Regulation of E-cadherin Expression by VHL and Hypoxia-Inducible Factor

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Abstract

Mutations in von Hippel-Lindau tumor suppressor gene (VHL) underlie the VHL hereditary cancer syndrome and also occur in most sporadic clear cell renal cell cancers (CCRCC). Currently, the mechanism(s) by which VHL loss of function promotes tumor development in the kidney are not fully elucidated. Here, we show that VHL inactivation in precancorous lesions in kidneys from patients with VHL disease correlates with marked down-regulation of the intercellular adhesion molecule E-cadherin. Moreover, in VHL-defective cell lines (RCC4 and RCC10) derived from sporadic CCRCC, reexpression of VHL was found to restore E-cadherin expression. The product of the VHL gene has multiple reported functions, the best characterized of which is its role as the recognition component of an ubiquitin E3 ligase complex responsible for mediating oxygen-dependent destruction of hypoxia-inducible factor-α (HIF-α) subunits. We show that HIF activation is necessary and sufficient to suppress E-cadherin in renal cancer cells. Given the fundamental role of E-cadherin in controlling epithelial behavior, our findings give insight into how VHL inactivation/HIF activation may lead to kidney cancer and also indicate a mechanism by which reduced oxygenation could alter E-cadherin expression in other cancers and influence normal homeostasis in other epithelia. (Cancer Res 2006; 66(7): 3567-75)

Introduction

von Hippel-Lindau (VHL) disease is an autosomal dominant multitymored syndrome, which includes an ~70% lifetime risk of clear cell renal cell carcinoma (CCRCC), multiple visceral cysts (affecting the kidney and also other organs), hemangioblastomas of the central nervous system and retina, and pheochromocytomas (1). In accordance with Knudson’s two-hit model for tumor suppressor action, disease manifestations involve somatic mutation or inactivation of the remaining normal allele. The great majority of sporadic CCRCC, which is the commonest form of kidney cancer, also involve biallelic inactivation of VHL. The VHL gene is situated at 3p25 and encodes two protein products of apparent molecular weight of 19 and 30 kDa (pVHL19 and pVHL30, referred to collectively as pVHL), both of which suppress tumor growth of VHL-defective CCRCC lines in xenograft experiments (1).

The best characterized function of pVHL is its role in regulating cellular responses to oxygen, through its role in the oxygen-dependent inactivation of the transcription factor hypoxia-inducible factor (HIF; ref. 2). In normal cells, HIF plays a central role in coordinating changes in gene expression in response to alterations in oxygen supply (3). In most normal tissues in vivo and in vitro under standard cell culture conditions, activation of the HIF pathway is usually minimal. However, when oxygen tension is reduced or when VHL is inactivated, HIF becomes stabilized and activated, inducing the transcription of a wide range of target genes. In general, HIF activation promotes adaptation of the cell, organ, or organism to hypoxia, by modulating a range of processes that include increases in glucose transport, glycolysis, and angiogenesis (1–3). Oxygen-responsiveness of the HIF transcriptional complex is mediated through the HIF-α subunits (the best characterized of which are HIF-1α and HIF-2α), which are continuously destroyed in the presence of oxygen (4, 5). This is achieved by oxygen-dependent enzymatic hydroxylation of specific prolyl residues in the central part of the HIF-α molecule, which then allows ubiquitination by a cognate E3 ligase complex in which pVHL acts as the recognition molecule, which then allows ubiquitination by a cognate E3 ligase complex in which pVHL acts as the recognition component (1–4). The enzymes responsible for HIF prolyl hydroxylation are a family of three dioxygenases termed PHD1, PHD2, and PHD3 (for prolyl hydroxylase domain-containing proteins; also known as EGLN1-3 and HPH1-3; ref. 4).

As a consequence of pVHL inactivation, the HIF pathway is constitutively active in both hereditary and sporadic VHL-defective CCRCC. Expression of pVHL in otherwise defective CCRCC-derived cell lines restores normal degradation of HIF-α subunits and also suppresses tumor growth in xenograft models (6). Importantly, overexpression of a modified HIF-2α molecule, which cannot be recognized by pVHL, is sufficient to override the tumor suppressor effects of VHL (6). Furthermore, knock down of HIF-2α in pVHL-negative CCRCC cell lines abrogates their tumorigenic potential (6). These observations show that suppression of HIF-2α is necessary and sufficient for pVHL’s action as a tumor suppressor in fully transformed CCRCC cells. Current challenges are to understand (a) the key downstream targets of HIF involved in tumorigenesis, (b) the extent to which these HIF-mediated changes in fully transformed cancer cells reflect tumor initiation and progression in the normal renal epithelium, and (c) the role of a number of other reported functions of pVHL that are not related to HIF (1, 7–12).

Intercellular adhesion in the context of epithelia is mediated by specialized structures termed intercellular junctions, which include desmosomes and hemidesmosomes, “gap” or communicative junctions, and the adherens junction and tight junction (13–15). The adherens junction is mainly composed of transmembrane calcium-dependent glycoproteins called cadherins (15). E-cadherin is the classic epithelial cadherin, and by dimerizing with other
E-cadherin molecules on adjacent cells, it seals the basolateral intercellular space, helping to maintain tissue integrity and architecture (15, 16). Besides this structural role, E-cadherin also exerts important effects on cell signaling, through the interaction of its cytoplasmic domain with the catenin family of proteins (15–17). Although α-catenin connects E-cadherin to the actin cytoskeleton, γ-catenin and particularly β-catenin have the ability to interact with the T-cell factor family of transcription factors, whose transcriptional activity they increase (17). By controlling the amount of β-catenin available in the cytoplasm, E-cadherin can regulate the expression of critical genes involved in cell death/proliferation (e.g., cyclin D1 and c-myc). Taken together, it is thus not surprising that loss of E-cadherin expression is a hallmark feature of cancers of epithelial origin (13, 18, 19).

In this report, we show, by analysing very early microscopic lesions of pVHL inactivation in the kidneys of patients with VHL disease, that pVHL inactivation strikingly reduces E-cadherin expression in human kidney tubular cells in vivo. In vitro studies using pVHL-defective cell lines obtained from sporadic CRCC tumors established that reexpression of VHL restores E-cadherin expression in fully transformed CRCC cells. To define the underlying mechanism, we used several approaches that show that the effect of pVHL loss on E-cadherin expression is mediated via activation of HIF.

Materials and Methods

Cell culture, chemicals, and antibodies. RCC4, RCC10, and 786-O cell lines and the corresponding stable transfectants with wild-type pVHL have been described previously (2, 20). RCC10 cells were generously provided by K. Plate and T. Acker (Institute of Neurology/Edinger Institute, Frankfurt University Medical School, Frankfurt, Germany). Cells were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) with penicillin/streptomycin, glutamine, and 10% fetal bovine serum.

Desferrioxamine and cobalt chloride (CoCl2) were purchased from Sigma (St. Louis, MO); dimethyl-oxalylglycine was purchased from Frontier Scientific (Logan, UT). For hypoxic experiments, cells were exposed to 1% (St. Louis, MO); dimethyl-oxalylglycine was purchased from Frontier

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Clinical material and immunohistochemistry. Surgical samples, including six nephrectomy specimens from five subjects with VHL disease, were analyzed (Table 1). Specimens were formalin fixed and embedded in paraffin wax; 3-μm serial sections mounted on Snowcoat X-tra slides (Surupath, Richmond, IL) were dewaxed in xylene and rehydrated using graded ethanol washes. For antigen retrieval, sections were immersed in preheated Dako target retrieval solution (Dako) and treated for 90 seconds in a pressure cooker (E-cadherin, AQP-2, and THP) or incubated for 5 minutes at room temperature with Proteinase K (Dako; GP-200). Sections analyzed contained both tumor and adjacent normal renal parenchyma acting as an internal control; in addition, substitution of the primary antibody with antibody diluent was used as a negative control. Antigen/antibody complexes were detected using the Envision system (Dako) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin for 30 seconds, dehydrated in graded ethanol washes, and mounted in DPX (Lamb, London, United Kingdom).

Immunoblotting. Cell lysis was done with urea-SDS buffer as described (2), supplemented with phenylmethylsulfonyl fluoride. Immunoblots were visualized with enhanced chemiluminescence (ECL) or ECL plus (Amer sham, Arlington Heights, IL).

Immunofluorescence microscopy. Cells were grown to confluence on coverslips in 24-well dishes, fixed for 10 minutes at −20°C with methanol, and washed with TBS. Alexa 488 conjugates (Molecular Probes, Eugene, OR) were used for visualization; coverslips were mounted using 4’,6-diamidino-2-phenylindole containing Vectashield (Vector Laboratories, Burlingame, CA). Samples were analyzed using an immunofluorescence microscope (Olympus BX41) equipped with IMAGE PRO software.

Real-time reverse transcription-PCR. Cells were grown to confluence, and total cellular RNA was isolated using RNA Bee (Biogenesis). Total RNA (2 μg/20 μL reaction) was retrotranscribed using an avian myeloblastosis virus retrotranscription kit (Roche, Indianapolis, IN). PCR was carried out using an Opticon 2 machine (MJ Research, Waltham, MA). Analysis of each experimental sample was in duplicate or triplicate. All real-time reverse transcription-PCR (RT-PCR) data are given as a value normalized to the level of β-actin expression in the same retrotranscription. β-Actin expression was not significantly altered by hypoxia, dimethyl-oxalylglycine, desferrioxamine, or CoCl2, nor did it differ between VHL-negative RCC and VHL cell lines.

Snail mRNA was measured using an “On Demand” Taqman assay from Applied Biosystems (Foster City, CA).

β-Actin, E-cadherin, PHD3, and vascular endothelial growth factor (VEGF) mRNA were measured using SYBR Green (AB gene) and the following primers: β-actin, 5′-CCCAGAGCAAGAGGG-3′ (forward) and 5′-GTCCA-GACGCCAGAGTG-3′ (reverse); E-cadherin, 5′-GACACAAGCCCGGAGATT-3′ (forward) and 5′-GGAACCTCTCTGCTGCTCA-3′ (reverse); PHD3, 5′-GATGCCT-GAGAAGAGGGC-3′ (forward) and 5′-CTGGCAAGAGGATTCGATGTG-3′ (reverse); VEGF, 5′-TGCCCAATGGTGCCAG-3′ (forward) and 5′-GTAAGGTGGTACGCGG-3′ (reverse).

RNase protection assay. 32P-labeled riboprobes were generated for E-cadherin (nucleotides 3411–3551; accession no. NM_004360) and U6 small nuclear (U6sn) RNA (nucleotides 1–107; accession no. X01366). Radiolabeled riboprobes were protected from RNase digestion by parallel hybridization to 25 μg of total RNA for E-cadherin and 125 ng total RNA for U6sn at 60°C.

Table 1. Details of the material analyzed from patients with VHL disease

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Sex</th>
<th>Mutation</th>
<th>Codon</th>
<th>No. lesions</th>
<th>Unicellular lesions</th>
<th>Multicellular lesions</th>
<th>Total area (cm²)</th>
<th>No. paraffin blocks</th>
<th>Selected block area (cm²)</th>
<th>No. lesions analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>36</td>
<td>48del.</td>
<td>Arg¹⁵⁵Leu</td>
<td>176</td>
<td>90</td>
<td>86</td>
<td>6.7</td>
<td>3</td>
<td>1.3</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>F</td>
<td>36</td>
<td>47del.</td>
<td>Ser²⁹Pro</td>
<td>44</td>
<td>30</td>
<td>14</td>
<td>5.3</td>
<td>4</td>
<td>0.2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>M</td>
<td>36</td>
<td>47del.</td>
<td>Ser²⁹Pro</td>
<td>38</td>
<td>17</td>
<td>21</td>
<td>5.6</td>
<td>6</td>
<td>0.8</td>
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<td>4</td>
</tr>
<tr>
<td>F</td>
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<td>40del.</td>
<td>Phe³⁶Cys</td>
<td>146</td>
<td>127</td>
<td>19</td>
<td>2.4</td>
<td>1.5</td>
<td>1.2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
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<td>64del.</td>
<td>Glu²⁵Lys</td>
<td>39</td>
<td>25</td>
<td>14</td>
<td>1.5</td>
<td>3</td>
<td>0.6</td>
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The sw recombinated after RNA digestion and analyzed on 8% polyacrylamide gels.

Small interfering RNA transfections. The concentration of small interfering RNA (siRNA) oligos was 100 nmol/L; for the combined HIF-1α and HIF-2α transfections, 50 nmol/L was used for each. Transfections were done in p60 culture dishes using LipofectAMINE 2000 (Invitrogen, San Diego, CA). Cells were transfected as a pool, and after 15 to 20 hours, were divided onto 24- or 6-well dishes or plated on coverslips. Cells were analyzed 3 to 5 days after transfection.

siRNA oligo sequences were HIF-1α (21), 5′-CUGAUGACCAGCAACU-3′ (sense) and 5′-UCAAGUUGCUGGUCAUCAGdTdT-3′ (antisense); HIF-2α (21), 5′-CAGCAUCUUUGAUAGCAGUdTdT-3′ (sense) and 5′-ACUGCUCUAAAGAUGCUGdTdT-3′ (antisense); Firefly luciferase (http://www.rockefeller.edu/labheads/tuschl/), 5′-CGUACGCGGAAUACUUCGAdTdT-3′ (sense) and 5′-AAGCUAAAGGUACACAAUdTdT-3′ (antisense).

Infection of HIF retroviral vectors. Viral supernatants were prepared by transfecting the Phoenix packaging cell line (Orbigen, San Diego, CA) using LipofectAMINE 2000. After the initial transfection, Phoenix cells were grown at 32°C. The supernatant was collected and filtered (0.45 μm), then supplemented with 0.25 volume fresh medium with 7.5 μg/mL Polybrene (Sigma), and added to target cells which had been plated the day before on p100 dishes at 30% to 40% confluence. After 20 hours, cells were washed, and fresh media were added for 20 hours before performing a second round of infection.

Constitutively active forms of HIF-1α (carrying the following substitutions: P402A and P564A) or HIF-2α (substitutions: P405A and P531A) were cloned into pBMN-I-EGFP (22). Infection efficiency (as checked by flow cytometry) was 90% in each experiment, with comparable efficiency for empty vector and those encoding HIF.

pVHL retroviral vectors. pCMVR-Neo empty or expressing pVHL30, were a kind gift from Wilhelm Krek (Institute of Cell Biology, Zurich, Switzerland) and Alexander Hergovich (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland; ref. 12). pCMVR-Neo containing pVHL19, pVHLV84L or pVHL L188V, were obtained by transferring inserts from pcDNA3 plasmids described previously (12, 23). Infections were done as above; cells were selected with G418.

Figure 1. Reduced E-cadherin expression in early foci of pVHL loss of function. A, immunohistochemistry of unaffected renal parenchyma from a case of sporadic CCRCC tumor. Top, serial sections stained for E-cadherin and the proximal tubule marker glycoprotein 200 (GP200). The glomerulus is indicated (G). Bottom, tubules displaying Tamm-Horsfall protein (THP) or Aquaporin 2 (AQP2), also express E-cadherin; THP and AQP2 are, respectively, expressed in the earliest and latest part of the distal tubule. B, serial sections of kidneys from patients with VHL disease immunostained for CAIX (left) and E-cadherin (right). Asterisks, normal distal tubules, in which the epithelium is strongly positive for E-cadherin. Arrows, region of pVHL loss of function, in which CAIX is expressed, and E-cadherin expression is decreased. C, immunohistochemistry of representative CCRCC labelled for CAIX (left) and E-cadherin (right). The dotted line delimits the interface between the normal renal parenchyma and the tumor.
Results and Discussion

Recently, we reported that in kidneys from VHL patients, it is possible to detect large numbers of premalignant foci showing loss of function of pVHL, besides the much smaller numbers of cysts and CCRCCs (24). These early lesions can be identified by labeling for the HIF target CAIX. Interestingly, these foci of loss of pVHL function mainly occur in a restricted portion of the kidney tubule, the distal tubule. Because E-cadherin has been reported to be expressed selectively in the distal part of the human kidney tubule (25), and given its importance in the control of epithelial properties and in limiting the progression of many tumors, we considered that early pVHL inactivation in the distal tubule might diminish E-cadherin expression as a candidate mechanism in the evolution of CCRCC.

We first verified the reported distribution of E-cadherin in normal renal parenchyma. As expected, E-cadherin was observed exclusively in the distal part of the renal tubule, including the cortical thick ascending limb, the distal convoluted tubule, and the collecting duct (Fig. 1A). Next, we examined E-cadherin and CAIX expression in serial sections of kidney material from patients with VHL disease (for clinical details and the number and characteristics of the early foci, see Table 1). Strikingly, the portions of the distal tubules that expressed CAIX showed a substantial decrease in E-cadherin (Fig. 1B, four lesions of different complexity are shown). Moreover, the CCRCC tumors found in these kidneys from VHL patients showed very little or no labeling for E-cadherin (Fig. 1C), which was also the case in samples obtained from sporadic CCRCC (Fig. 1C, the dotted line marks the limit between non affected renal parenchyma and the tumor). These experiments therefore show that in renal tubular epithelium loss of function of pVHL is associated with a marked decrease in the expression of E-cadherin.

We next examined E-cadherin expression in two pVHL-defective cell lines derived from sporadic CCRCC (RCC4 and RCC10) and corresponding sublines in which pVHL was stably reexpressed (referred to here as RCC4/VHL and RCC10/VHL). Notably, whereas pVHL-defective RCC4 and RCC10 cell lines expressed little or no

Figure 2. pVHL expression restores E-cadherin in CCRCC cell lines. A, immunoblots of RCC10 and RCC4 cells that lack pVHL function show high levels of HIF-1α, HIF-2α, and the HIF target GLUT1. These are suppressed in the pVHL-expressing sublines RCC10/VHL and RCC4/VHL. E-cadherin shows the opposite behavior. Lysates from the esophageal squamous cancer cell line H357 (Control) served as a positive control for E-cadherin expression. pVHL expression in RCC4/VHL and RCC10/VHL was tested by Western blotting; HeLa cells (a cervical carcinoma cell line) were used as a cell line expressing native pVHL (Control); RCC4/VHL contains similar levels of pVHL to HeLa cells. Very similar results were obtained when embryonic kidney 293 cells were used (data not shown). RCC10/VHL expressed the p30 isoform at a higher level. B, immunofluorescence microscopy for E-cadherin. Phase-contrast pictures were taken before fixing the cells (top) and show a more fibroblastoid phenotype in the absence of pVHL. C, real-time RT-PCR analysis of E-cadherin, PHD3, and VEGF mRNA in RCC10 and RCC10/VHL. Data for E-cadherin expression in pVHL-negative cells is expressed as a percentage of the value in pVHL-transfected cells (assigned as 100%), for three independent experiments. D, Rnase protection analysis of E-cadherin mRNA. U6snRNA was used as loading control.
E-cadherin, RCC4/VHL and RCC10/VHL showed substantial levels of E-cadherin protein on both immunoblotting (Fig. 2A) and immunofluorescence (Fig. 2B). To further investigate the link between pVHL status and E-cadherin, we then analyzed E-cadherin mRNA by quantitative real-time RT-PCR in RCC10 and RCC10/VHL. The level of E-cadherin mRNA was higher in RCC10/VHL (Fig. 2C); conversely, mRNA levels of VEGF and PHD3 (two well-characterized targets of HIF) were much higher in pVHL-negative cells. Similar results were obtained using a specific probe for human E-cadherin in an RNase protection assay (Fig. 2D). Thus, expressing pVHL in otherwise defective CCRCC cells can restore E-cadherin expression, and this operates at least in part through increasing the level of E-cadherin mRNA.

The most extensively characterized biochemical function of pVHL is its role in destruction of the α-subunits of HIF in the presence of oxygen (2). To examine whether the observed suppression of E-cadherin was mediated by HIF, we used several approaches. First, we studied the effects of expressing selected missense mutations of pVHL (pVHL V84L and pVHL L188V). These mutations are associated with isolated familial pheochromocytoma, referred to as type 2C VHL disease. We and others have shown that these mutant pVHL molecules regulate HIF normally (23, 26, 27). Introduction of these molecules provides a method for restoring HIF regulation without correcting all consequences of pVHL loss of function. RCC4 and RCC10 cells were infected with retrovirus coding for pVHL19, pVHL30, pVHL V84L and pVHL L188V or empty vector. pVHL19, pVHL30, pVHL V84L and pVHL L188V down-regulated both HIF-1 and HIF-2 protein levels and increased E-cadherin expression in both RCC4 and RCC10 cells (Fig. 3A). Restoration of E-cadherin expression was also verified by immunofluorescence microscopy and real-time RT-PCR analysis in RCC10-infected cells (Fig. 3B and C). The level of mRNA of PHD3 and VEGF was analyzed in parallel and showed the expected pattern for genes whose expression is increased by HIF activation.

The above results would be consistent with HIF mediating the suppression of E-cadherin in VHL-negative CCRCC cells. To further
explore this, we tested whether activating HIF in RCC10/VHL would down-regulate E-cadherin mRNA. First, we used 1% oxygen; as expected, this activated the HIF pathway potently, as evidenced by the induction of expression (measured by real-time RT-PCR) of VEGF (data not shown) and PHD3 mRNA (Fig. 4A). In parallel, hypoxia significantly reduced E-cadherin mRNA as measured by real-time RT-PCR (Fig. 4A). Reduction in E-cadherin mRNA by hypoxia was also observed in both RCC10/VHL and RCC4/VHL cells by RNase protection assay (Fig. 4B). Next, we used three different chemical agents that activate the HIF pathway by decreasing HIF hydroxylation (4): (a) the 2-oxoglutarate analogue dimethyl-oxalylglycine, (b) CoCl2, and (c) the iron chelator desferrioxamine. These agents reduced expression of E-cadherin mRNA and E-cadherin protein by immunoblotting (Fig. 4A and C).

To directly test the role of HIF in regulating E-cadherin expression, we infected RCC10/VHL cells with retroviral vectors encoding constitutively active forms of either HIF-1α or HIF-2α. These engineered HIF molecules contained mutations of the two prolyl residues, which are targets for oxygen-dependent hydroxylation (4); (a) the 2-oxoglutarate analogue dimethyl-oxalylglycine, (b) CoCl2, and (c) the iron chelator desferrioxamine. These agents reduced expression of E-cadherin mRNA and E-cadherin protein by immunoblotting (Fig. 4A and C).

Taken together, these results show that activation of HIF diminishes E-cadherin expression in the presence of pVHL. We next examined whether inhibition of HIF with siRNA would restore E-cadherin expression in pVHL-negative cells. Transient transfections of pVHL-negative RCC10 cells were done with siRNA duplexes targeting HIF-1α or HIF-2α; siRNA for firefly luciferase was used as a control. The efficacy and subunit selectivity of these duplexes was verified by Western blotting (Fig. 6A). As we described in another recent study (22), inhibition of HIF-2α was associated with a consistent increment in HIF-1α. Combined inhibition of both HIF-1 and HIF-2 was less efficient in down-regulating HIF-1 than HIF-2 (Fig. 6A). Inhibition of HIF-1 alone did not result in E-cadherin expression. Inhibition of HIF-2 resulted in detectable but rather variable expression of E-cadherin. Combined inhibition of HIF-1α and HIF-2α siRNA robustly increased the expression of E-cadherin as assessed by Western blotting (Fig. 6A), immunofluorescence (Fig. 6B), and real-time RT-PCR (Fig. 6C). Together, with the effects of expressing active HIF-α molecules, these results would suggest that both HIF-1α and HIF-2α contribute to suppression of E-cadherin in pVHL-negative CCRCC.

An important issue in understanding how the HIF pathway functions in normal physiology, and in cancer development, is the extent to which HIF-1 and HIF-2 have overlapping functions and regulate each other (22). HIF-1 in renal carcinoma cell lines increases expression of the proapoptotic protein BNIP3, whereas HIF-2 increases cyclin D1 expression, and both HIF-1 and HIF-2 contribute to the high level of Glut1 expression. In accordance with this, overexpression of a constitutively active form of HIF-2 but not

![Figure 4](https://example.com/f4.png)

**Figure 4.** Hypoxia, dimethyl-oxalylglycine (MMOG), desferrioxamine (DFO), and CoCl2 all reduce E-cadherin mRNA in CCRCC cells reexpressing pVHL. A, real-time RT-PCR analysis of E-cadherin and PHD3 mRNA in RCC10/VHL cells exposed to hypoxia (H; 1% oxygen, 60 hours), desferrioxamine (75 μmol/L, 48 hours), dimethyl-oxalylglycine (0.5 mmol/L, 48 hours), or CoCl2 (75 μmol/L, 48 hours). Between two and four independent experiments were analyzed depending on the stimulus. B, RNase protection assay showing that hypoxia (H; 1% oxygen, 48 hours) reduces E-cadherin mRNA in RCC10/VHL and RCC4/VHL. C, Western blotting showing reduced expression of E-cadherin protein in RCC10/VHL cells exposed to either hypoxia or dimethyl-oxalylglycine; Glut1 was used as a control HIF-responsive gene. Control corresponds to untreated cells cultured in normoxia.
HIF-1 induces tumorigenesis in pVHL-reconstituted CCRCC cells (6). Interestingly, some pVHL-negative CCRCC cell lines (e.g., 786-O) express only HIF-2 and not HIF-1. Taken together, this has suggested that evolution to renal carcinoma involves a progressive switch to HIF-2. Our data suggest that HIF-2 is more effective in decreasing E-cadherin than HIF-1, but that both molecules contribute to the decrease in expression in renal carcinoma cell lines. A significant caveat is that in the experiments with forced expression of active HIF molecule aspects of selectivity could be overcome.

Several aspects of our study are of interest. First, the observation that E-cadherin is expressed by renal carcinoma cell lines when complemented with pVHL supports our recent data, suggesting that CCRCC may originate from the distal rather than the proximal tubule (24). Although a proximal tubular origin has generally been accepted (29), there is other evidence consistent with the tumors arising from the distal tubule (30). Identifying the correct cell type of origin is a prerequisite for any meaningful comparison of CCRCC with normal epithelium, because the functional specialization and the genes that are expressed are quite different in the distal and proximal tubule. A second significant aspect of our study is that it provides a potential mechanism by which loss of pVHL function could contribute to tumor initiation, evolution, and phenotype, based on the extensive evidence that down-regulation of E-cadherin promotes progression and invasion in multiple cancers. To date, E-cadherin has received little attention in clear cell renal cell carcinoma, at least in part because this molecule is not expressed in the normal proximal tubule. Interestingly, E-cadherin has been shown to be subject to loss of heterozygosity and methylation in some renal carcinoma cell lines (including 786-O cells; ref. 31), which would be consistent with expression in the parental cell type in the distal tubule and selection for loss of E-cadherin function during cancer evolution, both through pVHL inactivation/HIF activation and other additional genetic or epigenetic modifications. It is, however, noteworthy that pVHL suppresses tumor formation by 786-O cells (6), although 786-O/VHL cells do not express E-cadherin in cell culture (data not shown; ref. 32). This establishes, at least in this cell model, that restoration of E-cadherin expression is not essential for suppression of xenograft growth by pVHL in a fully transformed CCRCC cell line.

A remaining question is how HIF reduces E-cadherin expression. Inactivation of E-cadherin in cancer is generally due to either gene methylation or transcriptional silencing, the latter being achieved through binding of transcriptional repressors to specific DNA sequences (termed E-boxes) in the proximal E-cadherin promoter (13). This raises the possibility that HIF is inducing the expression of a repressor, which in turn decreases the transcription of E-cadherin mRNA. Multiple transcriptional repressors of E-cadherin have been described (13, 33–35), among which SNAIL (SNAI1) is the best characterized. Using real-time RT-PCR, we examined the expression of SNAIL in VHL-negative RCC4, RCC10, and 786-O cell lines and their corresponding stable transfectants with pVHL. Interestingly, we found that SNAIL mRNA expression was increased by the absence of pVHL in all three CCRCC cell lines (Supplementary Fig. S1). This suggests that increased expression of SNAIL mRNA may be involved in down regulating E-cadherin in CCRCC, a possibility that we are currently investigating.

In summary, our study shows that pVHL inactivation leads to down-regulation of E-cadherin both in vivo and in vitro, and that these effects are at least in part mediated via HIF. Given the fact that the HIF response to hypoxia is seen in all cell types examined to date, our study raises the interesting possibility that E-cadherin expression in other settings besides renal epithelium and kidney...
Figure 6. Suppression of HIF in pVHL-negative CCRCC cells restores E-cadherin expression, which is localized to cell-cell contacts. A, pVHL-negative RCC10 cells were transfected with siRNA for either HIF-1α (100 nmol/L), HIF-2α (100 nmol/L), or both (50 nmol/L each). Western blotting for E-cadherin shows potent up-regulation in cells transfected with the combined HIF-1α and HIF-2α duplexes. Firefly luciferase siRNA (100 nmol/L) was used as a control. B, immunofluorescence for E-cadherin in pVHL-negative RCC10 cells transfected with either control siRNA, HIF-1α, or HIF-2α siRNA, or both. C, real-time RT-PCR analysis for E-cadherin mRNA of pVHL-negative RCC10 cells treated with siRNA oligos as indicated. Representative experiment, including the SD between the three replicate samples.

cancer may also be influenced by HIF. If so, this possibility could offer insight into why mutations in E-cadherin are infrequent in cancers in general, because HIF activation is very common in solid tumors and may thus provide a reliable epigenetic route to down-regulating E-cadherin efficiently. It would also explain why expression of E-cadherin in tumors is frequently patchy (as it commonly happens with areas of hypoxia) rather than homogeneous. In this context, we note a recent study reporting that hypoxia reduces E-cadherin expression in ovarian cancer cells in vivo and in vitro (36). Finally, it is tempting to speculate that oxygen gradients could also be a general mechanism to modulate E-cadherin expression (e.g., during embryonic development and normal epithelial differentiation).

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References

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