Gene Expression Profiling in a Renal Cell Carcinoma Cell Line: Dissecting VHL and Hypoxia-Dependent Pathways

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Abstract

The von Hippel-Lindau tumor suppressor, pVHL, is a key player in one of the best characterized hypoxia signaling pathways, the VHL-hypoxia-inducible factor (VHL-HIF) pathway. To better understand the role of VHL in the hypoxia signaling pathways of tumor cells, we used serial analysis of gene expression (SAGE) to investigate hypoxia-regulated gene expression in renal carcinoma cells (786-0), with and without VHL. The gene expression profiles of the cancer cells were compared to SAGE profiles from normal renal proximal tubule cells grown under both normoxia and hypoxia. The data suggest that the role of VHL as a tumor suppressor may be more complex than previously thought. Further, the data reveal that renal carcinoma cells have evolved an alternative hypoxia signaling pathway(s) compared with normal renal cells. These alternative hypoxia pathways demonstrate VHL-dependent and VHL-independent regulation. The genes involved in such pathways include those with potential importance in the physiological and pathological regulation of tumor growth and angiogenesis. Some of the genes identified as showing overexpression in the cancer cells, particularly those encoding secreted or membrane-bound proteins, could be potential biomarkers for tumors or targets for rational therapeutics that are dependent on VHL status.

Introduction

Most solid tumors larger than several cubic millimeters have undergone an angiogenic switch, a crucial step for tumor growth and metastasis. Thus, blocking angiogenesis could be an effective strategy to arrest tumor growth (1). Signals from both genetic mutations and environmental factors regulate this switch (2), with one of the most potent environmental signals being hypoxia (low oxygen tension). Although new blood vessel formation is stimulated by hypoxia, and attenuates the hypoxic environment, hypoxia continues to affect some regions of large solid tumors. Adaptation to hypoxia is of fundamental importance in developmental, physiological, and pathophysiological processes (3, 4). This adaptation to hypoxia depends in part on appropriate expression of many physiologically relevant genes that are regulated transcriptionally, post-transcriptionally, or post-translationally. Gene expression changes in response to hypoxia are important to many fundamental biological processes including apoptosis, stress adaptation, and angiogenesis. Genes the expression of which change in response to hypoxia include vascular endothelial growth factor (VEGF), which is a potent angiogenic growth factor that induces new blood vessel formation in both physiological and pathophysiological processes, and genes involved in the adaptation of energy metabolism in decreased oxygen environments such as glucose transporter 1 (GLUT1) and phosphoglycerate kinase 1 (PGK1) (3, 5). Enhanced glucose metabolism and angiogenesis are hallmarks of tumor growth and involve up-regulation of genes that are normally induced by hypoxia.

Hypoxia-inducible factor (HIF) is a critical transcriptional regulator of many hypoxia-associated genes, including GLUT1 and VEGF (6). HIF is a heterodimer comprised of HIF-α and ARNT, two basic helix-loop-helix proteins in the PAS family (5). There are at least two mammalian HIF-α isoforms, HIF-1α and HIF-2α, which are predominantly regulated by protein stability, although there is some evidence that HIF mRNA levels may also be affected by oxygen (7, 8). Under normoxia, HIF-α proteins are rapidly degraded through the ubiquitin-proteasome pathway (8–11). In contrast, under low oxygen tension, HIF-α proteins are stabilized (6).

Inactivation of the VHL tumor suppressor gene results in the development of von Hippel-Lindau disease, a hereditary cancer syndrome distinguished by highly angiogenic tumors of the kidney, retina, and central nervous system (12, 13). Several lines of evidence suggest that pVHL, the VHL gene product, is a multifunctional protein. The best characterized function of pVHL is its role as a component of the ubiquitin E3 ligase complex that targets HIF-α for ubiquitin-dependent proteasome degradation (14–17). Recent studies demonstrated that the VHL-dependent proteolytic degradation of both HIF-1α and HIF-2α occurs through enzymatic hydroxylation of specific prolyl residues within the HIF-α oxygen-dependent degradation domain (ODDD) (18–20).

In hypoxic cells, HIF-α degradation is suppressed leading to enhanced transcription of target genes, including pro-angiogenic genes. In renal cell carcinoma (RCC), a common manifestation
of VHL disease, HIF-α is overproduced throughout the tumor. A well-known transcriptional target of HIF-α, VEGF, is overexpressed in RCC tumors and likely contributes to the activation of the angiogenic switch in these and other tumors (21–24). The renal carcinoma cell line 786-0 lacks functional VHL protein, and although this cell type does not express HIF-1α, HIF-2α is constitutively expressed at high levels in these cells (25).

Elucidation of the molecular mechanisms involving hypoxia signaling pathways could have a profound impact on the diagnosis, prognosis, and treatment of tumors (26). Indeed, genetic disruption of the HIF-α hypoxia signaling pathway, or inhibition of HIF-α targets, effectively suppresses tumor growth (25, 27–29). Studies to address the molecular mechanisms by which cells adapt to hypoxia are heavily dependent on in vitro cancer cell line systems. It is unclear whether tumor cells and normal cells use the same hypoxia signaling pathways. It may be that tumors evolve alternative hypoxia signaling pathways to survive in an adverse environment, or perhaps to escape surveillance from normal growth checkpoints. Here, we investigated hypoxia-regulated gene expression in both normal and cancer cells at the transcriptional level. Using serial analysis of gene expression (SAGE) (30), we found that renal carcinoma cells, irrespective of VHL status, respond differently to hypoxia than normal renal tubule cells. We identified many hypoxia-inducible genes that could be key regulators of tumorigenesis and the angiogenic switch. We also show that hypoxia on individual gene expression (25). The data presented in this study should be interpreted cautiously because different cell lines may have evolved unique hypoxia response pathways.

Results and Discussion
SAGE of RCC 786-0 Cells Under Different Genetic and Environmental Conditions

A total of eight independent SAGE libraries was generated from eight different normal and tumor samples, as shown in Table 1. The samples varied in wild-type VHL gene expression and exposure to hypoxia. The parental 786-0 RCC line is defective in VHL expression. This line was engineered to express the wild-type VHL gene through infection with a retroviral vector carrying the VHL cDNA. SAGE libraries were made from the engineered 786-0 line grown under normoxia (V1; 786-0 VHL+/Nor.) and from the parental line lacking VHL, but infected with an empty retroviral vector and grown under normoxia (V2; 786-0 VHL−/Nor.). SAGE libraries were also made from these two cell lines grown under hypoxia conditions, respectively (V3; 786-0 VHL+/Hyp.).

| Table 1. SAGE Libraries of Cancer Cells (RCC 786-0) and Normal Cells |
|-------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Library | Cell Type | VHL Status | O2 Conditions | SAGE Tags |
| V1 | 786-0 | +VHL | Normoxia | 54,224 |
| V2 | 786-0 | −VHL | Normoxia | 51,759 |
| V3 | 786-0 | +VHL | Hypoxia | 51,000 |
| V4 | 786-0 | −VHL | Hypoxia | 55,368 |
| V5,6 | RPTEC | +VHL | Normoxia | 51,073; 56,395 |
| V7,8 | RPTEC | +VHL | Hypoxia | 30,760; 31,068 |

VHL Negatively Regulates Genes in 786-0 Cells

It has been shown that VHL negatively regulates many hypoxia-inducible genes through the VHL-HIF pathway. In addition, VHL has been shown to regulate the mRNA stability of some hypoxia-inducible genes (22), raising the possibility that VHL has effects on gene expression independent of HIF. Using standard molecular biology techniques, such as Northern blot analysis, others have examined the effects of VHL and hypoxia on individual gene expression (25). The data presented here use a genome-wide unbiased approach to extend the previous studies. Our analysis confirmed that the loss of pVHL and exposure to hypoxia induce a subset of the same genes (Fig. 1). Thirty-eight genes that are hypoxia inducible in the 786-0 VHL− cells are also up-regulated in 786-0 VHL− cells (Fig. 1 and Supplemental Materials). It is worth noting...
that GLUT1 is not among the 38 listed here because it is not induced at least 4-fold when compared to 786-0 VHL- cells grown under normoxia. This is largely due to the high basal expression of GLUT1 in the 786-0 VHL- cells (see Table 2c). Still, GLUT1 expression is induced 2-fold in the absence of VHL. Moreover, as shown previously (25), our data confirm that restoration of VHL expression rescues the ability of hypoxia to induce GLUT1 in renal carcinoma cells (3-fold in VHL-; 1.5-fold in VHL+). Interestingly, glial maturation factor β (GMFB), which is involved in the differentiation of brain cells and in the stimulation of neural regeneration (33), is among the genes induced in the absence of VHL or in the presence of hypoxia (Table 2b). Overexpression of genes involved in neuronal growth and differentiation is intriguing because other manifestations of VHL disease include the development of phaeochromocytomas and hemangioblastomas of the retina and central nervous system (12, 34).

Importantly, the vast majority (122 of 160 genes; Fig. 1) of genes induced by hypoxia in 786-0 VHL- cells are not up-regulated (at least 4-fold) in 786-0 cells by simply mutating VHL. This demonstrates that in a tumor cell, the loss of VHL is not equivalent to hypoxic exposure, and suggests that the VHL tumor suppressor may have an important distinct role from its participation in the hypoxia-inducible pathway.

Fig. 1 also shows that in the tumor cells, there are 154 genes that appear to be negatively regulated by VHL. These genes are up-regulated in 786-0 cells that lack VHL compared to those expressing VHL (Table 2a and Supplemental Materials). Among those genes are matrix metalloprotease 1 (MMP1) which encodes a protease that cleaves collagens (35). MMPs have previously been shown to be regulated by VHL (36). Numerous studies have demonstrated that these proteases play an important role in angiogenesis, morphogenesis, and tissue remodeling, processes which are all associated with the timely breakdown of extracellular matrix during tumorigenesis (37–39). This group also contains genes that are involved in cell growth and differentiation, including insulin-like growth factor binding protein 3 (IGFBP3) (40). This suggests that the VHL tumor suppressor functions by directly regulating genes that control cell growth and differentiation, as well as angiogenesis.

Gene Expression Changes in 786-0 Cells That Are Affected by VHL

pVHL controls expression of key transcriptional regulators such as HIF-α, thus a defect in VHL is expected to have profound effects on the expression of many genes. Fig. 2 illustrates that, as expected, the renal carcinoma 786-0 cells have undergone a multitude of gene expression changes when compared to normal renal cells. Using the RPTEC gene expression profile as a reference, we were able to dissect out VHL-dependent and VHL-independent changes in the cancer cell line. Seven hundred eighty-seven (416 + 371) genes show at least 4-fold overexpression in the 786-0 cells lacking VHL when compared to the RPTECs and 839 (468 + 371) genes show similar differential expression when VHL is reintroduced. It is possible that some of the 416 genes that are induced in the 786-0 cells lacking VHL would also be induced in RPTECs if they lacked wild-type VHL expression. However, 371 genes are overexpressed in the 786-0 cells regardless of VHL status. Overall, VHL status in this cancer cell line affects expression of more than 800 genes (416 + 468). This observation underscores the importance of VHL as a master regulator of numerous genes and pathways, and suggests that the role of pVHL as a tumor suppressor may be more complex than previously considered.

The 416 genes up-regulated in 786-0 cells lacking VHL include genes that encode growth factors and their receptors, proteins involved in cell-matrix interactions, and proteins that control cellular metabolism (Table 2c and Supplemental Materials). As expected, many of the genes in this group are hypoxia-inducible genes, including GLUT1, IGFBP3, and VEGF, all of which are known to be regulated by HIF-1α (5) and are therefore expected to be induced in the absence of VHL. (These genes are also up-regulated in the RPTECs grown under hypoxia with respect to RPTECs grown under normoxia; Table 2c.) It is noteworthy that although the 786-0 cells lack expression of HIF-1α, there is increased expression of these HIF-1α regulated genes when compared with normal cells. 786-0 cells constitutively express HIF-2α, and there is known overlap in the genes that are regulated by the HIF-α proteins (41). Importantly, HIF-1α and HIF-2α are also suspected to have unique targets because mice deficient for either gene do not show redundant phenotypes (42). It is also possible that these hypoxia-inducible genes may be normally repressed through a VHL-dependent pathway that is independent of HIF. Consistent with this, a previous study using this cell line showed that VHL negatively regulates VEGF expression largely by affecting the stability of the VEGF mRNA (22).

Hypoxia-overexpressed gene 3 (HOG3) was also expressed more highly in the 786-0 VHL- Nor. cells than in the RPTEC Nor. cells (Table 2c). HOG3 is a HIF-1-dependent hypoxia-inducible gene originally identified in human glioblastoma cells (43). HIG2 (hypoxia-inducible gene 2), which was previously identified as a hypoxia-inducible gene in cultured human cervical epithelial cells and in cervical tumor xenografts deprived of oxygen (44), was also induced in the 786-0 VHL- Nor. cells. As discussed above, overexpression of these genes is consistent with the role of VHL in negatively regulating HIF-responsive genes and suggests that HIG2 may also be regulated by HIF-α.

Four hundred sixty-eight genes are exclusively up-regulated in the 786-0 cells that have been engineered to re-express VHL, with respect to their expression in RPTECs (Fig. 2). Interestingly, many of these genes have been previously implicated in playing a functional role in angiogenesis and tumorigenesis (Table 2d and Supplemental Materials). These include syndecan 2 (SDC2) (45), neuropilin 1 (NRP1) (46), plasminogen activator urokinase receptor (PLAUR) (47–49), and integrin β3 binding protein (ITGB3BP) (50, 51). The role of VHL in negatively regulating genes involved in angiogenesis has been extensively studied. Our data suggest that VHL may also positively regulate genes that promote angiogenesis, at least in the context of a cancer cell.

HIF-α is regulated through prolyl hydroxylation by PH domain-containing (PHD) proteins 1, 2, and 3 (52, 53). In vitro studies using recombinant PHD proteins suggest that
Table 2. Differentially Expressed Genes in Renal Cells Grown Under Different Conditions

<table>
<thead>
<tr>
<th>SAGE Tag</th>
<th>Accession no. or UniGene ID</th>
<th>Gene Symbol (Name)</th>
<th>Ratio 1</th>
<th>Ratio 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>TAGAATTTTC</td>
<td>Mmp1; metalloprotease 1 (pitrilysin family)</td>
<td>4.28</td>
<td>3.28</td>
</tr>
<tr>
<td></td>
<td>GGTGAATCTC</td>
<td>IGFBP3; insulin-like growth factor binding protein 3 (EST)</td>
<td>4.28</td>
<td>1.11</td>
</tr>
<tr>
<td>b</td>
<td>CCACGTTCACA</td>
<td>ENO2; enolase 2 (γ, neuronal)</td>
<td>8.61</td>
<td>5.44</td>
</tr>
<tr>
<td></td>
<td>GTGCTTGAC</td>
<td>GMFB; glia maturation factor, β</td>
<td>7.50</td>
<td>6.56</td>
</tr>
<tr>
<td>c</td>
<td>GAGACTCCGT</td>
<td>GLUT1; solute carrier family 2 (facilitated glucose transporter), member 1</td>
<td>5.25</td>
<td>14.02</td>
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<tr>
<td></td>
<td>ACTGAGGAAAA</td>
<td>IGFBP3; insulin-like growth factor binding protein 3</td>
<td>6.63</td>
<td>5.18</td>
</tr>
<tr>
<td></td>
<td>CCAGAGTCTC</td>
<td>VEGF; vascular endothelial growth factor (EST)</td>
<td>4.33</td>
<td>5.39</td>
</tr>
<tr>
<td></td>
<td>GCCAGGTGTG</td>
<td>HOG3; hypothetical protein DKFZp434K1210</td>
<td>12.89</td>
<td>14.39</td>
</tr>
<tr>
<td></td>
<td>TGACTTAAAT</td>
<td>HIG2; hypoxia-inducible protein 2</td>
<td>9.16</td>
<td>18.68</td>
</tr>
<tr>
<td>a*</td>
<td>XGATGGTGAT</td>
<td>ADK3; adenylate kinase 3</td>
<td>2.11</td>
<td>7.17</td>
</tr>
<tr>
<td></td>
<td>CTTGAGTAC</td>
<td>ALDOC; aldolase C, fructose-bisphosphate</td>
<td>0</td>
<td>5.51</td>
</tr>
<tr>
<td></td>
<td>AAAAGATACT</td>
<td>P53Rb; Cbp/p300-interacting transactivator</td>
<td>0</td>
<td>4.85</td>
</tr>
<tr>
<td></td>
<td>GTGGTGCGAC</td>
<td>RA33; retinoic acid induced 3</td>
<td>3.90</td>
<td>17.70</td>
</tr>
<tr>
<td></td>
<td>TGTCATCACA</td>
<td>LOXL2; lysyl oxidase-like 2</td>
<td>10.82</td>
<td>9.99</td>
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<tr>
<td></td>
<td>ACATTCTAC</td>
<td>hypothetical protein FLJ23306 (EST)</td>
<td>2.11</td>
<td>12.56</td>
</tr>
<tr>
<td>d</td>
<td>CCAAAATGAA</td>
<td>SDC2; syndecan 2 (EST)</td>
<td>0</td>
<td>7.40</td>
</tr>
<tr>
<td></td>
<td>GTTGTTAATAA</td>
<td>ILKAP; integrin-linked kinase-associated serine/threonine phosphatase 2C</td>
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<td>6.11</td>
</tr>
<tr>
<td></td>
<td>TITTTGACCAA</td>
<td>NRP1; neuropilin 1; VEGF165 receptor</td>
<td>0</td>
<td>4.11</td>
</tr>
<tr>
<td></td>
<td>TACCCCATGGC</td>
<td>PLAUR; plasminogen activator, urokinase receptor</td>
<td>0</td>
<td>4.11</td>
</tr>
<tr>
<td></td>
<td>TATAAATGGT</td>
<td>ITGB3BP; integrin β3 binding protein (β3-endonexin)</td>
<td>0</td>
<td>4.11</td>
</tr>
<tr>
<td></td>
<td>TAATTTGAA</td>
<td>PHLA-1; EGL-9 (Caenorhabditis elegans) homologue 1</td>
<td>2.11</td>
<td>4.11</td>
</tr>
<tr>
<td>e</td>
<td>MTT349</td>
<td>TGFBI; transforming growth factor, β-induced, M, 68,000</td>
<td>24.74</td>
<td>15.98</td>
</tr>
<tr>
<td></td>
<td>TGCATCTGT</td>
<td>CCNB1; cyclin B1</td>
<td>9.64</td>
<td>7.38</td>
</tr>
<tr>
<td>f</td>
<td>GGAAGTGTG</td>
<td>TGFBI; transforming growth factor, β-induced, M, 68,000 (EST)</td>
<td>6.56</td>
<td>5.44</td>
</tr>
<tr>
<td></td>
<td>CGTGTATGT</td>
<td>ZFP36L1; zinc finger protein 36</td>
<td>5.44</td>
<td>7.61</td>
</tr>
<tr>
<td></td>
<td>TCCCATC</td>
<td>p53-induced protein PIGPC1; THW tumor suppressor</td>
<td>7.61</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>CCAGGTTCACA</td>
<td>ENO2; enolase 2 (γ, neuronal)</td>
<td>5.44</td>
<td>12.28</td>
</tr>
<tr>
<td>h</td>
<td>GAGCTTGGAG</td>
<td>ITGAE; integrin αE</td>
<td>4.74</td>
<td>5.30</td>
</tr>
<tr>
<td></td>
<td>CTTTGACG</td>
<td>EDN2; endothelin 2</td>
<td>4.74</td>
<td>5.44</td>
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<tr>
<td></td>
<td>AATAAAGACA</td>
<td>SERP1; stress-associated endoplasmic reticulum protein 1</td>
<td>7.58</td>
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<tr>
<td></td>
<td>AAACACCATG</td>
<td>PGMI; phosphoglucomutase 1</td>
<td>8.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACACAGCTGCA</td>
<td>CDC25B; cell division cycle 25B</td>
<td>6.63</td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>TCAGTGTTCG</td>
<td>HAX1; HS1 binding protein</td>
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<tr>
<td></td>
<td>CAGAATGTGA</td>
<td>hypothetical protein AF140225</td>
<td>4.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCCAGCTAT</td>
<td>ATP6V1; H+ transporting or FH1; FOXJ2 forkhead factor or CENPH; kinetochore protein CENP-H</td>
<td>6.63</td>
<td>6.56</td>
</tr>
<tr>
<td></td>
<td>TAGTAAGTCG</td>
<td>Homo sapiens mRNA; cDNA DKKZfp5860524</td>
<td>4.74</td>
<td>5.44</td>
</tr>
</tbody>
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Note: Complete SAGE data for the comparisons discussed in the text and shown in the figures can be found in the Supplemental Materials.

*UniGene Cluster ID.
Tumor hypoxia is a central issue in tumor physiology and cancer treatment because it is associated with adverse outcomes. Identifying genes specifically induced under hypoxic conditions may be functionally important in a variety of critical cellular pathways, including cell cycle arrest and regulation of angiogenesis. Thus, we compared the genes that are hypoxia inducible in the 786-0 VHL+ cells with the hypoxia-inducible genes in RPTECs to identify genes that are uniquely hypoxia regulated in a renal epithelial cell that has undergone malignant transformation.

Fig. 3 illustrates the genes that are induced at least 4-fold by hypoxia in the 786-0 VHL+ cells and in RPTECs. One hundred sixty genes are up-regulated by hypoxia in 786-0 VHL+ cells, whereas 414 genes are induced by hypoxia in the RPTECs (Supplemental Materials). It is noteworthy that only 7 genes are hypoxia inducible at least 4-fold in both RPTECs and in the 786-0 VHL+ cells. The vast majority of genes (153 of 160) that are hypoxia inducible in the 786-0 VHL+ cells are not induced at least 4-fold in RPTECs grown under hypoxia. As discussed above, VEGF is induced more than 5-fold in RPTECs grown under hypoxia, but because basal expression of VEGF is elevated in 786-0 VHL+ cells, the hypoxic induction is not as dramatic in these cells. The observation that greater than 95% of the hypoxia-inducible genes differ between the malignant cells and the normal renal cells suggests the establishment of an alternative hypoxia-sensitive pathway(s) in these tumor cells. The TGFβ-induced gene (TGFBI; Table 2f) is among the 153 hypoxia-inducible genes in 786-0 VHL+ cells but not in the RPTECs. TGFBI was also induced in the 786-0 cells regardless of VHL status (Table 2e), although the SAGE tags identified under these different conditions were distinct. Both tags are located in the 3’ UTR of the gene and therefore correspond to the same protein, although the alternative mRNAs may be regulated differently. TGFBI was previously reported to be elevated in adenomas and colorectal cancer (56, 57). TGFBI is overexpressed in the 786-0 cells with respect to RPTECs. Because two independent SAGE libraries were made from the RPTECs under each growth condition, the tag values were averaged before comparing. The gray intersection of the two circles denotes the genes that are up-regulated in both comparisons. The number of genes induced in each set is indicated. Letters in each section refer to representative genes from each set which can be found in the corresponding section of Table 2 and in the Supplemental Materials.
known to bind to collagens, and is believed to play an important role in cell-collagen interactions (58). Overexpression of this gene may therefore be a critical component in the tissue remodeling that accompanies tumorigenesis.

Recently, a set of hypoxia-overexpressed genes (HOGs) was identified in human glioblastoma cells using SAGE (43), and six of the HOGs were found to be controlled by HIF-1α (43). Interestingly, none of the six HOGs was induced in our 786-0 VHL + cells grown under hypoxia. As discussed above, the 786-0 cells lack HIF-1α expression so the failure to see HOG induction here may be explained by exclusive HIF-1α regulation of the HOGs. Alternatively, HOG expression may be cell type specific, although the induction of HOG3 in the absence of VHL (Fig. 2c and Table 2c) argues against this possibility for at least HOG. It is of interest to determine if the 153 genes hypoxia inducible in the 786-0 cells only when VHL is expressed are hypoxia inducible in other tumor types as well.

The seven genes that are hypoxia inducible in both RPTECs and in the 786-0 VHL + cells may be regulated by HIF-α proteins. Because HIF-2α is constitutively expressed in the 786-0 VHL + cells, regardless of oxygen pressure, we would expect to see higher expression of these seven genes in the 786-0 VHL + Nor. cells with respect to RPTECs grown under normoxia. Interestingly, this is only true for two of the seven genes, PAI-1 and a novel gene (no match for the SAGE tag in the public databases). This suggests that the other five genes are controlled through proteins other than HIF, and again separates the VHL and HIF-α gene-induction pathways.

Four hundred fourteen genes were induced at least 4-fold in the RPTECs grown under hypoxic conditions (Fig. 3). As expected, this group includes genes previously identified as hypoxic genes, such as GLUT1, IGFBP3, and VEGF (Table 2c, e, and Supplemental Materials) (5). HIF-1α mRNA was also detected at induced levels in the hypoxia-treated RPTECs (data not shown), which confirms that HIF-1α can be regulated at a transcriptional level (7, 8). In addition to the genes known to be hypoxia inducible, many of the detected genes are not well characterized and/or were not previously known to be regulated by hypoxia. Examples include retinoic acid induced gene 3 (RAI3) which encodes a putative G protein-coupled receptor (59), and lysyl oxidase-like gene 2 (LOXL2) which encodes an extracellular protein that may be involved in cell adhesion and senescence (60–62).

VHL-Independent Gene Expression in 786-0 Cells Under Hypoxic Conditions

VHL is a known regulator of the hypoxia pathway through HIF. A defect in VHL will result in the constitutive activation of the HIF-α pathway and lead to up-regulation of hypoxia-inducible genes, even under normoxic conditions. Interestingly, there are still 91 genes that are inducible by hypoxia in 786-0 VHL + cells (Fig. 4 and Supplemental Materials). Fig. 4 illustrates that 87 genes are hypoxia inducible in 786-0 cells only when VHL is defective, whereas 156 are only induced by hypoxia in the presence of VHL (Supplemental Materials). This suggests that these tumor cells have both VHL-dependent and VHL-independent mechanisms to respond to hypoxic stress. Among the 87 genes which are hypoxia inducible in 786-0 VHL + cells are genes known to be involved in cell-cell interactions and vascular remodeling (Table 2h and Supplemental Materials), including integrin αE (ITGAE) (63) and endothelin 2 (EDN2) (64, 65). Interestingly, EDN2 was previously identified as a hypoxia-inducible gene in hypopharyngeal tumor cells (66). As expected, this list also includes genes involved in the stress response (stress-associated endoplasmic reticulum protein 1; SERP1) (67), cellular metabolism (phosphoglucomutase 1; PGM1) (68), and cell growth control (cell division cycle 25B; CDC25B) (69). In addition, many of the genes encode proteins of unknown function that may prove to be important regulators of hypoxia-responsive pathways on further characterization.

Interestingly, four genes are induced by hypoxia in 786-0 cells regardless of VHL expression (Table 2b). These four genes are not among the 414 genes that are hypoxia inducible in normal kidney cells (RPTECs). The specificity of induction in renal cancer cells, regardless of VHL status, suggests that these four genes may be useful biomarkers for RCC and/or effective therapeutic targets. HAX-1, one of these four genes, encodes a protein known to interact with various proteins including the polycystic kidney disease protein (PKD2) (70). It has been proposed that HAX-1 connects PKD2 to the actin cytoskeleton. Our observation that HAX-1 expression increases in a hypoxic tumor environment suggests that this protein may contribute to the cell-matrix contact changes associated with tumor progression. Functional characterization of the genes that correspond to the remaining three tags in this group may be valuable in understanding the molecular changes associated with tumor hypoxia.

Real-Time Quantitative PCR of Genes Differentially Expressed in 786-0 Cells

To confirm the expression results observed by SAGE, real-time quantitative PCR was used to examine the expression of three of the genes that were differentially expressed in the cancer cells. Gene expression was analyzed in a set of independently grown 786-0 cells that had been stably transfected with a plasmid expressing VHL (WT8) or an empty vector. This confirmed the presence of VHL-expressing cells and non-expressing controls.

FIGURE 4. Hypoxia-responsive genes in 786-0 cells lacking VHL. The black circle on the left denotes the genes that are up-regulated >4-fold in 786-0 VHL - Hyp. cells compared to 786-0 VHL + Nor. cells; the white circle on the right indicates the genes that are up-regulated >4-fold in 786-0 VHL + Hyp. compared to 786-0 VHL - Nor. cells. The gray intersection of the two circles denotes the genes that are up-regulated in both comparisons. The number of genes induced in each set is indicated. Letters in each section refer to representative genes from each set which can be found in the corresponding section of Table 2 and in the Supplemental Materials.
vector (pRC3) (71), and compared to expression in normal RPTECs grown under normoxia. Fig. 5 shows that the overall expression patterns for the two technologies were similar. The ratios of gene expression levels detected by Real-time reverse transcription (RT)-PCR were within 2.5- to 3-fold of those detected by SAGE, for all but GLUT1 in the VHL- cells. Specifically, CCNB1 was highly expressed in both the WT8 and pRC3 samples. TGF-β1 was induced in both WT8 and pRC3, although it was most highly expressed in the VHL-deficient pRC3 cells, consistent with the SAGE data that showed dramatic overexpression in the 786-0 cells lacking VHL. GLUT1 showed robust expression in the absence of VHL, by both technologies, although the expression was 5-fold higher in the pRC3 VHL- RT-PCR sample than in the 786-0 VHL- SAGE sample. This may be due to the sensitivity of the technique, or perhaps to the method of normalization (see “Materials and Methods”). It is also possible that pRC3 expresses more GLUT1 than the line used for SAGE analysis.

In summary, the gene expression profile analyses of the RCC line 786-0 and the normal kidney cells, grown under different environmental conditions, demonstrated that 786-0 cells have evolved genetic alterations that affect cell growth and differentiation, as well as an altered response to hypoxia. Recently, GEM microarrays were used to study VHL-dependent gene expression in a different RCC line grown under both normoxia and hypoxia (72). These studies were limited to the 9182 genes spotted on the GEM array, and the expression profiles of normal renal tubule cells were not considered. Nevertheless, the findings were consistent with our results that not all VHL target genes are regulated by oxygen.

The data presented here of VHL and oxygen-dependent gene regulation in normal and malignant renal cells confirm and extend results by others using low-throughput expression analyses. Our data provide evidence for alternative hypoxia-response pathways that are either VHL-dependent or -independent. The genes involved in such pathways have potential importance in the physiological and pathological regulation of tumor growth, metabolism, and angiogenesis. Because renal cell tumors are highly vascularized, it is believed that the tumor cells may induce angiogenic factors such as VEGF and TGF-β, as our data clearly support. Some of the genes identified here as showing overexpression in the cancer cells, particularly those encoding secreted or membrane-bound proteins, could be potential biomarkers for tumors or targets for rational therapeutics. Moreover, our analysis identified genes that are inducible by hypoxia specifically in the cancer cells that have lost VHL expression, such as SERP1 and HAX1, which may prove to be valuable targets specific for mutant tumor cells. We, and others, have shown previously that SAGE is a highly reproducible technology that is representative of similar samples using other quantitative expression technologies (43, 56, 73–75). Thus, examination of gene expression in multiple tumors and cell systems, accompanied by functional characterization of some of the genes described here, could lead to important therapeutic targets that may be VHL specific. That is, some renal and other tumors that have lost VHL expression may be resistant to certain therapies. Understanding the molecular changes that accompany VHL loss may lead to effective new therapeutics.

Materials and Methods

Cells and Cell Culture

The human sporadic RCC cell line 786-0 contains a single VHL allele with a frameshift mutation at codon 104 resulting in no VHL protein (76). In addition, 786-0 cells lack HIF-1α expression but constitutively express HIF-2α (25). 786-0 cells were infected with either an empty vector (pBABE-Puro-HA) (77) or the vector carrying a wild-type VHL gene (pBABE-Puro-HA-VHL). This vector was constructed by inserting the VHL cDNA carried on the BamHI-EcoRI fragment from pRc/CMV-HA-VHL (71). The cell lines were maintained in DMEM (Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum. This pair of cells (786-0 VHL- and 786-0 VHL- ) was grown under both normoxic (5% CO₂/21% O₂) (samples V1 and V2, respectively) and hypoxic (i.e., 5% CO₂/
1% O$_2$/94% N$_2$) (samples V3 and V4, respectively) conditions for 48 h as described previously (22). Primary cultures of human RPTECs (Clonetics, San Diego, CA) were maintained in the supplier’s defined Renal Epithelial Cell Growth Medium which includes 10 ng/ml hEGF, 5 ng/ml insulin, 0.5 ng/ml hydrocortisone, 0.5% fetal bovine serum, 0.5 ng/ml epinephrine, 0.675 ng/ml triiodothyronine, 10 ng/ml transferrin, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B. Two independent samples of normal renal proximal tubule cells (RPTECs) were grown under both normoxia (samples V5 and V6) and hypoxia (samples V7 and V8) for 48 h to generate gene expression profiles from nonneoplastic cells. For real-time RT-PCR analysis, RNA was extracted from an independent set of 786-0 cells, which do or do not express VHL. pRc3 cells are defective for VHL, while WT8 cells have been stably transfected with a wild-type copy of VHL, as described (71).

SAGE Libraries and SAGE Analysis

Total RNA samples were isolated by either RNAzol B (Tel-Test Inc., Friendswood, TX) or TRizol (Invitrogen Life Technologies) according to the manufacturer’s recommendation. Poly(A)$^+$ RNA was extracted using the Oligotex mRNA Mini Kit (Qiagen, Inc., Valencia, CA) and the Dynabeads mRNA DIRECT Kit (Dynal, Oslo, Norway). SAGE libraries were generated and the tags sequenced as described (30). SAGE 3.0 software [http://www.sagenet.org/sage_protocol.htm] was used to identify tag sequences and to quantify the abundance of each tag. The gene identity and UniGene cluster assignment of each SAGE tag was obtained using the tag-to-gene “reliable” map (updated April 23, 2001) from [http://www.ncbi.nlm.nih.gov/pub/SAGE/map] and the table of UniGene clusters [http://www.ncbi.nlm.nih.gov/UniGene/] (UniGene Build #140; last update 9/25/01).

In total, over 380,000 SAGE tags were sequenced and more than 61,000 unique tags were identified. To compare expression levels of SAGE tags present in distinct libraries sequenced to different depths, tag values were normalized to represent at least 4-fold using normalized data when comparing the SAGE tag abundance in two or more libraries. To perform calculations where the denominator is zero, the denominator is taken as a value of 1.

Quantitative Real-Time PCR

Real-time RT-PCR was performed using the High-Capacity cDNA Archive Kit, TAQman Universal PCR Master Mix and TAQman Ribosomal RNA Control Reagent kit (Applied Biosystems, Foster City, CA), with primer-probe sets designed to amplify CCNB1, TGFβ1, and GLUT1. PCR primers were obtained from Integrated DNA Technologies (Coralville, IA).

The real-time PCR probes were obtained from Synthegen, LLC (Houston, TX). The probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) and at the 3' end with tetramethylrhodamine (TAMRA). The CCNB1 primers were 5'-GCCCTCTACCGTTACCCTTCA-3' and 5'-GCCCAAGTTATGTTGCTGCACC-3' with probe 5'-TCCAACCTTCTCACTTTGATGGCTCTCGA-3'. The TGFβ1 primers were 5'-TTGAGCCCTCTGGGCTG-3' and 5'-TGCGGCAACCTTTGACACCA-3'. The GLUT1 primers were 5'-GATCTGGGGCTGACCCATACCGGGTCTCAA-3' and 5'-GATCTGGGGCTGACCCATACCGGGTCTCAA-3'. The GLUT1 probes were 5'-TTGCCGGCTACGAGGCTG-3' and 5'-ACATGGGCGAAGGCTGCAC-3' with probe 5'-TGGGTGTGTACGTGCGCCCTGACC-3'. Gene expression was quantitated using the comparative Ct method described in User Bulletin #2 (Applied Biosystems, ABI PRISM 7700 Sequence Detection System) with 18S rRNA as reference. Reactions were run on the ABI Prism 7700 according to the manufacturer’s recommendations.

Acknowledgments

We thank Dr. Mark A. Goldberg for critical reading of the manuscript and helpful discussions and Drs. Clarence Wang and Viacheslav Akmaev for assistance in SAGE data analysis. We also thank Xiaohong (Sherry) Cao for help in preparing the manuscript.

References


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