Inhibition of HIF2α Is Sufficient to Suppress pVHL-Defective Tumor Growth

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Biallelic inactivation of the von Hippel–Lindau tumor suppressor gene (VHL) is linked to the development of hereditary (VHL-associated) and sporadic clear-cell renal carcinomas as well as other abnormalities. The VHL gene product, pVHL, is part of an E3 ubiquitin ligase complex that targets the α subunits of the heterodimeric transcription factor HIF (hypoxia-inducible factor) for degradation in the presence of oxygen. Here we report that a HIF2α variant lacking both of its two prolyl hydroxylation/pVHL-binding sites prevents tumor inhibition by pVHL in a DNA-binding dependent manner. Conversely, downregulation of HIF2α with short hairpin RNAs is sufficient to suppress tumor formation by pVHL-defective renal carcinoma cells. These results establish that tumor suppression by pVHL is linked to regulation of HIF target genes.

Introduction

von Hippel–Lindau (VHL) disease is caused by heterozygous germline inactivation of the VHL tumor suppressor gene, which resides on chromosome 3p25 (Kaelin 2002). The cardinal feature of this hereditary cancer syndrome is the development of multiple vascular tumors, called hemangioblastomas, in the central nervous system and retina, as well as an increased risk of clear-cell carcinoma of the kidney and pheochromocytoma. Tumor development in VHL disease is linked to somatic inactivation or loss of the remaining wild-type VHL allele, leading to loss of the wild-type VHL gene product, pVHL. In the kidney, this event occurs very early, as it has been documented in epithelial cells lining premalignant renal cysts (Zhuang et al. 1995; Lubensky et al. 1996; Mandriota et al. 2002). Consistent with Knudson’s two-hit model, somatic VHL mutations are also common in sporadic clear-cell renal carcinomas and hemangioblastomas. Conversely, restoration of pVHL function is sufficient to suppress tumor formation by pVHL-defective renal carcinoma cells in vivo (Iliopoulos et al. 1995; Gnarra et al. 1996; Schoenfeld et al. 1998).

pVHL is the substrate recognition module of an E3 ubiquitin ligase complex that contains elongin B, elongin C, Cul2, and Rbx1 (also called ROC1 or Hrt1) (Kaelin 2002). This complex targets the α subunits of the heterodimeric transcription factor HIF (hypoxia-inducible factor) for polyubiquitination and hence proteasomal degradation. There are three human HIFα proteins (HIF1α, HIF2α, and HIF3α). Enzymatic hydroxylation of conserved prolyl residues within these proteins by members of the egg-laying-defective nine (EGLN) family is required for their recognition by pVHL (Kaelin 2002). This posttranslational modification is inherently oxygen-dependent. Accordingly, HIFα subunits are normally unstable in the presence of oxygen, but are stabilized under low-oxygen (hypoxic) conditions. In contrast, cells lacking wild-type pVHL fail to degrade HIFα subunits in the presence of oxygen, and thus hypoxia-inducible gene products are constitutively overproduced. Among these proteins are vascular endothelial growth factor (VEGF) and platelet-derived growth factor B, implicated in angiogenesis; phosphoglycerate kinase and glucose transporter 1 (GLUT1), involved in glucose uptake and metabolism; and transforming growth factor α (TGFα), which can establish a mitogenic autocrine loop with the epidermal growth factor (EGF) receptor (EGFR) (Iliopoulos et al. 1996; Knebelmann et al. 1998; Maxwell et al. 1999; de Paulsen et al. 2001).

Tumor-derived pVHL mutants are typically defective with respect to HIF polyubiquitination in vivo, and the HIF target genes cited above are implicated in tumorigenesis. Thus, correlative data and biological plausibility support a role for HIF in pVHL-defective tumor formation. Nonetheless, emerging genotype–phenotype correlations in VHL disease suggest that pVHL has multiple functions. For example, pVHL mutants associated with a low risk (type 2A VHL disease) and high risk (type 2B disease) of renal cell carcinoma are similarly defective with respect to HIF regulation (Clifford et al. 2001; Hoffman et al. 2001). Interestingly, individuals with Chuvash polycythemia are homozygous for a hypomorphic VHL allele that is quantitatively defective with respect to HIF regulation, which leads to overproduction of erythropoietin in vivo but not tumor formation (Ang et al. 2002). Moreover, forced activation of HIF target genes has not led to tumor formation in the animal models tested so far (Vincent et al. 2000; Elson et al. 2001; Rebar et al. 2002). Conversely, some pVHL mutants that retain the ability to...
regulate HIF are linked to familial pheochromocytoma (type 2C VHL disease) (Clifford et al. 2001; Hoffman et al. 2001). Collectively, these findings suggest that tumor formation following pVHL inactivation reflects the loss of multiple pVHL functions in a context-dependent manner.

In this report we provide data that strengthen our earlier conclusion that inhibition of HIF2α is necessary for pVHL-dependent suppression of renal carcinoma tumor formation in vivo (Kondo et al. 2002). Moreover, we provide evidence that inhibition of HIF2α is likewise sufficient to suppress tumor formation by VHL(-/-) renal carcinoma cells in vivo. Collectively, these results indicate that HIF2α is a critical downstream target of pVHL with respect to suppression of renal carcinogenesis.

**Results and Discussion**

**Inhibition of HIF2α Target Genes Is Necessary for Tumor Suppression by pVHL**

Hydroxylation of HIF1α Pro564 or HIF2α Pro531 generates a pVHL-binding site (Ivan et al. 2001; Jaakkola et al. 2001; Yu et al. 2001). We previously showed that a HIF2α variant in which Pro531 was replaced by alanine (HIF2α P531A) escaped recognition by pVHL and induced the expression of HIF target genes in vivo (Kondo et al. 2002). Moreover, HIF2α P531A abrogated pVHL-dependent tumor suppression in vivo, implying that HIF is functionally downstream of pVHL and that inhibition of HIF is necessary for tumor suppression by pVHL (Kondo et al. 2002). Shortly thereafter, it was shown that hydroxylation of HIF1α Pro404 (corresponding to HIF2α Pro405) creates a second potential pVHL-binding site within HIF1α (Masson et al. 2001). Although we could not detect a physical interaction between pVHL and HIF2α P531A (Kondo et al. 2002), the identification of a second potential pVHL-binding site left open the possibility that the biological effects of HIF2α P531A were due, at least partly, to perturbation of pVHL function as a result of direct binding. If true, this would undermine the conclusions described above. Moreover, we had not established whether the biological effects of HIF2α P531A required that it bind to DNA, as would be expected if its oncogenic effects were due to transcriptional activation of specific hypoxia-inducible promoters. To this end, we repeated our earlier experiments using retroviral vectors encoding HIF2α P405A:P531A or HIF2α P405A:P531A:bHLH*. The latter contains a five amino acid substitution within the HIF2α basic helix-loop-helix (bHLH) domain that leads to loss of DNA-binding capability (Kondo et al. 2002).

786-O renal carcinoma cells lack wild-type pVHL and overproduce HIF2α (Iliopoulos et al. 1995; Maxwell et al. 1999). HIF1α is not detectable in these cells (Maxwell et al. 1999). Reintroduction of wild-type pVHL into 786-O cells by stable transfection does not affect cell growth in vitro under standard cell culture conditions (Figure 1B). In contrast, but in keeping with our earlier results with HIF2α (Kondo et al. 2002), HIF2α P405A:P531A restored the ability of WT8 cells to form large tumors in vivo in nude mouse xenograft assays (Figure 1C). HIF2α P405A:P531A:bHLH* did not promote tumor formation by WT8 cells, implying that tumor formation by WT8 cells is pVHL-dependent.

![Figure 1. HIF2α Overrides Tumor Suppression by pVHL](http://biology.plosjournals.org)

(A) 786-O subclones that were transduced to produce wild-type pVHL (WT8) or with an empty plasmid (PRC3) cells, as well as WT8 cells infected with an empty retrovirus (Empty) or retroviruses encoding the indicated HIF2α variants [(P-A)² = P405A:P531A and * = bHLH mutation] were grown in the presence of 21% or 1% oxygen and immunoblotted (IB) with the indicated antibodies.

(B) In vitro proliferation of WT8 cells infected with the indicated retroviruses.

(C) Tumor weights approximately 9 wk after subcutaneous implantation of WT8 cells infected with the indicated retroviruses in nude mice. Number of tumors analyzed is shown in parentheses. Error bars = one standard error. DOI: 10.1371/journal.phylo.0000083.g001

Neither HIF2α P405A:P531A nor HIF2α P405A:P531A:bHLH* affected the proliferation of WT8 cells in vitro under standard cell culture conditions (Figure 1B). In contrast, but in keeping with our earlier results with HIF2α P531A (Kondo et al. 2002), HIF2α P405A:P531A restored the ability of WT8 cells to form large tumors in vivo in nude mouse xenograft assays (Figure 1C). HIF2α P405A:P531A:bHLH* did not promote tumor formation by WT8 cells, implying that tumors
promotion by HIF2α P405A:P531A is linked to its ability to act as a sequence-specific DNA-binding transcriptional regulator. These results, together with our earlier findings, indicate that inhibition of HIF2α is necessary for tumor suppression by pVHL.

Loss of HIF2α Is Sufficient to Suppress pVHL-Defective Tumor Growth In Vivo

To ask whether inhibition of HIF2α is likewise sufficient for tumor suppression by pVHL, we set out to inhibit HIF2α in VHL(−/−) renal carcinoma cells using short hairpin RNAs (shRNA). We tested five HIF2α shRNAs based on 19mer sequences that are unique to HIF2α according to GenBank. Two such shRNAs (#2 and #3) decreased HIF2α protein levels, as determined by anti-HIF2α immunoblot analysis and by diminished activity of a cotransfected HRE–luciferase reporter plasmid, when transiently introduced into 786-O cells (data not shown). Infection of 786-O cells with retroviruses encoding shRNA #2 or #3, but not the parental retrovirus, led to decreased steady-state levels of HIF2α protein as well as decreased levels of GLUT1, which is encoded by a HIF-responsive gene (Figure 2A). Downregulation of HIF2α did not affect cell growth in vitro, but was sufficient to impair tumor growth in vivo (Figure 2B–2D). The former observation is consistent with the finding that pVHL does not inhibit cell proliferation under standard cell culture conditions and argues against the idea that the latter was due to nonspecific toxicity. Moreover, these in vivo effects could be prevented by coadministration of a retrovirus encoding an HIF2α mRNA with silent third-base mutations within the shRNA recognition site (MT*) and were not observed with retroviruses encoding a scrambled HIF2α shRNA or luciferase shRNA (data not shown). Thus, tumor suppression by the HIF2α shRNA was unlikely to reflect a spurious interaction with an unintended target.

To ask whether these findings could be extended to other VHL(−/−) renal carcinoma cell lines, we repeated these
experiments in A498 VHL(−/−) renal carcinoma cells. Tumor formation by these cells in nude mice is diminished following restoration of pVHL function (Loneragan et al. 1998). In keeping with the results obtained with 786-O cells, downmodulation of HIF2α levels with shRNA did not affect A498 cell growth in vitro (data not shown), but dramatically inhibited tumor growth in vivo (Figure 4A). It is noteworthy that both 786-O cells and A498 cells produce HIF2α and not HIF1α (Maxwell et al. 1999). It will be important in the future to ask whether disruption of HIF2α is sufficient to suppress tumor formation by pVHL-defective cells that produce both HIF2α paralogs. In this regard, studies of renal precursor lesions in VHL patients suggest that HIF2α is more oncogenic than HIF1α (Mandriota et al. 2002). It is tempting to speculate that loss of HIF1α expression in some pVHL-defective renal carcinoma cells confers a selective advantage in vivo, perhaps related to the ability of HIF1α to induce apoptosis in some settings (Carmeliet et al. 1998).

Several histological renal carcinoma variants have been recognized, including clear-cell carcinoma and papillary (chromophil) carcinoma. VHL mutations are common in the former, but not in the latter (Gnarra et al. 1994; Takahashi et al. 2002). Interestingly, the small A498 tumors that did form in the presence of HIF2α shRNA consisted of malignant cells forming tubulopapillary structures, corresponding to papillary (chromophil) renal carcinoma histology, whereas the empty vector tumors consisted primarily of sheets of clear cells, as would be seen in typical clear-cell renal carcinoma, with interspersed areas displaying papillary features (Figure 4B). This suggests that dysregulation of HIF2α is causally linked to the clear-cell pattern and is consistent with the tight linkage between VHL mutations and this renal carcinoma subtype.

Most of the work performed so far with respect to the oncogenic effects of HIF has focused exclusively on HIF1α, where both prooncogenic and antioncogenic effects have been reported (Maxwell et al. 1997; Carmeliet et al. 1998; Ryan et al. 1998, 2000; Hopfl et al. 2002). Likewise, loss of pVHL is prooncogenic in a restricted subset of human tissues (Kaelin 2002). In the mouse, loss of pVHL promotes hemangioma development in the liver, but inhibits tumor formation by embryonic stem cells (Haase et al. 2001; Mack et al. 2003). These observations conform to the emerging paradigm that the same mutation can be either prooncogenic or antioncogenic, depending on the molecular and cellular context. Therefore, one must be cautious in extrapolating our findings beyond human clear-cell renal carcinomas.

Loss of pVHL in the human kidney gives rise to premalignant renal cysts (Zhuang et al. 1995; Lubensky et al. 1996; Mandriota et al. 2002). It is presumed that additional mutations at non-VHL loci are required for conversion to frank renal cell carcinomas. It will therefore be of interest to determine whether dysregulation of HIF is sufficient to produce renal cysts. In this regard, TGFα, which is encoded by a HIF target gene, is a potent renal mitogen and is sufficient to induce renal cysts in the mouse (Lowden et al. 1994; Chailler and Briere 1998; Ramp et al. 2000; de Paulsen et al. 2001). On the other hand, our data do not exclude the possibility that the development of renal pathology following pVHL loss in humans reflects a complex interplay between dysregulated HIF2α and loss of a second pVHL function. pVHL has been implicated in control of cell-cycle, differentiation, and extracellular matrix formation, although the extent to which these activities are due to control of HIF is not known (Kaelin 2002). A number of non-HIF pVHL-binding partners have, however, been reported, including atypical protein kinase C members, VDU1, SP1, and fibronectin (Kaelin 2002).

**Therapeutic Implications**

Our findings strengthen the notion that inhibition of HIF2α might be therapeutically useful in pVHL-defective clear-cell renal carcinoma. On the other hand, sequence-specific DNA-binding transcription factors have not proven to be attractive drug targets to date. For this reason, it will be important to determine which HIF2α target genes are necessary for its oncogenic activity. Among the known HIF targets, the abovementioned TGFα and its cognate receptor, EGFR, are frequently overproduced in renal carcinoma and are suspected to establish an autocrine loop (Myldlo et al. 1989; Lager et al. 1994; Knebelmann et al. 1998; de Paulsen et al. 2001). A number of EGFR are presently in clinical trials (Fabbro et al. 2002). Likewise, overproduction of VEGF is common in renal cell carcinoma and likely contributes to tumor angiogenesis in this setting (Walke et al. 1991; Brown et al. 1993; Takahashi et al. 1994; Nicol et al. 1997; Ramp et al. 1997). Drugs directed against VEGF or its receptors are also
being tested in humans (Fabbro et al. 2002). In a recent Phase II study, a neutralizing VEGF antibody was shown to delay disease progression in metastatic renal carcinoma (Yang et al. 2003) and offers hope that rational combinations of small molecules directed against HIF targets will alter the natural history of this disease.

Materials and Methods

Plasmids. pBABE-puro-HA-HIF2α P405A/P531A was generated by two-step PCR. The pcDNA3.0-HA-HIF2α P531A (Kondo et al. 2002) insert was first amplified with primer A (5'-GGCCGGATCCCGACC- CATGACA-3') and primer B (5’-TCTCAGGGATCCGACCTG-3') or primer C (5’-CAGCTGCTGATCCCCCCAGGA-3') and primer D (5’-GCGGCCGCAATTGTCAGGTGGCCTGGTC-3'). Aliquots of these two PCRs were then mixed and amplified with primers A and D. The resulting PCR product was digested with BamHI and MunI and ligated into pbABE-puro-HA-Vector cut with BamHI and EcoRI. In parallel, similar reactions were carried out with pcDNA3.0-HA-HIF2α P531A/AbHLL* (Kondo et al. 2002) as the PCR template to make pBABE-puro-HA-HIF2α P405AP531AbHLL* (conversion of amino acids residues 24-29, CRKRKS to ACAASA).

Short interfering RNAs (siRNAs) corresponding to two unique HIF2α 19mer sequences (5', 5'-GAGAAGGTCGAAAAGGTG-3' and 3', 5'-GAGAAGGTCGAAAAGGTG-3') downregulated HIF2α protein levels and HIF-dependent transcriptional activity. Synthetic oligonucleotides spanning the #2 siRNA sequence (5’-GATCCCCGACAGGTCTGCTCCTTTTTGGAAA-3') and the #3 sequence (5’-GATCCCCGACAGGTCTGCTCCTTTTTGGAAA-3') were annealed by incubation in 30 mM HEPES–KOH, 2 mM Mg–acetate for 4 min at 95°C followed by 10 min at 70°C. The resulting duplex oligonucleotides were phosphorylated with T4 polynucleotide kinase (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol and ligated into pRETRO-SUPER vector (Brummelkamp et al. 2002) cut with BglII and HindIII. The resulting plasmid was designated as pBABE-hydro-HA-HIF2α siRNA recognition site mutant for #3 siRNA (5’-GAGAAGGTCGAAAAGGTG-3') (HIF2α MT) was generated by two-step PCR. The pcDNA3.0-HA-HIF2α MT insert was first amplified with primers A and D and primer E (5’-GATCCCCGACAGGTCTGCTCCTTTTTGGAAA-3’) or primer F (5’-GGAAGGTCGAAAAGGTG-3’) and primer D. Aliquots of these two PCRs were then mixed and amplified with primers A and D. The resulting PCR product was digested with BamHI and MunI and ligated into pBABE-puro-HA-Vector cut with BamHI and EcoRI. All plasmids were authenticated by DNA sequencing. pGL2-VEGF control (Promega Corporation, Madison, Wisconsin, United States) and SuperSignal West Pico chemiluminescent Western blotting reagent (Pierce, Rockford, Illinois, United States) were used for detection of Western blotting results.

Cell culture. Renal carcinoma cell lines (786-O and A498) and Phoenix cells (a generous gift of Dr. Gary Nolan, Department of Molecular Pharmacology, Stanford University, Stanford, California, United States) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Cells were maintained in a humidified atmosphere of 5% CO2 in air at 37°C.

Acknowledgments

This work was sponsored by the National Cancer Institute and the Murray Foundation. The authors have declared that no conflicts of interest exist.

Author contributions. KK and W.G.K conceived and designed the experiments, KK, W.G.K., and ML performed the experiments. KK, ML, and W.G.K. analyzed the data. KK contributed reagents/materials/analysis tools. W.G.K and W.G.K wrote the paper.

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