Tumorigenesis and Neoplastic Progression

Clusterin Is a Secreted Marker for a Hypoxia-Inducible Factor-Independent Function of the von Hippel-Lindau Tumor Suppressor Protein

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Germline mutations in the von Hippel-Lindau (VHL) tumor suppressor gene predispose people to renal cancer, hemangioblastomas, and pheochromocytomas in an allele-specific manner. The best documented function of the VHL gene product (pVHL) relates to its ability to polyubiquitinate, and hence target for destruction, the α subunits of the heterodimeric transcription factor hypoxia-inducible factor (HIF). pVHL mutants linked to familial pheochromocytoma (type 2C VHL disease), in contrast to classical VHL disease, appear to be normal with respect to HIF regulation. Using a simple method for identifying proteins that are differentially secreted by isogenic cell line pairs, we confirmed that the HIF targets IGBP3 and PAI-1 are overproduced by pVHL-defective renal carcinoma cells. In addition, cells lacking wild-type pVHL, including cells producing type 2C pVHL mutants, were defective with respect to expression and secretion of clusterin, which does not behave like a HIF target. Decreased clusterin secretion by pVHL-defective tumors was confirmed *in vivo* by immunohistochemistry. Therefore, clusterin is a secreted marker for a HIF-independent pVHL function that might be especially important in pheochromocytoma development. (Am J Pathol 2006; 168:574–584; DOI: 10.2353/ajpath.2006.050867)

von Hippel-Lindau disease, caused by heterozygous germline inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene, presents clinically as a hereditary cancer syndrome.^{1,2} Tumor development in this setting is due to somatic inactivation of the remaining wild-type VHL allele in a susceptible cell.³ The classical tumors observed in VHL disease are retinal and central nervous system (usually within the cerebellum or spinal cord) blood vessel proliferations called hemangioblastomas. These tumors, although benign, cause significant morbidity and mortality because of mass effect. Some VHL families also exhibit an increased risk of clear cell renal cell carcinoma or pheochromocytoma. In keeping with the Knudson 2-hit model, many sporadic hemangioblastomas and renal cell carcinomas are also due to VHL inactivation, as a result of either somatic mutations or hypermethylation.^{3,4} For reasons that are still unclear, somatic VHL mutations are rare in sporadic pheochromocytomas absent an occult germline VHL mutation, despite the fact that certain germline VHL mutations confer an increased risk of this tumor.

Genotype-phenotype correlations have emerged in VHL disease. VHL families can be subdivided into type 1 (low risk of pheochromocytoma) and type 2 (high risk of pheochromocytoma). Type 2 disease can be subdivided into type 2A (low risk of renal cell carcinoma) and type 2B (high risk of renal cell carcinoma).¹ Some VHL families exhibit an increased risk of pheochromocytoma without

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the other hallmarks of VHL disease (type 2C). Almost all type 2 patients harbor *VHL* missense mutations, whereas null *VHL* alleles cause type 1 disease. This suggests that complete loss of pVHL function is incompatible with pheochromocytoma development or that pheochromocytoma in this setting reflects a mutant pVHL gain-of-function.

The best understood function of pVHL relates to its ability to target the transcription factor hypoxia-inducible factor (HIF) for polyubiquitination and hence destruction.³ HIF consists of a labile α subunit and a stable β subunit. In the presence of oxygen, HIF α subunits are enzymatically hydroxylated on specific prolyl residues. pVHL is the substrate recognition unit of an E3 ubiquitin ligase complex and binds directly to hydroxylated HIF. In cells that are hypoxic, or lack pVHL, HIF escapes destruction and is free to activate HIF target genes such as vascular endothelial growth factor (VEGF), Glut1, and transforming growth factor- α . To date, all pVHL mutants linked to hemangioblastoma or renal cell carcinoma development are defective with respect to HIF regulation. In renal carcinoma nude mouse xenograft assays, suppression of HIF2 α target genes is both necessary and sufficient for tumor suppression by pVHL.^{5–8} Collectively, these observations suggest that HIF2 α is a critical downstream target of pVHL.

Several observations, however, suggest that pVHL has important functions in addition to its ability to down-regulate HIF. First, there is no evidence that forced activation of HIF target genes is sufficient to cause tumor growth and some evidence that it is not. For example, forced production of HIF1 α in the skin or muscle leads to the elaboration of seemingly normal blood vessels without tumors.^{9,10} Second, both type 2A and type 2B pVHL mutants are defective with respect to HIF regulation, suggesting that a second, HIF-independent, function of pVHL relevant to kidney cancer is differentially altered by type 2A and 2B *VHL* mutations.^{11,12} Third, type 2C mutants retain the ability to down-regulate HIF, implying that a HIF-independent function is important for the development of pVHL-defective pheochromocytomas.^{11,12} Finally, Chuvash polycythemia patients do not appear to be tumor prone and yet are germline homozygotes for a *VHL* allele that is hypomorphic with respect to HIF regulation.^{13,14} Absence of tumor development in this setting might, among several possibilities, be due to preservation of a HIF-independent pVHL function.

A number of additional biochemical functions have been ascribed to pVHL, such as binding to atypical PKC family members, SP1, fibronectin, tubulin, RNA polymerase II subunits, VDU1 (and VDU2), VBP1, VHL α K, and MSH4^{15–28}. There is evidence, for example, that pVHL can direct the polyubiquitylation of PKC family members and certain RNA polymerase subunits,^{20,22,29} can regulate transcriptional elongation through its association with elongin B and elongin C,³⁰ can regulate microtubule stability through its interaction with tubulin,¹⁹ and can regulate extracellular matrix assembly.^{26,31,32} How and whether these different activities relate to tumor suppression by pVHL is still unknown.

We initially aimed to identify secreted proteins that reflect *VHL* gene status in hopes of developing a biomarker that could be used to monitor pVHL-defective tumors *in vivo*. Two of the first three markers we identified, IGFBP3 and PAI-1, are encoded by HIF-responsive genes^{33,34} and, accordingly, were increased in pVHL-defective cells. Control of PAI-1 mRNA by pVHL has already been described.^{35–37} The third marker, clusterin, was not under HIF control and was decreased in pVHL-defective tumor cells. Notably, every tumor-derived pVHL mutant examined, including those linked to type 2C disease, was defective with respect to clusterin production. These findings indicate that induction of clusterin reflects a HIF-independent pVHL function that may be important for tumor suppression, especially with respect to the development of pheochromocytoma. In addition to serving as a marker for this function, clusterin secretion might contribute to tumor suppression by pVHL because it has been shown to inhibit cell proliferation and promote apoptosis in certain settings.^{38–41}

Materials and Methods

Plasmids

The *Bam*HI-*Eco*RI fragment from pRc-CMV-HA-VHL⁴² was subcloned into pBabe-puro to create pBabe-puro-HA-VHL. pBabe-puro-HA-HIF2 α P531A and pBabe-puro-HA-HIF2 α P531A bearing a basic helix-loop-helix (bHLH) mutation were described previously.⁵

Cell Culture

786-O and A498 renal cell carcinoma subclones stably transfected with either pRc-CMV (clones pRC3 and pRCB3, respectively) or pRc-CMV-HA-VHL (clones WT8 and WTD10, respectively)^{42,43} and 786-O subclones stably transfected to produce type 2C pVHL mutants (R64P, F119S, and L188V)¹² were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum supplemented with 1 mg/ml of G418 at 37°C in a humidified 10% CO₂-containing atmosphere.

Retroviruses

Retroviral plasmids were transfected into the Phoenix packaging cell line using FuGene (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Tissue culture supernatant was harvested 48 hours later, passed through a 0.45- μ m filter, and added to cells in the presence of 4 μ g/ml polybrene. Infected cells were selected by growth in the presence of puromycin (1.5 μ g/ml).

Cell Proliferation Assays

Renal cell carcinoma cells (10⁴) were plated in 60-mm plastic dishes in DMEM containing 10% fetal bovine serum supplemented with 1 mg/ml of G418 and allowed to

adhere for 12 hours. The cells were then washed twice with phosphate-buffered saline, and the medium was changed to DMEM containing 10 or 2.5% fetal bovine serum or Opti-MEM supplemented with 1 mg/ml of G418. At various time points thereafter, viable cells, as determined by exclusion of trypan blue, were counted using a hemocytometer.

Antibodies

Polyclonal anti-HA (Y-11), anti-clusterin (sc-6419), and anti-PAI-1 (sc-8979) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Glut1 antibody was obtained from Alpha Diagnostic Inc. (San Antonio, TX). Polyclonal anti-IGFBP3 and VEGF polyclonal antibodies were obtained from R&D Systems (Minneapolis, MN). Monoclonal Anti-HIF2 α antibody (clone ep190b) was obtained from Novus Biologicals (Littleton, CO). Anti-tubulin monoclonal antibody (cloneB1.2.5) was obtained from Sigma (St. Louis, MO).

Immunoblot Analysis

Cells were lysed in EBC buffer (50 mmol/L Tris [pH 8.0], 120 mmol/L NaCl, and 0.5% Nonidet P-40) supplemented with complete mini protease cocktail (Roche Molecular Biochemicals). Equivalent amounts of protein, as determined by the Bradford method, were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and wet-transferred to polyvinylidene difluoride filters (Bio-Rad, Hercules, CA). The filters were blocked in Tris-buffered saline with 5% nonfat milk for 1 hour in room temperature and probed with the indicated antibodies in Tris-buffered saline (10 mmol/L Tris [pH 8] and 150 mmol/L NaCl) supplemented with 4% bovine serum albumin. Bound antibody was detected using Supersignal West Pico (Pierce, Rockford, IL).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were plated in 6-well plates (10^5 cells/well) and allowed to adhere for 24 hours before media replacement. At various time points thereafter, aliquots of media were removed, and VEGF and IGFBP3 ELISAs were performed using commercially available kits according to the manufacturer's instructions (R&D Systems). Three independent wells were used for each set of experimental conditions.

Analysis of Proteins Secreted in the Supernatant (Trichloroacetic Acid [TCA] Precipitation)

Cells (2×10^6) were plated in a 100-mm dish in DMEM containing 10% fetal bovine serum supplemented with 1 mg/ml of G418. Twenty-four hours later, the cells were washed twice with phosphate-buffered saline, and the medium was changed to 10 ml of Opti-MEM supplemented with 1 mg/ml G418. Seventy-two hours later, the tissue culture supernatant from three plates was pooled,

clarified by centrifugation at $2100 \times g$ for 10 minutes at 4°C, and transferred to a 50-ml Oak Ridge Teflon FEP tube (Nalgene, Rochester, NY). Ice-cold TCA was added to a final concentration of 20%, mixed well, and left on ice for 20 minutes. Proteins were precipitated by centrifugation at $8400 \times g$ for 30 minutes at 4°C. The supernatant was discarded, and the pellet was washed once with ~ 15 ml of ice-cold acetone followed by centrifugation at $8400 \times g$ for 30 minutes at 4°C. The pellet was air dried in a fume hood for 1 hour and dissolved in 100 μ l of EBC lysis buffer (300 mmol/L Tris [pH 8.0], 120 mmol/L NaCl, and 0.5% Nonidet P-40) supplemented with complete mini protease cocktail. The amount of protein precipitated was determined by the Bradford method before one-dimensional or two-dimensional gel analysis.

Two-Dimensional Protein Electrophoresis

Proteins were dissolved in the urea sample buffer provided in the MK-1 kit (Kendrick Labs, Madison, WI). Two-dimensional electrophoresis was performed according to the method of O'Farrell⁴⁴ by Kendrick Labs as follows. Isoelectric focusing was performed in glass tubes with an inner diameter of 2.0 mm using 2.0% pH 3.5 to 10 ampholines (LKB/Pharmacia) for 9600 volts/hour. After equilibration for 10 minutes in buffer 0 (10% glycerol, 50 mmol/L dithiothreitol, 2.3% SDS, and 0.0625 mol/L Tris [pH 6.8]), the tube gel was sealed to the top of the stacking gel of an SDS-10% polyacrylamide slab gel (0.75 mm thick). Electrophoresis was performed for about 4 hours at 12.5 mA/gel. The following proteins (Sigma) were added as molecular weight standards for the second dimension: myosin (220,000 d), phosphorylase A (94,000 d), catalase (60,000 d), actin (43,000 d), carbonic anhydrase (29,000 d), and lysozyme (14,000 d). After staining, the gels were dried between sheets of cellophane paper with the acid edge to the left.

Northern Blot Analysis

RNA was isolated using Nucleospin RNA II kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol, resolved using a 1.2% agarose/formaldehyde gel (10 μ g/lane), and blotted onto a nylon membrane (Schleicher & Schuell, Germany) by capillary transfer. Hybridization was done using ExpressHyb hybridization solution (Clontech). The following probes were used: Avall fragment of IGFBP3 cDNA, FokI fragment of PAI-1 cDNA, and BglI and HindIII fragment of glyceraldehyde-3-phosphate dehydrogenase cDNA. The clusterin probe was made by polymerase chain reaction (PCR) amplification of a clusterin cDNA using primers 5'-GTGCAATGAGACCAT-GATGG-3' and 5'-CAGGTAGTGGTAGGTATCCT-3').

VHL Genotyping

Genomic DNA was prepared from the freshly frozen tumor specimens by proteinase K/phenol-chloroform extraction. All three exons of the VHL gene were amplified by PCR using three sets of previously described prim-

Figure 1. Characterization of 786-O subclones grown in synthetic media. **a:** 786-O *VHL*^{-/-} human renal cell carcinoma cells stably transfected with plasmids encoding hemagglutinin-tagged wild-type pVHL (WT8) or empty vector (pRC3) were grown in DMEM containing 10% fetal calf serum (■), 2.5% fetal calf serum (○), or Opti-MEM (●). The number of living cells, as determined by trypan blue exclusion, was determined at the indicated time points. **b:** WT8 (open bars) or pRC3 cells (gray bars) were grown in DMEM containing 10% fetal calf serum or Opti-MEM. At the indicated time points, VEGF concentration in the supernatant was measured by ELISA. VEGF concentration (picograms/milliliter) was normalized to total cell protein. Error bars = 1 SE. **c:** WT8 or pRC3 cells were grown in Opti-MEM. The conditioned media, as well as unconditioned Opti-MEM, were precipitated with TCA, resolved by SDS-PAGE under nonreducing or reducing conditions, and immunoblotted with anti-VEGF polyclonal antibody.

ers⁴⁵ followed by sequencing. The institutional review board of the Kyoto University Graduate School of Medicine approved this study.

VHL Hypermethylation Assays

DNA was treated with sodium bisulfite as described previously.⁴⁶ Briefly, 2 µg of DNA was incubated with 3 mol/L NaOH in a final volume of 20 µl for 15 minutes at 37°C. Freshly prepared 4.8 mol/L sodium bisulfite (278 µl) and freshly prepared 100 mmol/L hydroquinone (2 µl) were added, and the sample underwent 20 cycles of denaturation at 95°C for 30 seconds and incubation at 55°C for 15 minutes. The sample was desalted using the QIAquick PCR purification kit (Qiagen) and desulfonated by incubation with 3 mol/L NaOH for 15 minutes at 37°C. The DNA was ethanol-precipitated and resuspended in 50 µl of water. The methylation status in the CpG island of VHL

Figure 2. Regulation of IGFBP-3 and PAI-1 by pVHL. **a:** 786-O subclones (WT8 or pRC3), as well as 786-O cells infected with a retrovirus encoding wild-type pVHL (786-O + V) or with an empty virus (786-O + Empty) were grown in Opti-MEM for 3 days. Secreted proteins were precipitated with TCA, resolved by SDS-PAGE, and detected by silver staining. Mass spectrometry sequencing of the band indicated by the arrow revealed peptides from IGFBP-3 and PAI-1. **b:** 786-O subclones (WT8 or pRC3) or A498 *VHL*^{-/-} human renal cell carcinoma cells stably transfected with plasmids encoding wild-type pVHL (WTD10) or empty vector (pRCB3) were grown in Opti-MEM for 3 days. Secreted proteins were precipitated with TCA, resolved by SDS-PAGE, and immunoblotted with anti-IGFBP3 or anti-PAI-1 antibodies. **c:** WT8 or pRC3 cell were grown in DMEM containing 10% fetal bovine serum. At the indicated time points, IGFBP3 or PAI-1 concentration in the supernatant was examined by ELISA. The concentrations of both proteins (nanograms/milliliter) were normalized to total cell protein. Error bars = 1 SE.

gene was determined by methylation-specific PCR as described previously.⁴⁷

Immunohistochemistry

Immunohistochemical analysis was performed on formalin-fixed tissue obtained from the archives of Brigham and Women's Hospital, from Kyoto University Hospital, and from consultation. Formalin-fixed, paraffin-embedded tissue blocks were cut into 4-µm-thin sections, transferred to glass slides, and pretreated with a digital timed pressure cooker and Target Retrieval solution (Dakocytomation, Carpinteria, CA) before incubation with anti-clusterin antibody (1:4000 dilution; clone 41D; Upstate Cell Signaling) for 40 minutes at room temperature. Bound antibody was detected using the EVISION+ HRP detection system (Dakocytomation). Under these conditions, signals were not observed when duplicate samples were processed in parallel with the primary antibody omitted (data not shown). Anaplastic large cell lymphoma and normal adrenal glands were used as positive controls. Immunoreactivity was further graded semiquantitatively as follows: 1, no staining; 2, only membrane staining; 3, 1 to 10% cells staining, weak; 4, 1 to 10% cells staining, strong; 5, 11 to 50% cells staining, weak; 6, 11 to 50% cells staining, strong; 7, >51% cells staining, weak; and 8, >51% cells staining, strong.

Figure 3. Two-dimensional gel analysis of IGFBP-3 and PAI-1. WT8 and pRC3 cells were grown in Opti-MEM. Secreted proteins were precipitated with TCA, resolved by two-dimensional-gel electrophoresis, and detected by silver staining (**a**) or by immunoblot analysis with the indicated antibodies (**b** and **c**). In **a**, the WT8 and pRC3 silver-stained gels were scanned, pseudo-colored green and red, respectively, and superimposed *in silico*. Note the series of red spots at ~43 kD (PAI-1) and ~34 kD (IGFBP-3).

In addition, samples were scored for Golgi clusterin staining (positive or negative) and for coarse granular clusterin staining (positive or negative). All tumors were scored independently by two of the authors (P.A. and V.N.) without knowledge of the *VHL* genotype. Statistical significance was determined with Fisher's exact test.

Results

We used a simple approach to identify proteins that are differentially secreted by matched (isogenic) renal carcinoma cell line pairs that do or do not contain wild-type pVHL. To reduce the complexity of conditioned media, cells were grown in synthetic serum-free media. In pilot experiments, we found that human renal carcinoma cell

Figure 4. pVHL-dependent secretion of clusterin revealed by two-dimensional gel analysis. WT8 and pRC3 cells were grown in Opti-MEM. Secreted proteins were precipitated with TCA, resolved by two-dimensional-gel electrophoresis, and detected by Coomassie blue staining or by anti-clusterin immunoblot (IB) analysis. For unclear reasons, clusterin was not easily seen on silver-stained gels (Figure 3).

lines, such as 786-O *VHL*^{-/-} cells stably transfected with a plasmid encoding wild-type pVHL (clone WT8) or with an empty expression plasmid (clone pRC3), proliferate for at least 3 days when grown in synthetic media (Opti-MEM) (Figure 1a; data not shown). Moreover VEGF, which is the product of a HIF-responsive gene and is negatively regulated by pVHL,^{48–51} was overproduced by pRC3 cells grown in either conventional media or Opti-MEM (Figure 1b). Next, conditioned media from cells grown in Opti-MEM was concentrated by TCA pre-

Figure 5. Regulation of clusterin by pVHL is HIF independent. **a:** Northern blot analysis of 786-O and A498 subclones using the indicated probes. **b:** 786-O and A498 subclones were grown in the presence of 1% or 21% oxygen for 24 hours in Opti-MEM. Whole-cell extracts (WCE) and conditioned media after TCA precipitation (Supernatant) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. **c:** pRC3 and WT8 cells infected to produce HIF2 α P531A, HIF2 α P531A with a bHLH mutation or with empty vector were grown in Opti-MEM for 3 days. Secreted proteins were precipitated by TCA, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies.

cipitation, resolved by SDS-polyacrylamide gel electrophoresis under reducing or nonreducing conditions, and immunoblotted with an anti-VEGF antibody. As expected, increased amounts of VEGF were detected in the pRC3-derived media pellet compared with the WT8-derived media pellet, suggesting that TCA precipitation can be used to concentrate and measure differentially secreted proteins (Figure 1c).

Next, conditioned media from matched pVHL(+) and pVHL(−) cell line pairs grown in Opti-MEM were concentrated by TCA precipitation, resolved by one-dimensional or two-dimensional gel electrophoresis, and analyzed by silver or Coomassie blue staining. Bands (or spots) that reproducibly differed in abundance as a function of pVHL status were excised and identified using mass spectrometry. Some differences between WT8 and pRC3 cells might be due to heterogeneity within the original 786-O cell population, rather than due to pVHL status because both are clonal derivatives of 786-O cells. For this reason, we also compared polyclonal 786-O cells infected with a retrovirus encoding pVHL (786-O + VHL) to polyclonal 786-O cells infected with an empty retrovirus (786-O + Empty) and A498 VHL−/− cells stably transfected with a plasmid encoding wild-type pVHL (clone WTD10) to A498 cells stably transfected with an empty expression plasmid (clone pRCB3).

The abundance of an approximately 40-kd protein was reproducibly increased in the conditioned media of pVHL(−) cells compared with pVHL(+) cells (Figure 2a). Mass spectrometry of the corresponding protein band revealed the presence of both IGFBP-3 and PAI-1. Increased production of IGFBP-3 and PAI-1 in the conditioned media of pVHL(−) cells was confirmed by immunoblot analysis (Figure 2b) and ELISA (Figure 2c). Because these two proteins co-migrated under these one-dimensional gel conditions, conditioned media from pVHL(−) and pVHL(+) cells were next resolved by two-

Figure 6. Type 2C pVHL mutants fail to up-regulate clusterin. **a:** Whole-cell extracts prepared from WT8, pRC3, or 786-O subclones stably transfected with plasmids encoding hemagglutinin (HA)-tagged type 2C pVHL mutants (R64P, F119S, and L188V) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Note that type 2C mutants down-regulate HIF2 α and the canonical HIF target Glut1. **b:** Northern blot analysis of the 786-O clones in **a** with the indicated probes. Note that type 2C mutants down-regulate the canonical HIF target VEGF but are grossly impaired with respect to clusterin.

dimensional gel electrophoresis and silver stained or transferred to nitrocellulose. Silver-stained images were pseudocolored red (pRC3) or green (WT8) and superimposed *in silico* (Figure 3a). The spots corresponding to IGFBP-3 and PAI-1 were confirmed by immunoblot analysis using antibodies that are specific for these two proteins (Figure 3, b and c). The identification of increased IGFBP-3 and PAI-1 in the conditioned media of pVHL(−) cells further validated our assay, because it was established before that both are regulated by HIF and inhibited by pVHL.^{35–37}

In two-dimensional gels, we noted a series of spots that were increased in conditioned media of pVHL(+) cells (Figure 4). These spots corresponded to clusterin as determined by mass spectrometry and anti-clusterin immunoblot analysis (Figure 4; data not shown). Increased clusterin production by pVHL(+) cells relative to pVHL(−) cells was due to increased clusterin mRNA accumulation (Figure 5a). To determine whether alter-

Figure 7. Decreased clusterin staining in pVHL-defective renal carcinoma. Hematoxylin and eosin staining (**top**) and anti-clusterin staining (**bottom**) of clear cell renal carcinoma with wild-type (**left**) or mutant (**right**) *VHL*. Magnification, $\times 400$.

ations in clusterin production were an indirect manifestation of changes in HIF, we measured clusterin secretion in cells in which HIF activity was modulated by changes in oxygen or by genetic approaches. As expected, both HIF2 α and a canonical HIF target, the Glut1 glucose transporter, were induced by hypoxia in pVHL(+) cells but not in pVHL(–) cells (Figure 5b). Likewise, secretion of both PAI-1 and IGBP-3 was induced by hypoxia in pVHL(+) cells but not in pVHL(–) cells, in keeping with the abovementioned knowledge that they are both under HIF control (Figure 5b). In contrast, secretion of clusterin was not reproducibly altered by changes in ambient oxygen (Figure 5b; data not shown).

Next, we analyzed pVHL(+) cells (WT8) that were infected with a retrovirus encoding HIF2 α P531A, which escapes recognition by pVHL and transcriptionally activates HIF target genes.⁵ WT8 cells infected with a DNA-binding defective version of HIF2 α P531A (P531A + bHLH)⁵ or an empty retrovirus served as negative controls. As expected, both PAI-1 and IGFBP3 were induced by HIF2 α P531A, in a DNA binding-dependent manner, to levels approximating those seen in pVHL(–) cells (pRC3) (Figure 5c). In contrast, none of these retroviruses consistently affected clusterin production.

Some naturally occurring *VHL* alleles cause familial pheochromocytoma without the classical signs of VHL disease (type 2C VHL disease). Type 2C pVHL mutants retain the ability to down-regulate HIF and its downstream targets.^{11,12} In contrast, we found that clusterin production was not restored in 786-O cells stably transfected to produce type 2C pVHL mutants such as pVHL R64P, F119S, or L188V (Figure 6). Every disease-associated pVHL mutant we have examined to date is quantitatively defective with respect to promoting clusterin secretion (data not shown). In contrast, clusterin levels in *VHL*+/+ ACHN and Caki renal carcinoma cells were similar to those in *VHL*–/– cells engineered to produce wild-type pVHL (data not shown). Collectively, these results indicate that clusterin secretion is a biomarker for a HIF-independent function that is lost by tumor-associated pVHL mutants.

To ask whether pVHL regulates clusterin expression *in vivo*, we analyzed 63 clear cell renal carcinomas with known *VHL* status for clusterin expression by immunohistochemical analysis. Of these tumors, 34 had *VHL* alterations (33 mutations and 1 hypermethylation) (*VHL* mutant) and 29 had wild-type *VHL*. *VHL* mutant tumors displayed a profound decrease in clusterin staining rela-

Table 1. Correlation of Clusterin Expression to VHL Gene Status in Renal Cell Carcinomas

Pattern of staining	VHL mutant	VHL wild type
No staining (1)	13 (38.2%)	0
Only membrane staining (2)	3 (8.8%)	1 (3.4%)
1 to 10% cells staining, weak (3)	15 (44.1%)	5 (17.2%)
1 to 10% cells staining, strong (4)	0	0
11 to 50% cells staining, weak (5)	2 (5.8%)	4 (13.7%)
11 to 50% cells staining, strong (6)	1 (2.9%)	8 (27.5%)
>51% cells staining, weak (7)	0	6 (20.6%)
>51% cells staining, strong (8)	0	5 (17.2%)
Total number of cases	34 (100%)	29 (100%)

tive to wild-type tumors (Figure 7; Tables 1 and 2; $P < 0.0001$). VHL mutant tumors were also less likely to exhibit positive Golgi staining for clusterin (Table 3; $P < 0.008$). Similarly, clusterin staining was reduced in pheochromocytomas harboring *VHL* mutations (Figure 8; data not shown). Therefore, the influence of pVHL on clusterin secretion in cell culture experiments is mirrored *in vivo*.

Discussion

Using a simple method for identifying differentially secreted proteins, we found that IGBP3 and PAI-1 are up-regulated and clusterin is down-regulated after pVHL inactivation. Increased secretion of IGBP3 and PAI-1 by pVHL-defective cells is in keeping with the knowledge that they are HIF-responsive gene products^{33,34} and with earlier studies that observed increased IGFBP3 and PAI-1 mRNA levels in cells lacking wild-type pVHL.^{35–37} In contrast, our studies suggest that clusterin expression is not under the control of oxygen or HIF. Moreover, pVHL mutants linked to type 2C VHL disease (pheochromocytoma only) retain the ability to suppress HIF and are impaired with respect to clusterin induction. Every disease-associated pVHL mutant we have examined to date is defective in this assay. Therefore, clusterin secretion is a marker for a HIF-independent pVHL function that might contribute to tumor suppression by pVHL, especially with respect to pheochromocytoma.

Clusterin is ubiquitously expressed, with high levels in VHL target organs such as brain, liver, kidney, and adrenal medulla, and exists in both intracellular and secreted forms.^{52,53} Its predominant form is a secreted heterodimeric glycoprotein of 75 to 80 kd and is highly conserved across species, showing 70 to 80% identity at the amino acid level among mammals. Clusterin expression tends to increase with senescence or during cellular transformation, although whether such increases are causal with respect to these two cellular phenotypes is

Table 3. Correlation of Clusterin Expression to VHL Gene Status in Renal Cell Carcinomas

Pattern of staining	VHL mutant	VHL wild type
Golgi positivity (9)	2	10
Coarse granular (10)	8	7

unclear.⁵³ A variety of functions have been ascribed to clusterin, including both anti-apoptotic and pro-apoptotic activities in a context-dependent manner. For example, clusterin promotes chemoresistance in various models (anti-apoptotic activity), including renal carcinoma cells, whereas loss of clusterin promotes survival of hypoxic neurons (pro-apoptotic activity)^{53–56}. The latter is intriguing given the role of pVHL in oxygen sensing and also suggests that loss of clusterin might promote survival within the hypoxic zones of pVHL-defective solid tumors. Recent studies indicate that clusterin inhibits nuclear factor κ B activity through stabilization of I κ B.⁴¹ Therefore, loss of clusterin expression offers one explanation for why pVHL-defective tumor cells exhibit increased nuclear factor κ B signaling and resistance to tumor necrosis factor- α .^{57,58} Clusterin has also been shown to decrease prostate cancer cell proliferation and neuroblastoma cell invasion *in vitro*.^{39–41} Therefore, it is conceivable that clusterin contributes to tumor suppression by pVHL in addition to serving as a marker for its integrity.

In one study, clusterin was found to be overexpressed in approximately 50% of nonpapillary renal cell carcinomas and to be associated with a poor prognosis.⁵⁹ Although *VHL* status was not examined in this study, this number is consistent with earlier findings that *VHL* is mutated in a similar proportion of sporadic nonpapillary renal cell carcinomas.⁶⁰ Based on our work, we would predict that the tumors characterized by clusterin “overexpression” would be those that retained wild-type pVHL. If true, this would be concordant with earlier studies showing that *VHL* +/+ renal cancers have a bad prognosis relative to renal cancers with *VHL* mutations.⁶¹

Clusterin is up-regulated by activated membrane-bound receptors such as epidermal growth factor receptor and nerve growth factor receptors and by oncoproteins such as B-Myb and Src.^{53,62} Control of clusterin transcription is complex and involves transcription factors AP1, AP2, Sp1, NF1, and B-Myb. It will be important to determine whether pVHL regulates clusterin transcription, mRNA stability, or both and to determine which factor(s) links pVHL status to the production of clusterin mRNA.

It is currently unknown why certain germline *VHL* mutations predispose to pheochromocytoma and yet somatic *VHL* mutations are rare in sporadic pheochromocytoma.

Table 2. Correlation of Clusterin Expression to VHL Gene Status in Renal Cell Carcinomas

Pattern of staining	VHL mutant	VHL wild type
1, 2, and 3 (no staining, membranous or weak positivity in $\leq 10\%$ of the cells)	31 (91.1%)	6 (20.6%)
5, 6, 7, and 8 (positivity in $>10\%$ of the cells)	3 (8.8%)	23 (79.3%)

Figure 8. Decreased clusterin staining in pVHL-defective pheochromocytoma. Hematoxylin and eosin staining (**top**) and anti-clusterin staining (**bottom**) of normal adrenal gland (**left**), pheochromocytoma with wild-type (**middle**), or mutant (**right**) *VHL*. Magnification, $\times 400$.

cytoma (that is, in pheochromocytomas developing in the absence of a germline *VHL* mutation), in apparent violation of the Knudson two-hit model. In this regard, it is interesting that type 2C mutants are defective with respect to the production of two secreted proteins, clusterin and fibronectin, that might plausibly be linked to tumor suppression. In the germline setting, *VHL* haploinsufficiency might lead to impaired secretion of such proteins within an organ such as the adrenal medulla. In the sporadic setting, loss of secretion by a rare cell would presumably not lead to a phenotype because of normal protein secretion by its neighbors. Interestingly, *NF1* is another gene linked to hereditary pheochromocytoma, and a role for haploinsufficiency has been demonstrated in mouse tumors developing after *NF1* inactivation.⁶³

It is possible that measurement of secreted biomarkers such as IGFB3, PAI-1, and clusterin in body fluids (for example, blood and urine) might be useful for monitoring pVHL-defective renal carcinomas. An immediate question is whether the IGFB-3 and PAI-1 isoforms secreted by renal cancer cells can be distinguished from those present in normal serum, which are largely derived from other organs such as the liver. In addition, identification of new *VHL* biomarkers might ultimately lead to appreciation of novel pVHL functions and hence new mechanistic insights into tumor suppression by pVHL and the basis for the genotype-phenotype correlations observed in *VHL* disease.

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