

Inhibition of Hypoxia-Inducible Factor Is Sufficient for Growth Suppression of VHL^{-/-} Tumors

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Abstract

The von Hippel-Lindau tumor suppressor protein (pVHL) is a substrate receptor for the mammalian SCF-2 E3 ubiquitin ligase complex that targets several substrates for ubiquitination and proteasomal degradation. Among these targets are the α -regulatory subunits of the hypoxia-inducible factor (HIF). VHL^{-/-} cells constitutively overexpress hypoxia-inducible genes through both transcriptional and posttranscriptional mechanisms and form tumors when injected into nude mice. Reintroduction of pVHL into VHL^{-/-} cell lines restores normal oxygen-dependent regulation of these genes and suppresses tumor formation in the mouse xenograft assay. We report here that short hairpin RNA-mediated inactivation of HIF phenocopies the effects of pVHL reintroduction with respect to decreased expression of hypoxia-inducible genes, decreased ability to promote vascular endothelial cell proliferation *in vitro*, and tumor growth suppression *in vivo*. In addition, HIF inactivation abrogated the cellular response to hypoxia, indicating that HIF is the only pVHL target required for this response. These data suggest that deregulation of hypoxia-inducible genes in VHL^{-/-} cells can be attributed mainly to deregulation of HIF and validate HIF as a therapeutic anticancer drug target.

Introduction

The von Hippel-Lindau tumor suppressor protein (pVHL) is the substrate receptor of a mammalian E3 ubiquitin ligase complex consisting of Cullin-2, Elongins B/C, and the RING finger protein Rbx-1 (1, 2). Germ line mutations in the *VHL* gene predispose affected individuals to a variety of hyper-vascular tumors including renal cell carcinomas (RCC), hemangioblastomas, and pheochromocytomas (3). The *VHL* gene is also somatically inactivated in the majority of sporadic RCC and hemangioblastoma cases (4). Several substrates of pVHL have been reported thus far, including the α -regulatory

subunits of hypoxia-inducible factor (HIF), the subunits of the RNA polymerase II complex, and the activated form of protein kinase C- δ (5–7). The relative importance of each substrate for tumorigenesis as mediated by loss of VHL function is currently under investigation.

A hallmark of the VHL^{-/-} cells is the constitutive overexpression of hypoxia-inducible genes under normoxic conditions (8, 9). This up-regulation has been attributed to both transcriptional and posttranscriptional events (8–10). Reintroduction of pVHL into VHL^{-/-} cells reconstitutes normal regulation of these genes and suppresses the ability of VHL^{-/-} cell lines to grow as tumors in the mouse xenograft assay without altering their *in vitro* growth characteristics (9, 11).

Regulation of HIF activity by pVHL provides a link between *VHL* being a tumor suppressor gene and the function of pVHL as the substrate recognition component of an ubiquitin ligase complex. Under normoxic conditions, pVHL binds to the α -regulatory subunits of HIF leading to their polyubiquitination and proteasomal degradation (5, 12). This interaction depends on hydroxylation of HIF on conserved proline residues by a family of HIF prolyl hydroxylases (HPHs; 13, 14). Hypoxia inactivates the HPHs and thereby disrupts the pVHL-HIF interaction. Once stabilized, HIF can be detected in the nucleus within seconds. Tumor-associated VHL mutations inhibit the ability of the SCF-2^{VHL} complex to ubiquitinate HIF, resulting in HIF stabilization and constitutive overexpression of hypoxia-inducible genes (5, 12). Many of the growth and angiogenic factors not regulated in the absence of pVHL are HIF target genes (*e.g.*, *VEGF*, *PDGF*, *GLUT-1*, and *MMP*) and up-regulation of these genes is generally considered part of the malignant phenotype (15–18). However, these genes are regulated by factors other than HIF because basal expression is observed even in pVHL-expressing cell lines. Moreover, pVHL targets multiple proteins for proteasomal degradation and hypoxia-inducible genes are also subject to posttranscriptional regulation. It is therefore important to evaluate the role of HIF with regard to the ability of pVHL to mediate regulation of hypoxia-inducible genes and tumor suppression.

It is currently not clear whether HIF activity is itself critical for tumor growth. Overexpression of HIF1 α in the epidermis of transgenic mice leads to the formation of mature vessels only, without evidence of tumorigenesis (19). Carmeliet *et al.* (20) reported that HIF1 α ^{-/-} embryonic stem cells generate aggressive vascularized tumors, albeit in a latent fashion, when injected in syngeneic animals. Moreover, normoxic stabilization of HIF1 α was not sufficient to reproduce tumorigenesis in pVHL-reconstituted RCC cells (21). Despite these observations, there is mounting evidence that HIF is critical for tumor growth. HIF1 α is overexpressed in a variety of human tumors

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but not in the corresponding surrounding normal tissue (22, 23) or in benign hyperplastic lesions (24). Contrary to the report by Carmeliet *et al.*, Ryan *et al.* found that HIF1 α ^{-/-} mouse embryonic stem cells exhibit a significantly reduced capacity to form teratocarcinomas when injected into syngeneic animals compared with HIF1 α wild-type controls (25). Lastly, *in vivo* disruption of HIF with the transcriptional coactivator p300 may lead to tumor suppression (26). Such conflicting reports suggest that the relative importance of HIF in mediating tumor angiogenesis may be tissue specific or depend on the genetic background of the tumor.

Hypoxia-inducible gene expression is regulated by HIF2 α in the VHL^{-/-} RCC cell line 786-O while HIF1 α is not expressed (5, 27). It has recently been shown that overexpression of a HIF2 α , but not HIF1 α , mutant that escapes pVHL-mediated degradation in this cell line overrode the ability of reintroduced pVHL to suppress tumor formation, indicating that ubiquitin-dependent degradation of HIF is necessary for pVHL-mediated tumor suppression (28). Notwithstanding the limitations of overexpression gain-of-function experiments, we wanted to investigate whether inactivation of HIF2 α is sufficient for tumor suppression in VHL^{-/-} 786-O cells. In this report, we explore the effects of short hairpin RNA (shRNA)-mediated HIF2 α inhibition on tumor suppression in these cells using the mouse xenograft assay.

Results

shRNA-Mediated Inhibition of HIF2 α in VHL^{-/-} Cells

The human RCC cell line 786-O harbors an inactivating mutation in one VHL allele while the second allele is deleted. This VHL^{-/-} cell line constitutively expresses the HIF2 α but not the HIF1 α isoform (5, 27). This cell line has been used extensively in the past to decipher critical functions of pVHL. In previous work, we showed that 786-O cells form tumors when injected into nude mice and that stable reintroduction of pVHL in these cells suppresses tumor formation in the mouse xenograft assay (11).

To evaluate the role of HIF2 α in VHL^{-/-} tumor formation, we engineered 786-O-derived clones in which HIF2 α has been stably inactivated by shRNA interference (shRNAi). Two types of vectors were used: (a) a pSuperRetro (pSR) vector in which the expression of HIF-targeting shRNAi is placed under the control of the H1 RNA polymerase III promoter and (b) vector pTU6IIa in which a U6 promoter directs the synthesis of shRNAi. In experiments presented below, we used the pSR-based HIF2 α knockdown clone pSR1 and pTU-based clones pTR1, pTR2, and pTR3. Control clones were engineered by stable transfection of 786-O cells with empty corresponding plasmids: pSV1 for pSR and pTV1 and pTV2 for pTUIIa plasmids. A genome-wide search identified HIF2 α as the only target of the oligonucleotides used for shRNAi construction. Because the value of “scrambled” shRNAi is still under discussion in the field, we elected to instead provide evidence of shRNAi specificity by targeting two different portions of the HIF2 α gene using different vector systems. We have previously engineered 786-O derivative clones that stably express hemagglutinin-tagged wild-type pVHL (WT8) or harbor empty plasmid vector as control (PRC3; 11).

HIF2 α expression was examined in the HIF2 α knockdown clones by Western blot analysis. Lysates from PRC3 and WT8 clones were included as corresponding positive and negative controls. HIF2 α expression was dramatically decreased in the 786-O clones transfected with shRNAi-encoding vectors but not empty control plasmids (WB: HIF2 α in Fig. 1B). To assay for HIF activity, we transiently transfected the HIF knockdown or control clones with a hypoxia response element (HRE)-driven firefly luciferase reporter. Clones with decreased HIF2 α protein showed dramatically decreased HRE reporter activity compared with control clones as measured by normalized luciferase activity (Fig. 1A). These experiments confirm that clones engineered to express shRNA directed against HIF2 α decreased HIF2 α protein expression and HIF reporter activity.

Inhibition of HIF2 α in VHL^{-/-} Cells Phenocopies the Effect of pVHL Reintroduction on Hypoxia-Inducible Genes

Deregulation of hypoxia-inducible genes in VHL^{-/-} cells was attributed to both transcriptional and posttranscriptional events (8–10). Subsequent to these observations, pVHL was shown to be a substrate receptor for a SCF-like E3 ubiquitin ligase complex that targets the α -regulatory subunits of HIF for degradation. The regulation of hypoxia-inducible genes could,

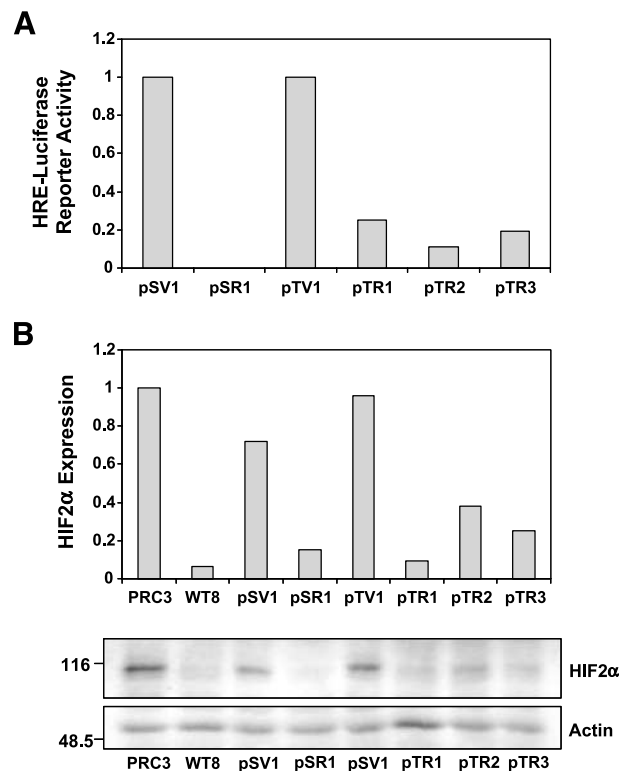


FIGURE 1. Inhibition of HIF2 α in VHL^{-/-} cells by shRNAi. VHL^{-/-} 786-O cells were stably transfected with pSR or pTU6 plasmids expressing shRNA targeting HIF2 α or control empty vector. **A.** Normalized HRE-driven luciferase activity in shRNAi *versus* control clones. **B.** Western blot analysis for HIF2 α expression in HIF2 α shRNAi and pVHL-reconstituted clones *versus* vector-only control clones. Bar graph quantitates relative HIF2 α levels normalized for actin loading control.

at least in part, be explained by regulation of HIF by pVHL. Because it is known that pVHL targets other proteins besides HIF for degradation, it is important to evaluate the role of HIF with regard to the ability of pVHL to mediate regulation of hypoxia-inducible genes and tumor suppression.

To this end, lysates obtained from HIF knockdown or control clones were examined for the expression of *Glut-1*, a known HIF2 α target gene (29). The shRNAi-encoding clones displayed decreased *Glut-1* expression (WB: *Glut-1* in Fig. 2B) compared with control clones. In addition, tissue culture supernatants conditioned by the HIF knockdown clones contained significantly reduced levels of secreted vascular endothelial growth factor (VEGF), a second endogenous HIF target, compared with control clones (Fig. 2A). *Glut-1* and VEGF expression from WT8 and PRC3 cells are shown for comparison. shRNAi-mediated inhibition of HIF and restoration of pVHL function in VHL $-/-$ cells decreased the expression of at least these two hypoxia-inducible genes to comparable levels, although the levels were always slightly higher in the HIF knockdown clones. We attribute this to incomplete inhibition of HIF activity in the clones. These data suggest that pVHL regulates hypoxia-inducible genes predominantly through its regulation of HIF stability. It is notable that

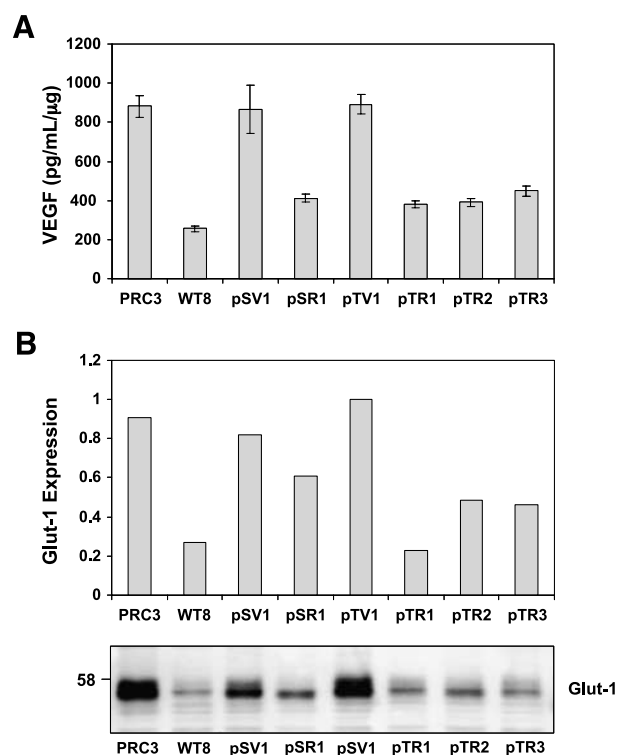


FIGURE 2. shRNAi of HIF2 α decreases expression of HIF target genes. **A.** ELISA for secreted VEGF concentration in tissue culture supernatants of HIF2 α shRNAi and pVHL-reconstituted clones *versus* vector-only control clones. *Bars*, SEM. Differences in VEGF expression between pVHL-reconstituted or HIF2 α knockdown clones and their respective control clones were significant ($P < 0.01$). **B.** Western blot analysis for *Glut-1* expression in the HIF2 α shRNAi clones was performed using unboiled samples with one-tenth of the lysate volume loaded in Fig. 1. Bar graph quantitates relative *Glut-1* expression normalized for actin loading control (shown in Fig. 1).

the degree of *Glut-1* down-regulation in pSR1 and pSV1 shRNAi clones does not correlate linearly with the degree of HIF reduction. This possibly reflects clonal differences resulting from additional cellular factors influencing *Glut-1* promoter activity in addition to HIF2 α .

To further examine this hypothesis, we subjected the HIF knockdown or control clones to hypoxia along with WT8 and PRC3 cells. The effects of hypoxia were compared with the corresponding clones cultured under normoxia. We found that hypoxia had no effect on HIF2 α expression (Fig. 3B) or VEGF secretion (Fig. 3A) in any of the HIF knockdown clones, whereas both were increased in the pVHL-reconstituted WT8 cells. All differences in VEGF expression between pVHL-reconstituted or HIF knockdown clones and their respective control clones were statistically significant ($P < 0.01$), whereas differences in VEGF expression within a single clone following exposure to hypoxia was significant for the pVHL-reconstituted WT8 clone only ($P < 0.05$). We conclude that HIF is the predominant factor regulating hypoxia-inducible genes in VHL $-/-$ cells, at least under the experimental conditions used in this study.

Inhibition of HIF2 α Does Not Affect Growth *In Vitro*

Reintroduction of pVHL into 786-O cells has little effect on *in vitro* cell growth (8). Therefore, inhibition of HIF2 α in the absence of pVHL is likewise expected to have little effect on *in vitro* cell proliferation. However, because any effect on cell growth would complicate interpretation of tumor growth suppression in the mouse xenograft assay, we tested all HIF knockdown clones for changes in proliferation as measured by cleavage of the tetrazolium salt WST-1. We found that no difference in proliferation between HIF knockdown and control clones (Fig. 4).

Inhibition of HIF2 α Suppresses Angiogenesis *In Vitro*

The tumor suppression observed by reintroducing pVHL into VHL $-/-$ cells is attributed to disrupting tumor angiogenesis by blocking the release of proangiogenic factors. We therefore decided to assay the ability of conditioned tissue culture supernatant from PRC3, WT8, pSV1, pSR1, pTV1, and pTR1 cells to induce human umbilical vein endothelial cell (HUVEC) proliferation as an *in vitro* assay for changes in the angiogenic potential of these biological fluids. We found that inhibition of HIF activity decreased the ability of conditioned supernatant to induce HUVEC proliferation to a degree similar to the reintroduction of pVHL (Fig. 5). We therefore hypothesize that decreasing HIF activity in 786-O cells will decrease tumor vascularization *in vivo*.

Inhibition of HIF2 α Suppresses Tumorigenesis of VHL $-/-$ Cells

Lastly, we examined whether HIF2 α inhibition alters the tumorigenic profile of VHL $-/-$ cells. The HIF2 α shRNAi-expressing clones pSR1, pTR1, pTR2, and pTR3, along with the corresponding control clones pSV1, pTV1, and pTV2, were injected s.c. into nude mice. PRC3 and WT8 clones were also injected as positive and negative controls for the mouse xenograft assay. Mice were observed over a period of 6–8 weeks,

by which time tumors reached maximal size. Figure 6 presents the average mass of excised tumors formed by each clone. The tumors that formed in sites injected with the HIF knockdown clones were dramatically smaller than those from control injections. In many cases, in fact, no tumors formed at all. Thus, clones derived from two different vector systems expressing shRNAs targeting distinct HIF2 α sequences, but not vector-only control clones, led to tumor suppression. The degree of tumor suppression by the inhibition of HIF2 α was similar to the degree of tumor suppression achieved by reintroduction of pVHL (Fig. 6, A and B).

Discussion

The VHL^{-/-} human RCC cell line 786-O forms tumors in the nude mouse xenograft assay. We showed previously that reintroduction of pVHL into these cells markedly inhibited the ability of these cells to grow as tumors in nude mice (8, 9) without altering their growth characteristics *in vitro*. These observations led to the hypothesis that the cellular microenvi-

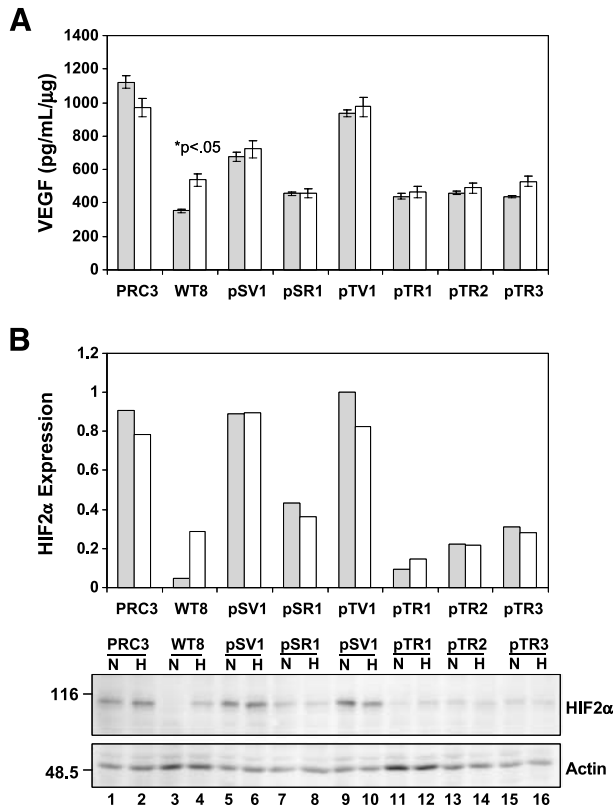


FIGURE 3. HIF2 α inhibition prevents response to hypoxia. **A.** ELISA for secreted VEGF concentration in tissue culture supernatants of HIF2 α shRNAi and pVHL-reconstituted clones *versus* respective vector-only control clones under normoxic (21% O₂, gray bars) and hypoxic (1% O₂, white bars) conditions. Bars, SEM. Differences in VEGF expression between pVHL-reconstituted (WT8) or HIF2 α knockdown clones (pSR1, pTR1, pTR2, and pTR3) and their respective control clones (pRC3, pSV1, and pTV1) were significant ($P < 0.01$), whereas differences in the hypoxic induction of VEGF were significant for the pVHL-reconstituted WT8 cells only ($P < 0.05$). **B.** Western blot analysis for HIF2 α expression in cells under normoxic and hypoxic conditions (denoted as N and H, respectively). Bar graph quantitates relative HIF2 α levels normalized for actin loading control. **A** and **B.** Gray bars, normoxia; white bars, hypoxia.

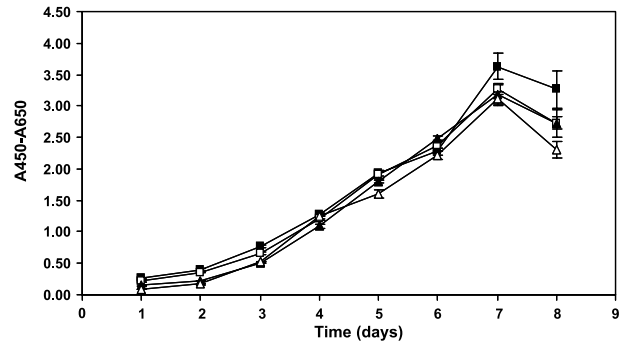


FIGURE 4. *In vitro* cell proliferation is not affected by HIF2 α -directed shRNAi. Cell proliferation measured via cleavage of the tetrazolium salt WST-1 at the indicated time points. Bars, SEM. Closed squares, PRC3; open squares, WT8; closed triangles, pTV1; open triangles, pTR1. Similar results were obtained for pSV1, pSR1, pTV2, pTR2, and pTR3 cells (data omitted for the sake of clarity).

ronment participates in pVHL-mediated tumor suppression possibly through regulation of hypoxia-inducible genes and angiogenesis (11). RCC cells lacking wild-type pVHL inappropriately overproduce hypoxia-inducible mRNA under both hypoxic (1% ambient O₂) and normoxic (21% ambient O₂) conditions (8, 9). Reintroduction of pVHL into these cells restores the normal profile of hypoxia-inducible mRNA regulation and leads to tumor suppression.

The overexpression of hypoxia-inducible genes in VHL^{-/-} cells has been attributed to transcriptional and posttranscriptional events (8, 9), implying that the effects of pVHL could be attributed to targeting multiple substrates for degradation (6, 7). HIF transactivates a family of angiogenic and growth factors such as VEGF, platelet-derived growth factor- β , and transforming growth factor- α . It is therefore conceivable that not only constitutive HIF up-regulation explains the hypervascular nature of VHL-associated lesions but also HIF itself may act as an oncogene that directly contributes to malignant transformation in this context. However, to the best of our knowledge, there is no case in which a direct mutation in the HIF locus serves as an initiating event for tumorigenesis. Here, we show that HIF is the

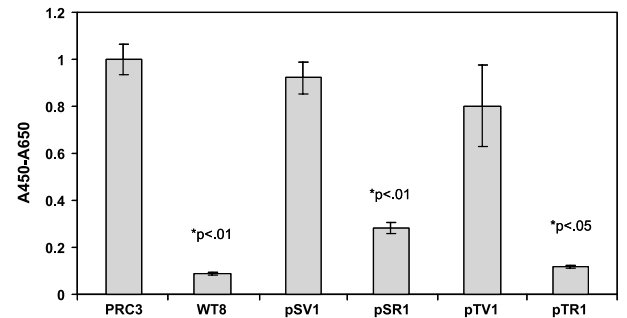


FIGURE 5. HIF2 α -directed shRNAi reduces the overall proangiogenic activity of conditioned supernatant. HUVEC proliferation as measured by WST-1 cleavage in the presence of conditioned supernatant from PRC3, WT8, pSV1, pSR1, pTV1, and pTR1 clones. Differences between shRNAi and pVHL-reconstituted clones *versus* vector-only control clones were significant. Bars, SEM.

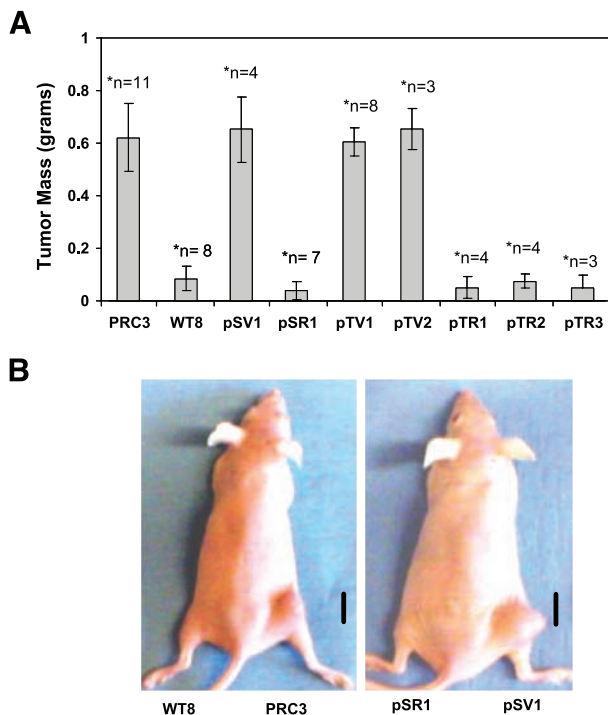


FIGURE 6. HIF2 α -directed shRNAi suppresses tumor formation. **A.** Average tumor mass resulting from cells injected into the flank of nude mice. *n*, number of injection sites for each clone. All differences in tumor mass relative to the corresponding vector-only control cell injections were statistically significant ($P < 0.01$). Bars, SEM. **B.** Representative mice injected with cells in which pVHL function was reconstituted (WT8) versus HIF activity was inhibited (pSR1) along with corresponding control injections.

principal mediator of hypoxia-inducible gene deregulation in the VHL $^{-/-}$ renal cell line 786-O and, most notably, that HIF inhibition is sufficient to suppress the growth of these cells as tumors when injected into the flank of nude mice.

We should emphasize that our data address the role of HIF2 α in a RCC cell line that expresses only the HIF2 α isoform. As mentioned in the introduction of this article, the role of HIF1 α and HIF2 α isoforms may be cell type specific. The HIF1 α and HIF2 α isoforms display different but overlapping tissue distribution patterns. It is currently not known to what extent the functions of HIF1 α and HIF2 α overlap if expressed in the same cellular context. Overexpression of a transcriptionally active HIF2 α mutant, which escapes regulation by pVHL, overrides the tumor suppression effect of pVHL (28). Interestingly, the equivalent HIF1 α mutant isoform failed to do so in the same cell line (21). HIF2 α functions were preferentially linked to protecting cells from hypoglycemia, whereas HIF1 α functions were shown to confer protection from hypoxia and hypoglycemia (30, 31). In immortalized mouse fibroblasts, it was shown that the HIF1 α , but not HIF2 α , isoform was subject to hypoxia-dependent regulation (32). Other tumor-derived VHL $^{-/-}$ human RCC cell lines constitutively overexpress HIF2 α either alone or in combination with HIF1 α (5). By using cell lines that express both isoforms, we are currently examining the relative contribution of each isoform to VHL $^{-/-}$ tumor development.

In the experiments described above, HIF activity was down-regulated prior to the “angiogenic switch” required for tumor growth (33). Tumor angiogenesis is initiated by stimulation and formation of immature, leaky vessels—a phenomenon known as “pruning.” The development of mature vessels occurs in a subsequent multistep process. Our experiments support the notion that HIF inhibition is sufficient for prevention of VHL $^{-/-}$ tumors. Whether HIF inhibition is also sufficient for the regression of established tumors is a subject of current investigation.

Immunohistochemical studies suggest that HIF is constitutively overexpressed not only in VHL $^{-/-}$ tumors but also in VHL $^{+/+}$ tumors (15). Tumor-associated mutations, such as PTEN inactivation and HER-2/*neu* overexpression, stimulate the phosphoinositide 3-kinase pathway and promote HIF translation (34–36). Mutant p53 has also been implicated in HIF stability: wild-type, but not mutant, p53/mdm2 complexes directly bind to and promote HIF ubiquitination and degradation (37). In addition, the hypoxic conditions associated with solid VHL $^{+/+}$ tumors are likely to inactivate cellular HPHs and lead to HIF stability and activation independently from other tumor-associated mutations. Determining if HIF inhibition also leads to tumor prevention and/or regression in VHL $^{+/+}$ tumors is currently under investigation.

Complete inhibition of HIF activity was not necessary for effective tumor suppression. It is important to note that the HIF knockdown clones used in this study did not completely abolish HIF expression. Nevertheless, we observed a similar degree of tumor suppression for all HIF knockdown clones *in vivo* as well as a similar effect of HUVEC proliferation *in vitro*. We attribute this to HIF being a nodal point in the control of several proangiogenic and growth factors and hypothesize that the coordinated decrease in expression of all these factors in combination generates a synergistic effect that is sufficient for tumor suppression.

The experiments reported herein were performed in a prototypic VHL $^{-/-}$ cell line, 786-O, a favorite model cell system for investigating the role of pVHL function; nevertheless, they represent data from a single cell line. It will be necessary to examine whether HIF inhibition is sufficient to suppress growth of other VHL $^{-/-}$ tumor cell lines and to modulate HIF activity *in vivo* using appropriate animal tumor models linked to HIF deregulation. In addition, the discovery and application of highly specific HIF inhibitors *in vivo* will complement the evaluation of HIF as molecular target for drug development (38).

In summary, although HIF is not the only pVHL substrate, the experiments presented in this report provide evidence that HIF is the main mediator of the effect of pVHL on hypoxia-inducible genes. In addition, inhibition of HIF appears sufficient for suppression of VHL $^{-/-}$ tumors. This supports the hypothesis that HIF is a bona fide target for antiangiogenic and anticancer drug development and, as such, justifies its further molecular and pharmacologic evaluation.

Materials and Methods

Plasmids

Plasmid pRC/CMV-HA VHL was described previously (11). Plasmid pSR was purchased from OligoEngine, Inc. (Seattle, WA).

The pSR HIF2 α shRNAi was generated by ligating the following annealed oligonucleotides into *Bgl*II/*Hind*III-digested pSR vector: 5'-GATCCCCGACAAGGTCTGCAAAGGGTTT-CAAGAGAACCCTTTCAGACCTTGTCTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGACAAGGTCTGCAAAA GGGTTCTCTTGAAACCCTTTCAGACCTTGTCTGGG-3'. Positive inserts were screened for the presence of a 360-bp *Eco*RI/*Hind*III fragment and confirmed by luciferase activity in transient cotransfection experiments using U2OS cells along with a stabilized HIF2 α (P531A) mutant and a HRE-driven luciferase reporter construct (data not shown). Plasmid pTU6IIa (pTU) was a gift from Dr. James DeCaprio. pTU HIF2 α shRNAi was generated by ligating the following annealed oligonucleotides into *Age*I/*Xho*I-digested pTU vector: 5'-CCGGGGGCTGTGTCTGAGAAGAGTCTCTTCACTTCTCAGACACAGCCCCCTTTTTC-3' and 5'-TCGAGAAAAAAGGGGGCTGTGTCTGAGAAGAGTGAAGA-GACTCTTCTCAGACACAGCCC-3'. Positive inserts were screened by the introduction of the nonpalindromic *Ear*I site located within the hairpin loop of the shRNAi and confirmed using the same reporter assay described above (data not shown).

Four copies of the sequence defining the HRE (5'-CCA-CAGTGCATACGTGGGCTCCAACAGGTCCTCT-TCCCTCCCATGCA-3') were cloned in tandem into pGL-basic vector (Promega, Madison, WI) between the *Kpn*I and the *Xho*I restriction sites upstream of the luciferase reporter gene. This promoter is transactivated specifically by HIF2 α , hypoxia, and chemical mimetics of hypoxia (cobalt chloride and desferrioxamine) and is down-regulated by dominant-negative HIF isoforms (data not shown).

Cell Culture

All cell lines used in this study are summarized in Table 1. The human RCC cell line 786-O lacks wild-type pVHL (4, 11) and constitutively expresses the HIF2 α isoform only (5). Stable expression of 786-O cells with hemagglutinin-tagged pVHL or empty vector control generated clones WT8 and PRC3,

respectively (11). Cells were grown in DMEM (Media Tech, Herndon, VA) supplemented with 10% heat-inactivated Fetal Clone I (Hyclone Laboratories, Logan, UT) and 2 mM L-glutamine (Invitrogen, Life Technologies, Inc., Carlsbad, CA) under appropriate drug selection. WT8 and PRC3 cells were grown in geneticin (G418, 1 mg/ml; Life Technologies), stable transfectants with pSR plasmids in puromycin (1.25 μ g/ml), and stable transfectants with pTU plasmids in 10 μ g/ml blasticidin. All experiments regarding HIF2 α and Glut-1 expression, as well as secretion of VEGF in the tissue culture supernatant, were performed at 70–80% confluency. Cells were cultured under either normoxic conditions (5% CO₂, 21% O₂, 74% N₂) in a humidified incubator at 37°C or hypoxic conditions (5% CO₂, 1% O₂, 94% N₂) in an ESPEC triple gas incubator (Tabai-Espec Corp., Osaka, Japan).

Transfections

The HRE-directed firefly luciferase plasmid was cotransfected with plasmids encoding Renilla luciferase under the control of a constitutive SV40 promoter using FuGENE6. Firefly luciferase activity was normalized for Renilla counts.

Western Blot Analysis

Plates were washed with PBS, scraped, and centrifuged to pellet the cells. The cell pellet was lysed for 10 min, at 4°C, with a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP40, 20 μ g/ml trypsin inhibitor, 10 μ g/ml leupeptin, 0.2 mM Na₃VO₄, 5 μ g/ml pepstatin A, 10 μ g/ml aprotinin, 100 mM NaF, and 100 μ g/ml phenylmethylsulfonyl fluoride. The protein content of the clarified lysates was determined by Bradford assay (Bio-Rad, Hercules, CA). For HIF2 α expression analysis, 100 μ g of protein were loaded per lane, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride (Bio-Rad) membrane. The blot was cut in half and the upper portion was probed with monoclonal anti-HIF2 α antibody (Novus, Inc., Littleton, CO) while the lower was probed for actin as a loading control. For Glut-1 analysis, 10 μ g of unboiled total protein from the same sample set were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with polyclonal anti-Glut1 (Alpha Diagnostics, San Antonio, TX) antibody.

VEGF ELISA

VEGF concentration in the tissue culture supernatant was measured by VEGF ELISA kit according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN) and normalized for total protein using the Bradford assay. Error bars represent 1 SEM of measurements from three independent samples.

In Vitro Cell Proliferation Assay

Equal cell numbers were plated in 96-well plates and their proliferation was quantified by a colorimetric assay based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases according to the manufacturer's instructions (Roche, Nutley, NJ). Error bars represent 1 SEM from eight independent measurements.

Table 1. Description of Cell Lines Used in This Study

Clone	Description	Reference
786-O	Established VHL-/- RCC cell line	(39)
PRC3	786-O-derived clone stably transfected with empty pcDNA3 vector	(11)
WT8	786-O-derived clone stably transfected with pcDNA3::HA-VHL30	(11)
pSV1	786-O-derived clone stably transfected with empty pSR vector	This work
pSR1	786-O-derived clone stably transfected with pSR targeting HIF2 α	This work
pTV1	786-O-derived clone stably transfected with empty pTU6IIa vector	This work
pTV2	786-O-derived clone stably transfected with empty pTU6IIa vector	This work
pTR1	786-O-derived clone stably transfected with pTU6IIa targeting HIF2 α	This work
pTR2	786-O-derived clone stably transfected with pTU6IIa targeting HIF2 α	This work
pTR3	786-O-derived clone stably transfected with pTU6IIa targeting HIF2 α	This work

HUVEC Proliferation Assay

Equal HUVEC numbers were plated in a 96-well plate. Twenty-four hours later, medium was replaced with tissue culture supernatant that had been conditioned by the HIF2 α knockdown or control cell lines. HUVEC proliferation was measured by WST-1 cleavage as described above at the end of 48-h growth in conditioned medium. Error bars represent 1 SEM from three independent experiments.

Nude Mouse Xenograft Assay

The assay has been described previously (11). Briefly, 1×10^7 cells were injected s.c. through a 26-G syringe into the flank of National Cancer Institute *nu/nu* mice and the animals were scored for tumor development and tumor size at each site of injection. Matched HIF2 α shRNAi (left flank) and control clones (right flank) were injected into the same animal. Mice were sacrificed and the tumors were weighed when the largest tumor reached 1.5 cm in maximal diameter or when the animals were distressed.

Statistical Significance

Where indicated, statistical significance was determined using the Student's *t* test (two-tailed distribution of paired samples).

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