylases. Peter’s group then showed that eliminating the sole EglN family member in *C. elegans* using RNAi, but not other dioxygenases, induced HIF (Epstein *et al.*, 2001). They went on to confirm that human EglN1, EglN2, and EglN3 could all hydroxylate HIF1 $\alpha$  (Epstein *et al.*, 2001). Drs. Rick Bruick and Steven McKnight came to the same conclusion shortly thereafter using a very similar strategy in *Drosophila* cells (Bruick and McKnight, 2001). Later work by Jacques Pouyssegur indicated that EglN1 is the major enzyme responsible for regulating HIF $\alpha$  turnover in human cells (Berra *et al.*, 2003).

We quickly finished our paper describing the biochemical purification of EglN1 as the HIF $\alpha$  prolyl hydroxylase (Ivan *et al.*, 2002). Partly to enhance the novelty of our biochemical paper, we included data showing that a variety of structurally unrelated prolyl hydroxylase inhibitors from Fibrogen could, as I suspected, inhibit EglN1 in biochemical assays and stabilize HIF in cell culture experiments (Ivan *et al.*, 2002). This then launched medicinal chemistry efforts at Fibrogen and elsewhere to make more specific EglN inhibitors.

The development of EglN inhibitors was enhanced by a powerful mouse model created by a postdoctoral fellow in my laboratory, Michal Safran (Safran *et al.*, 2006). Michal made a reporter consisting of the HIF1 $\alpha$  oxygen-dependent degradation domain fused to firefly luciferase, which she then inserted into the murine *ROSA26* locus (to ensure ubiquitous expression). Firefly luciferase expression in mice can be imaged non-invasively by monitoring photon emissions after administration of luciferin. In pilot experiments, Michal observed clear signals over the thymus and kidneys in her reporter mice breathing room air, consistent with the knowledge that these organs are hypoxic at rest (Safran *et al.*, 2006). She also confirmed that the reporter was systemically induced when mice were switched to 8% oxygen (Safran *et al.*, 2006). Using her mouse, she quickly identified which of the Fibrogen compounds we had been given were bioactive *in vivo* after administration by oral gavage, in which organs, and for how long (for example, as in Figure 11). This helped identify promising scaffolds for further medicinal chemistry efforts. Michal did a proof-of-concept study showing that an early Fibrogen EglN inhibitor could induce erythropoietin and red blood cell production in functionally anephric mice, setting the stage for the eventual development of Fibrogen's clinical candidate, Roxadustat (Safran *et al.*, 2006).



**Figure 11.** Stabilization of HIF $\alpha$  with orally available EglN inhibitor. Immunoblot of HL-1 Cardiomyocytes (left panel) and bioluminescent imaging of HIF1 $\alpha$ -Luciferase mice (right panel) treated with EglN inhibitor FG-4497 in media or by oral gavage, respectively. The right panel also includes a schematic for the reporter, which consists of the HIF1 $\alpha$  oxygen-dependent degradation domain fused to firefly luciferase, inserted into the *ROSA26* locus (first described in {Safran, 2006 #2948}). From: Olenchok, B.A., *et al.*, EGLN1 Inhibition and Rerouting of alpha-Ketoglutarate Suffice for Remote Ischemic Protection. *Cell*, 2016. **164** (5): p. 884–95.

Consistent with *EglN1* being the major HIF regulator amongst the 3 *EglN* paralogs, *EglN1*<sup>-/-</sup> mouse embryos are not viable, whereas *EglN2*<sup>-/-</sup> mice and *EglN3* are grossly normal (Takeda *et al.*, 2006). To circumvent the *EglN1*<sup>-/-</sup> embryonic lethality, a postdoctoral fellow in my laboratory, Andy Minamishima, made mice where he could conditionally inactivate *EglN1* using a floxed *EglN1* allele and a tamoxifen-inducible Cre recombinase (Minamishima *et al.*, 2008). He showed that inactivation of *EglN1* in adult mice caused massive polycythemia (the same conclusion was reached by Guo-Hua Fong working in parallel (Takeda *et al.*, 2008)) due to increased renal erythropoietin expression (Minamishima *et al.*, 2008).

During fetal life the liver is the major source of erythropoietin. Shortly after birth, however, the hepatic *erythropoietin* locus is silenced, and the kidney becomes the major source of erythropoietin. This has both medical and economic implications. For example, over 20 million Americans have chronic kidney disease and 2–4 million of them will be anemic due, at least partly, to a relative deficiency of erythropoietin. To ask if the hepatic erythropoietin locus could be reactivated in adults by manipulating *EglN* activity, Andy eliminated *EglN1*, *EglN2*, and *EglN3* – either singly, in every pairwise combination, or all three – in the livers of adult mice and measured hepatic *erythropoietin* mRNA and serum erythropoietin (Minamishima and Kaelin, 2010). Eliminating all 3 paralogs led to robust and sustained induction of hepatic *erythropoietin* mRNA levels and circulating erythropoietin, thus explaining Michal's earlier results in functionally anephric mice (Figure 12) (Minamishima and Kaelin, 2010).

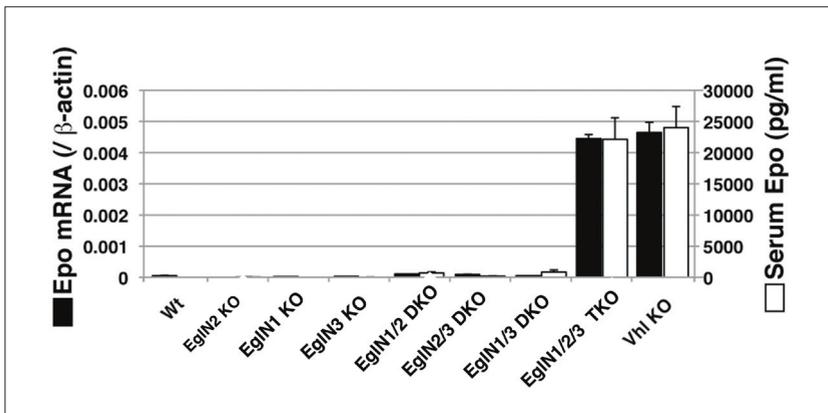


Figure 12. Reactivation of hepatic *EPO* locus after *EglN* inactivation. Hepatic erythropoietin (Epo) mRNA levels (solid bars), as determined by real-time RT-PCR, and serum Epo (open bars) in mice with the indicated genotypes. N= 3 mice per group. Note that the *EglN1* and *VHL* knockouts were liver-specific. From: Minamishima, Y.A. and W.G. Kaelin, Jr., *Reactivation of hepatic EPO synthesis in mice after PHD loss*. *Science*, 2010. **329** (5990): p. 407.

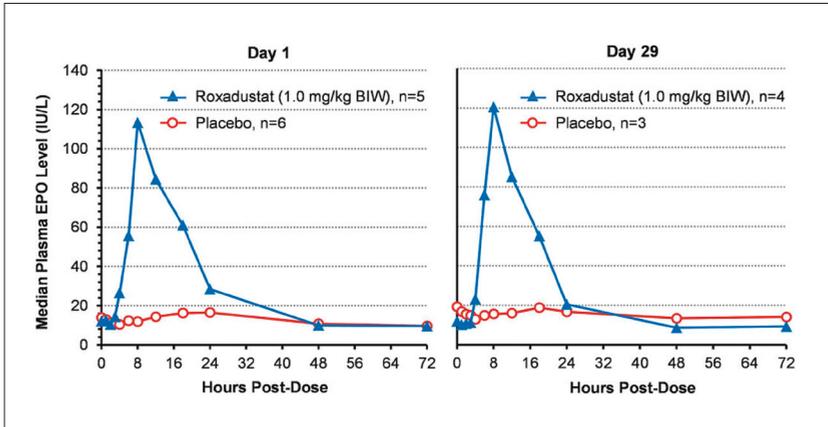


Figure 13. Induction of serum EPO in chronic kidney disease patients taking Roxadustat. From: Besarab, A., *et al.*, *Randomized placebo-controlled dose-ranging and pharmacodynamics study of roxadustat (FG-4592) to treat anemia in nondialysis-dependent chronic kidney disease (NDD-CKD) patients*. *Nephrol Dial Transplant*, 2015. **30** (10): p. 1665–73.

Early work by us and others showed that EglN3 is induced by HIF and that EglN2 and EglN3 can partially compensate for loss of EglN1 (Minamishima *et al.*, 2009). Andy found that acute inactivation of EglN1 transiently induced hepatic *erythropoietin* mRNA levels, which were then quickly dampened (presumably by EglN3) (Minamishima and Kaelin, 2010). Likewise, we collaborated with Alnylam Pharmaceuticals and showed that nanoparticle-based deliver of *EglN1* siRNAs in mice transiently induced erythropoietin (Querbes *et al.*, 2012).

At least four orally available prolyl hydroxylase inhibitors, including Roxadustat (Fibrogen), Vadadustat (Akebia), Daprodustat (GSK) and Molidustat (Bayer), have advanced to late stage clinical trials for the treatment of anemia in the setting of chronic kidney disease (Gupta and Wish, 2017). Roxadustat, the most advanced of these, has been approved in China and Japan based on positive phase 2 and phase 3 data (Figures 13 and 14). The United States new drug application was submitted in December of 2019 (Besarab *et al.*, 2015, Chen *et al.*, 2019, Kaplan, 2019). We and others have also shown that prolyl hydroxylase inhibitors are tissue protective in preclinical models of regional ischemia (Olenchock *et al.*, 2016).

#### KIDNEY CANCER TREATMENTS EMERGING FROM STUDIES OF PVHL AND HIF

Inactivation of the *VHL* gene is the initiating genetic event in ccRCCs arising in *VHL* patients, but is not sufficient to cause ccRCC. Loss of the remaining wild-type *VHL* allele in the kidneys of *VHL* disease patients

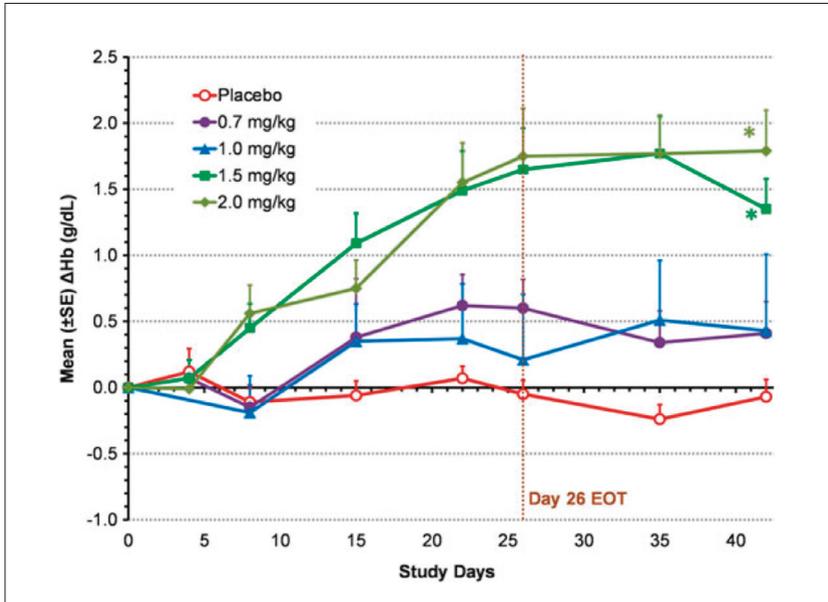


Figure 14. Increased red blood cell production in chronic kidney disease patients taking Roxadustat. From: Besarab, A., et al., *Randomized placebo-controlled dose-ranging and pharmacodynamics study of roxadustat (FG-4592) to treat anemia in nondialysis-dependent chronic kidney disease (NDD-CKD) patients*. *Nephrol Dial Transplant*, 2015. **30** (10): p. 1665–73.

causes the development of preneoplastic renal cysts. Additional stereotypical cooperating events, such as loss of the *PBRM1* or *BAP1* tumor suppressor genes, are required for progression to ccRCC (Kaelin, 2015). Dr. Charles Swanton and colleagues have done deep sequencing of multiple spatially distinct regions of ccRCCs and used the mutant allele frequencies from those regions from a given patient to infer their evolutionary histories (Gerlinger et al., 2012) (Gerlinger et al., 2014) (Turajlic et al., 2018, Mitchell et al., 2018). Here it again appears that biallelic *VHL* inactivation is the usual initiating or “truncal” mutation in most ccRCCs. Despite (or perhaps because of) this genetic complexity, our reconstitution experiments suggested that at least a subset of ccRCCs require an ongoing loss of pVHL function to grow as tumors in nude mice.

An open question in 2000 was whether HIF dysregulation was causal, or merely correlative, with respect to pVHL-defective tumors. It seemed plausible that it was causal, although HIF biology is very complex. HIF regulates many genes, some of which are likely to promote tumor growth (e.g. VEGF), and others that are likely to constrain tumor growth (e.g. the mTOR inhibitor REDD1 (Reiling and Hafen, 2004, Brugarolas et al., 2004)). We had already shown that restoring pVHL function

in *VHL*<sup>-/-</sup> ccRCC cells suppressed their ability to form tumors in nude mice (Iliopoulos *et al.*, 1995). A postdoctoral fellow in my laboratory, Keiichi Kondo, then showed that such tumor suppression by pVHL could be overridden by reintroducing a HIF2 $\alpha$  variant that escapes recognition by pVHL because of proline to alanine substitutions at its two potential prolyl hydroxylation sites (HIF2 dPA mutant) (Kondo *et al.*, 2002). Importantly, this was a specific property of HIF2 $\alpha$ . Conversely, Keiichi showed that eliminating HIF2 $\alpha$  with shRNAs suppressed tumor growth by two different *VHL*<sup>-/-</sup> ccRCC cell lines, as did my former postdoctoral fellow, Othon Iliopoulos, in his own laboratory (Kondo *et al.*, 2003) (Zimmer *et al.*, 2004). By then we were already growing leery of potential off-target effects with shRNAs, but Keiichi confirmed that the HIF2 shRNA effects were on-target because he could rescue them with an shRNA-resistant HIF2 $\alpha$  mRNA (Kondo *et al.*, 2003). Billy Kim showed that transgenic expression of HIF2 $\alpha$  dPA, but not HIF1 $\alpha$  dPA, phenocopied VHL inactivation in the liver and skin (Kim *et al.*, 2006). In the former he observed vascular lesions that very loosely resemble hemangioblastomas. And Lianjie Li showed that VHL families with a high risk of developing ccRCC have *VHL* alleles that cause higher levels of HIF than those families with a low risk of developing ccRCC (Li *et al.*, 2007).

Another postdoctoral fellow in the laboratory, Chuan Shen, took this a bit further. She showed that restoring wild-type HIF1 $\alpha$  expression in *VHL*<sup>-/-</sup> ccRCC cell lines that lacked HIF1 $\alpha$  suppressed their ability to form tumors in nude mice, while eliminating HIF1 $\alpha$  in *VHL*<sup>-/-</sup> ccRCC cell lines that retained wild-type HIF1 $\alpha$  promoted their tumor forming capability (Shen *et al.*, 2011). The differential effects of HIF1 $\alpha$  and HIF2 $\alpha$  in ccRCC were also noted by Drs. Celeste Simon and Peter Ratcliffe (Gordan *et al.*, 2008, Gordan *et al.*, 2007, Covelto *et al.*, 2005, Raval *et al.*, 2005, Mandriota *et al.*, 2002).

Dating back to the mid-1990s we knew that inactivation of pVHL upregulated VEGF and PDGF, providing a potential explanation for the highly angiogenic nature of VHL-associated neoplasms. VEGF is a mitogen and survival factor for endothelial cells, while PDGF stimulates pericytes. The work of Dr. Eli Keshet and coworkers showed that VEGF was particularly important for newly sprouting endothelial cells that lacked pericyte coverage (Benjamin *et al.*, 1998, Benjamin and Keshet, 1997).

I couldn't believe my luck that a number of pharmaceutical companies were, by the late 1990s, developing drugs that could inhibit VEGF itself (e.g. bevacizumab from Genentech) or the VEGF receptor KDR (e.g. SU5416 from Sugen and PTK787 from Novartis/Schering). Better still, some of the KDR inhibitors also fortuitously inhibited the PDGF receptor. It seemed like God was smiling on me. I began arguing that if VEGF inhibitors were going to work against any solid tumor, they would work

against VHL-associated tumors such as kidney cancers and hemangioblastomas. In other words, I thought these tumors offered the best chance to demonstrate proof of concept for this class of drugs in cancer. I was particularly optimistic about these drugs in hemangioblastomas because there were reasons to think that hemangioblastomas were genetically simpler than kidney cancers and because there were preclinical data to suggest that local deregulation of VEGF was sufficient (and one hoped necessary) to produce such vascular lesions (Benjamin and Keshet, 1997).

During this time I was consulting for Novartis, which had partnered with Schering to develop PTK787 (Dreves *et al.*, 2000). I advocated for a randomized trial of PTK787 versus placebo in kidney cancer. Unfortunately, the Novartis clinical development team was interested in a commercially more attractive cancer, such as colon cancer, and were leery of kidney cancer because so many prior drugs had failed in kidney cancer trials. Novartis also invoked the conventional wisdom that randomized phase 2 trials lack sufficient statistical power to draw meaningful conclusions. Ultimately, they ran two large randomized trials in colon cancer (which failed), but they did support a single arm trial of PTK787 in kidney cancer. A few responses were seen (de Bazelaire *et al.*, 2008). More remarkably, however, the time to progression data appeared to be remarkably better than one would have expected for a similar cohort of patients. Novartis convened a panel of clinical key opinion leaders (KOLs) who reviewed the data (I would later deduce that almost all of these KOLs were by then working with other VEGF inhibitors). The KOLs said the data were indeed exciting, but that it was formally possible the data were driven by patient selection or chance. This effectively ended PTK787 as a kidney cancer drug.

It became clear to me that small biotechnology companies might be less conservative than large, well-established, drug companies. Through the VHL family alliance I learned of a VHL patient who had lost one eye and was now legally blind because of a retinal hemangioblastoma adjacent to the optic nerve in her remaining eye. I worked with an ophthalmologist at the Joslin Diabetes Center, Dr. Lloyd Aiello, Jr., to treat her with an early KDR inhibitor from Sugen called SU5416 (Aiello *et al.*, 2002). She had dramatic improvement in her vision, likely because of decreased vessel leakiness (VEGF is also known as vascular permeability factor), but her hemangioblastoma did not shrink (Aiello *et al.*, 2002). Later attempts by others to treat VHL-associated hemangioblastomas with KDR inhibitors likewise produced modest results (Girmens *et al.*, 2003, Jonasch *et al.*, 2011, Migliorini *et al.*, 2015).

Sometime in the late 1990s I shared a car to Logan Airport with Rick Klausner, who at that time was still NCI director. I thought the NCI could do the type of clinical experiment that was needed to test whether VEGF

inhibitors could alter the natural history of kidney cancer. He enthusiastically agreed. The NCI did a clinical trial of bevacizumab in patients with metastatic kidney cancer, randomizing patients to placebo, low dose, or high dose bevacizumab (I don't actually know if my discussion with Rick was instrumental in this regard) (Yang *et al.*, 2002, Yang *et al.*, 2003). The fact that a placebo arm was ethically justifiable underscores the dearth of treatment options for kidney cancer patients at that time. The tumor shrinkage, or objective response, rates for the bevacizumab arms was very modest. Only 10% of patients on the high dose bevacizumab arm met the criteria for partial responses (Yang *et al.*, 2002, Yang *et al.*, 2003). But there was a clear, dose-dependent, improvement in time to progression in the patients who received bevacizumab (Yang *et al.*, 2002, Yang *et al.*, 2003). Plotting tumor size over time, in so-called "spider plots", you could clearly see that bevacizumab altered the natural history of these tumors (Yang *et al.*, 2002, Yang *et al.*, 2003). I was thrilled.

I loved this trial for two other reasons. One of the placebo patients actually had a partial response (spontaneous tumor regressions, although rare, have been reported in kidney cancer). Had this been a single arm trial of some new drug, this patient might have created false hope. More importantly, one of the early patients on the trial had massive hematuria and was taken to the NCI emergency room. The code was broken, at which point it was learned that this was also a placebo patient. Of course, there was a 2/3 chance the patient would have been getting bevacizumab. It gives me chills to think that if this patient been on bevacizumab the development of VEGF inhibitors might have been significantly delayed for safety concerns.

Another company, Onyx Pharmaceuticals (in collaboration with Bayer), had developed a Raf kinase inhibitor called BAY 43-9006 (sorafenib). In phase 1 trials it appeared that kidney cancers might be particularly sensitive to BAY 43-9006, which did not make sense given what was known about Raf and kidney cancer at that time (Awada *et al.*, 2005). Frank McCormick, a founder at Onyx, was familiar with our work. He suggested they test sorafenib against KDR. Sorafenib proved to be a fairly effective KDR inhibitor and was then developed as a kidney cancer drug (Ratain *et al.*, 2006, Escudier *et al.*, 2007). Sugen produced additional KDR inhibitors, including the drug now called sunitinib (Motzer *et al.*, 2007). Both sorafenib and sunitinib were approved for the treatment of kidney cancer based on positive phase 3 clinical trial data (Escudier *et al.*, 2007, Motzer *et al.*, 2007). Currently there are 7 approved KDR inhibitors for kidney cancer (avastin, sorafenib, sunitinib, axitinib, pazopanib, cabozantinib, levantinib) (Choueiri and Motzer, 2017). The objective response rates of these drugs differ somewhat, possibly due to differences in their ability to inhibit KDR, PDGFR, and other off-targets (e.g. cabozantinib

also inhibits MET, which has also been linked to kidney cancer growth and VHL biology (Koochekpour *et al.*, 1999, Bommi-Reddy *et al.*, 2008, Nakaigawa *et al.*, 2006, Pennacchietti *et al.*, 2003)).

Although VEGF inhibitors have become mainstays of kidney cancer treatment, not all patients respond to them and all will eventually become resistant to them. It seemed likely, a priori, that targeting HIF2 would be a more effective ccRCC treatment than targeting any single HIF2-responsive protein (e.g. VEGF). The dogma, however, was that DNA-binding transcription factors such as HIF2, with the exception of the steroid hormone receptors (which have hydrophobic ligand-binding pockets), were undruggable.

In ~2010, however, I was approached to join the scientific advisor board of a small biotechnology start-up company called Peloton Therapeutics in Dallas, Texas. One of the cofounders of this company, Steven McKnight at the University of Texas, Southwestern (UTSW), had originally cloned HIF2 $\alpha$  (Tian *et al.*, 1997) and was aware of our work on HIF2 $\alpha$  in ccRCC. Two of his UTSW colleagues, Rick Bruick and Kevin Gardner, had identified a potentially druggable pocket in the HIF2 $\alpha$  PAS B domain (Scheuermann *et al.*, 2009). Moreover, they identified chemicals that could bind to this pocket and, in so doing, induce an allosteric change in HIF2 $\alpha$  such that it could no longer bind to its partner, ARNT (Rogers *et al.*, 2013, Scheuermann *et al.*, 2013, Scheuermann *et al.*, 2009).

These chemicals were then outlicensed to form Peloton Therapeutics. The Peloton chemists did a brilliant job making these UTSW chemicals more drug-like: increasing their potency, specificity, and bioavailability (Wallace *et al.*, 2016). They provided us with a tool compound, PT2399, which was highly similar to their initial clinical candidate, PT2385.

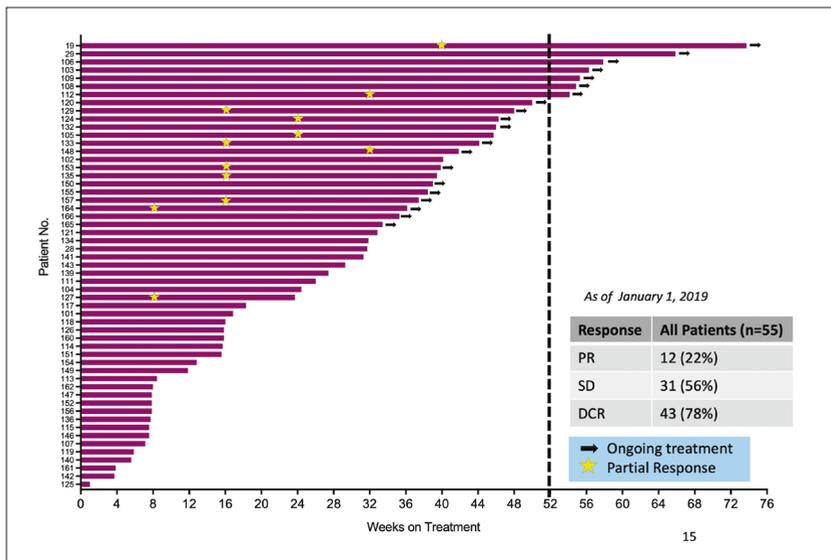
A major challenge in pharmacology, especially when doing so-called “down assays” (e.g. decreased viability, decreased proliferation, or decreased tumor growth), is to make sure that chemical-induced phenotypes are on-target and not off-target. Hyejin Cho, a postdoctoral fellow in my laboratory, showed that treating *VHL* $-/-$  ccRCC cell lines with PT2399 decreased HIF2-responsive mRNAs, decreased soft agar colony formation, and decreased tumor growth in nude mouse xenograft assays (Cho *et al.*, 2016). Importantly, Hyejin showed that these effects were largely rescued by a PT2399-resistant version of HIF2 $\alpha$  that had been created by Bruick and Gardner, thus establishing that the PT2399 effects were on-target (Cho *et al.*, 2016). The activity of this compound in preclinical ccRCC models was also reported by my former postdoctoral fellow, Dr. James Brugarolas, after he started his own laboratory (Chen *et al.*, 2016).

To our surprise, we discovered that some *VHL* $-/-$  ccRCC lines are insensitive to genetic (CRISPR/Cas9-mediated) and pharmacologic (PT2399) inhibition of HIF2 (Cho *et al.*, 2016). These same lines, when tested, are insensitive to restoration of pVHL function (unpublished

data). It is possible that some of these lines lost HIF2-dependence over time in culture. Alternatively, some cooperating genetic events in ccRCC (i.e. ensuing mutations after *VHL* mutation) might render ccRCC insensitive to pVHL and loss of HIF.

The first HIF2 inhibitor to be tested in the clinic, PT2385, demonstrated some activity in heavily pretreated ccRCC patients (Courtney *et al.*, 2018). This compound, however, has a metabolic liability that caused unacceptable variability in pharmacokinetics (Courtney *et al.*, 2018). PT2977 is a more potent HIF2 inhibitor with more favorable pharmacokinetic properties. It is about to enter Phase 3 testing in ccRCC based on very promising Phase 2 data in patients who have failed standard of care agents (Figure 15) (Jonasch *et al.*, 2019).

Approximately 50 *VHL* patients with measurable kidney tumors who had not received prior medical therapy have also been treated with PT2977. These patients were being followed in active surveillance programs in an attempt to delay or avoid multiple surgeries. As expected, many of these patients have other tumors, such as hemangioblastomas.



**Figure 15.** Swimmers plot of patients with metastatic kidney cancer treated with PT2399. Each horizontal bar represents an individual patient with metastatic kidney cancer and how long they remained on therapy once they began treatment with HIF2 inhibitor PT2399. Arrows indicate patients who were still on therapy at the time of this analysis. Yellow stars indicate patients who had a partial response (PR) by RESIST criteria. SD = Stable disease. DCR = disease control rate. From: 1. Jonasch, E., *et al.*, *A first-in-human phase I/II trial of the oral HIF-2 $\alpha$  inhibitor PT2977 in patients with advanced RCC*. 2019 European Society for Medical Oncology annual meeting, ESMO 2019, 27 Sept–1 Oct 2019 in Barcelona, Spain (911PD), 2019.

The response data for these patients have not been made public yet (and certainly have not undergone peer review). However, discoverable social media posts from these patients are very encouraging. With luck the natural history of the disease described by Collins, von Hippel, and Lindau will be favorably altered.

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