Chapter 2:

Fibronectin is a hypoxia-independent target of the tumor suppressor VHL

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The von Hippel-Lindau (VHL) tumor suppressor gene regulates the extracellular matrix by controlling fibronectin deposition. To identify novel VHL-target genes, we subjected mRNA from VHL deficient RCC cells (786-0-pRC) and a transfectant re-expressing wildtype VHL (786-0-VHL) to differential expression profiling. Among the differentially expressed genes, we detected that fibronectin is upregulated in the presence of VHL, while not affected by hypoxia. Thus regulation of fibronectin deposition by VHL occurs at the transcriptional level, irrespective of oxygen levels.

Introduction
Von Hippel-Lindau disease is caused by inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene, and is characterized by the formation of hypervascularized neoplasms, including virtually all sporadic and inherited renal cell carcinomas (RCCs) (1,2). VHL functions as the substrate recognition moiety of the E3 ubiquitin ligase complex, VCB-CUL2 E3 that targets the α-subunit of the hypoxia-inducible factor (HIF) for degradation by the proteasome under normoxic conditions (3). HIF is a transcription factor regulating genes encoding proteins that function to increase O₂ delivery, allow metabolic adaptation and promote cell survival (4). RCCs lacking functional VHL exhibit high levels of stabilized nuclear HIF, resulting in excessive transcription of HIF target genes, the best documented of which being the vascular endothelial growth factor (VEGF) (5,6). HIF-independent functions of VHL have been described in recent literature. VHL binds the extracellular matrix protein fibronectin; VHL-deficient cells show impaired fibronectin deposition possibly contributing to tumorigenesis and directly influencing the behavior of tumor cells (7). We and others have previously demonstrated that VHL appears to control extracellular matrix (ECM) degradation by regulating both metalloproteinases –2 and –9 and their inhibitors (8), as well as the urokinase-type plasminogen activator system (9,10). In addition, by promoting the assembly of actin and vinculin, VHL was found to affect cytoskeletal organization, focal adhesion formation and cell motility (11). VHL influences effects on proliferation, cell cycle progression, as well as alterations of the extracellular matrix interactions. Furthermore, fibronectin gene (FN1) expression has been correlated with increased tumor cell growth and invasiveness of VHL-defective RCCs (12).

To identify novel VHL-target genes, we subjected VHL-deficient RCC cell line (786-0-pRC) and transfectants re-expressing wildtype VHL cDNA (786-0-VHL) to differential expression profiling and identified a group of 16 genes that were either negatively or positively regulated by VHL. Of these genes two interesting features were noticed. The first was a group of genes positively regulated by VHL. Our results confirmed and expanded on earlier reports using differential hybridization (11, 13-16). Interestingly, we identified the gene encoding fibronectin, FN1, as a gene positively regulated by VHL, whose transcription levels are unaltered by hypoxia. These data support the hypothesis that VHL also regulates transcription in a HIF-independent manner. Secondly, our data suggest that VHL regulates fibronectin mRNA levels. This knowledge, taken together with the fact that VHL binds fibronectin, increases our understanding of how VHL regulates fibronectin deposition.

Material and Methods
Cell lines
Stable transfectants of 786-0 cells expressing vector backbone alone (786-0-RC) or
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**Table 1. VHL target genes**

<table>
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<tr>
<th>Accession number</th>
<th>Gene ID</th>
<th>Cy3/Cy5 (Log2 ratio)</th>
<th>Biological function</th>
</tr>
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<tr>
<td><strong>Upregulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA099195</td>
<td>GDIB</td>
<td>1.2</td>
<td>Intracellular vesicle transport</td>
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<td>AA136799</td>
<td>GADD45</td>
<td>0.7</td>
<td>Stress response; cell cycle arrest; cell death</td>
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<tr>
<td>AA195589</td>
<td>FN1</td>
<td>1.6</td>
<td>Cellular adhesion</td>
</tr>
<tr>
<td>H07071</td>
<td>VCAM1</td>
<td>0.8</td>
<td>Cellular adhesion</td>
</tr>
<tr>
<td>R81846</td>
<td>FRIL</td>
<td>0.8</td>
<td>Metal transport; RNA stability</td>
</tr>
<tr>
<td>W69515</td>
<td>MKK2</td>
<td>0.8</td>
<td>Stress response; nuclear export; cell growth</td>
</tr>
<tr>
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<td>GR75</td>
<td>0.7</td>
<td>Stress response; cell growth; cellular aging</td>
</tr>
<tr>
<td>AA023029</td>
<td>PPP5</td>
<td>0.7</td>
<td>Mitosis; RNA biogenesis; transcription</td>
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<td>AA151568</td>
<td>TEGT</td>
<td>0.7</td>
<td>Cell death</td>
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<tr>
<td>H02641</td>
<td>VHL</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
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<td></td>
<td></td>
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<tr>
<td>AA037034</td>
<td>COXA</td>
<td>-1.0</td>
<td>Metabolic response; electron transfer</td>
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<tr>
<td>H86642</td>
<td>CERU</td>
<td>-1.0</td>
<td>Metabolic response; copper</td>
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<tr>
<td>N31417</td>
<td>IGFBP3</td>
<td>-2.3</td>
<td>Cell growth</td>
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<td>N40420</td>
<td>CGD1</td>
<td>-0.8</td>
<td>Cell growth; cell cycle regulator</td>
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<td>N91060</td>
<td>VEGF</td>
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<td>Angiogenesis; cell growth</td>
</tr>
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<td>N31209</td>
<td>NUP153</td>
<td>-0.8</td>
<td>Nuclear-cytoplasmic transport</td>
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<td>R92231</td>
<td>TLE1</td>
<td>-0.7</td>
<td>Cell differentiation; cell fate</td>
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wild type VHL (786-0-VHL) (obtained from W. Kaelin, Dana Farber Cancer Institute, USA) were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA), L-glutamine (2 μM), penicillin (50 IU/ml), and streptomycin sulphate (50 μg/ml) (Invitrogen, Carlsbad, CA, USA).

RNA isolation

Total RNA was isolated using RNAzol B as described by the manufacturer (Campro Scientific, Veenendaal, The Netherlands) and dissolved in DEPC-treated milliQ H2O. Poly A+ RNA was extracted and purified using the oligotex mRNA Mini kit (Qiagen, Valencia, CA, USA).

Microarray analysis

1 μg of poly A+ RNA of each sample was reverse transcribed into cDNA using a reverse transcription kit (Invitrogen, Carlsbad, CA, USA) in combination with an oligo-dT15 primer (Invitrogen, Carlsbad, CA, USA) and amino allyl containing dUTPs (Sigma, St. Louis, MO, USA). cDNAs were fluorescently labeled with either Cy3 or Cy5 (Amersham Biotech, Buckinghamshire, UK). Chroma-spin columns were used to remove unincorporated Cy-dyes (Clontech, Palo Alto, CA, USA). Labeled cDNAs (100 ng per sample in hybridization buffer) were subjected to competitive hybridization on a 1.7K human microarray (obtained from the Microarray Facility of University of Toronto; http://www. uhnres.utoronto.ca/services/microarray). Hybridization buffer contained 25% formamide, 2.5X SSC, 0.2% SDS, 100 μg/ml herring sperm DNA and 200 μg/ml E. coli tRNA. A coverslip (25X25 mm) was applied to the microarray after which 30 μl of hybridization mix was applied to hybridize in a hybridization chamber (Corning, New York, USA) overnight in a 42°C water bath. After hybridization, microarrays were washed at room temperature in 1XSSC with 0.2% SDS for four minutes, 0.1XSSC with 0.2% SDS for four minutes, and 0.1XSSC for four
minutes, successively. Subsequently, microarrays were dried by centrifugation (5 min. at 124g) and scanned using a confocal laser scanner (Packard Scanarray 4000XL from Perkin Elmer, Boston, MA, USA). Data were analyzed using Imagenes software (Biodiscovery Inc., El Segundo, CA, USA).

RT-PCR analysis
One and a half µg of total RNA was reverse transcribed to obtain cDNA for reverse transcription polymerase chain reaction (RT-PCR) using SuperScript II Rnase H- Reverse Transcriptase (according to manufacturer; Invitrogen, Carlsbad, CA, USA). Gene-specific cDNA fragments were amplified by 25 cycles of PCR consisting of 1 min. at 95°C, 1 min. at 55°C and 2 min. at 72°C. PCR fragments were analyzed by DNA gel electrophoresis. The following primers were used for RT-PCR: VEGF forward primer: 5’-cgaaacagtaaccttctctgc, reverse primer: 5’-ccaccttc-ggtgattctgc (127 bp product), Glucose Transporter-1 (GLUT-1) forward primer: 5’-gatactcagcgatagacc, reverse primer: 5’-gtacacaccgatgatgaagc (114 bp product), Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) forward primer: 5’-atccctctcccc-gagttc, reverse primer: 5’-ccctctctctgattgtgg (136 bp product), FN1 forward primer: 5’-caacctgctgctgatgatgacc, reverse primer: 5’-ggatggtgcatcaatgca (138 bp product), 18S forward primer: 5’-agttggtggagcatttgtc, reverse primer: 5’-attgctcaatctcggggtgg (142 bp product)

Western analysis
Approximately 1x10^6 cells were lysed in 400 µl lysis buffer (20 mM Tris-HCl pH 8.0; 1% Triton-X-100; 140 mM NaCl; 10% glycerol; 0.005% Bromo-Phenol-Blue; 8% β-mercaptoethanol) containing the ‘complete’ cocktail of protease inhibitors (Roche, Basel, Switzerland). Cell remnants were removed by centrifugation (5 min. at 16,100 g at 4°C) and cleared lysates were stored at –20°C. Approximately 20 µg of protein per lane were analyzed by SDS-PAGE followed by western blotting using a polyclonal antibody specific for IGFBP3 (1:200; kind gift from Dr. J. van Doorn, WKZ Utrecht, the Netherlands), and monoclonal antibodies for fibronectin (1:500; M010, FN30-8, TaKaRa Biomedicals, Shiga, Japan) and for VHL (1:500; IG32, BD-Pharmingen, San Diego, USA), respectively. Antibodies were diluted in PBS containing 5% non-fat dried milk and 0.1% Tween-20. Swine anti-rabbit or rabbit anti-mouse peroxidase was used as a third antibody and enhanced chemiluminescence (Perkin Elmer Life sciences, Boston, MA, USA) was used for detection.

IGFBP3 and fibronectin detection assays
Secretion levels of IGFBP3 in conditioned medium were determined as described previously (17). Secretion levels of fibronectin were determined by sandwich enzyme-linked immuno-sorbent assay (ELISA) (18) using a polyclonal antibody specific for fibronectin and a monoclonal antibody against fibronectin (respectively M010 and FN30-8, TaKaRa Biomedicals, Shiga, Japan). Rabbit anti-mouse peroxidase was used as a third antibody for detection and quantification.

![Figure 1: Fibronectin (FN1) gene expression is positively regulated by VHL.](image)

Gene expression levels of CERU, IGFBP3, FN1, VEGF, VHL, and GAPDH were determined by RT-PCR analyses. All panels show specific products for the indicated genes. Left lanes represent VHL-mutant cell line 786-0-RC (RC); right lanes represent the same cell line stably overexpressing VHL, 786-0-VHL (VHL). The housekeeping gene GAPDH functions as a control for loading. Previously reported in the literature, VEGF, CERU, and IGFBP3 are all negatively regulated by VHL. FN1 expression, however, is regulated by VHL in the opposite manner.
Real-Time PCR
Real-time PCR was performed using the iCy-cler iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). An optical 96 well reaction plate (Bioplastics, North Ridgeville, OH, USA) and iCycler iQ sealing tape (Bio-Rad, Hercules, CA, USA) was used for PCR amplification. The final reaction mixture of 15 μl consisted of diluted cDNA, 1 X PCR buffer (Amersham Biotech, Buckinghamshire, UK), 0.05 mM dNTPs (Amersham Biotech, Buckinghamshire, UK), 100 μM forward primer, 100 μM reverse primer and 0.375 units Taq polymerase (Amersham Biotech, Buckinghamshire, UK). For detection of the PCR product in real-time, the SYBR Green I fluorophore (Roche, Basel, Switzerland) was used in a final 10,000-fold dilution. The cDNA was further diluted 6-fold or 2,000-fold prior to PCR amplification of GLUT-1, IGFBP-3 and FN1 or 18S, respectively. Gene-specific cDNA fragments, using the same primers as mentioned before, were amplified by 40 cycles of PCR consisting of 30 sec. at 94°C, 30 sec. at 58°C and 30 sec. at 72°C. For each assay, specific targets were amplified in triplicate with their respective standard curve. The equation of the standard curve was used to determine the starting quantity of the samples with the iCycler analysis software. All experimental samples were normalized to 18S quantities.

Results and discussion
Identification of VHL-regulated genes by microarray analysis
In an attempt to identify novel VHL-target genes involved in tumorigenesis, we subjected mRNA from a VHL effective renal carcinoma cell line (786-0-pRC) and transfectants re-expressing a wildtype VHL allele (786-0-VHL) to differential expression profiling. A glass-chip based cDNA array representing about 1700 distinct human genes was spotted in duplicate (obtained from the Microarray Facility of the University of Toronto: http://www.uhnres.utoronto.ca/services/microarray). The cDNA made from purified mRNA from 786-0-VHL cells was labeled with Cy3 and mixed with Cy5 labeled cDNA transcribed from 786-0-pRC purified mRNA. After normalization, the Cy3/Cy5 ratio of each gene individually was determined from three independent experiments and overlapping sets of genes were selected. In this way 9 genes, with an average log2 ratio above 0.7, were considered positively regulated by VHL, whereas 7 genes, that had an average log2 ratio of -0.7 or below, were considered negatively regulated (Table I). The expression of the GAPDH gene was not regulated by VHL (not shown). In addition, the average Cy3/Cy5 log2 ratio of the VHL gene (as expected above 0.7) was used as an internal control as a measure of the reliability of the observed changes.
RT-PCR was performed as an independent test to confirm the differential expression of some of the genes identified by DNA microarray. As shown in Figure 1, the expression of FN1 and VHL was shown to be higher in 786-0-VHL cells than in 786-0-pRC cells. The opposite was true for CERU, IGFBP3 and VEGF. The expression of GAPDH was taken as an internal control. In conclusion, similar expression patterns of VHL-target genes were found when measured by these two independent methods.

Using the Gene Ontology Consortium classification of biological processes, these target genes could be classified according to their biological function (Table I). In this way, VHL was shown to be a negative regulator of genes involved in cell proliferation such as IGFBP3 and cyclinD1. Moreover, VHL appeared to positively regulate GADD45, a gene involved in cell cycle arrest, as well as FN1 and VCAM1 (encoding the vascular cell adhesion molecule), both of which have roles in cell adhesion. Literature searches revealed that eight of the VHL-target genes identified in our study (VEGF, CERU, COXA, IGFBP3, cyclin D1, GADD45, VCAM1, and FN1) have been previously implicated in the development of RCC (19-23). We further focused on fibronectin as this protein is reported to interact with VHL and is believed to be regulated in a post-transcriptional manner by VHL. However, the transcriptional regulation of FN1 by VHL has not yet been reported.

Role of VHL in regulation of fibronectin

Levels of fibronectin mRNA increased 3.7-fold in the presence of VHL as measured by repeated real-time PCR analyses (Fig. 2), IGFBP3 was used to study the behavior of genes down-regulated by VHL. Protein expression levels were determined in whole cell lysates of 786-0-VHL versus 786-0-pRC cells by western analysis. Figure 3A correlates higher fibronectin levels with VHL in 786-0-VHL cells. In contrast, increased levels of IGFBP3 were detected in 786-0-pRC cells as compared to 786-0-VHL cells. Conditioned medium of 786-0-pRC and 786-0-VHL cells recapitulated the differential secretion of fibronectin and IGFBP3 (Fig. 3B). 786-0-VHL cells secreted 15-fold more fibronectin than 786-0-pRC cells. Accordingly, IGFBP3 secretion was relatively high (20-fold) in cultured medium from 786-0-pRC cells when compared to that of 786-0-VHL cells. Together, these data imply that aberrant production and secretion of fibronectin and IGFBP3 is directly related to VHL expression in RCC cell lines. It is worthy of noting that fibronectin mRNA levels are remarkably less sensitive to re-expression of VHL than detectable secreted protein levels (approximately 4-fold, discussion below). Fibronectinisanextracellularglycoproteinthat binds to and signals through heterodimeric cell surface receptors known as integrins (24). Loss of fibronectin matrix assembly has been recognized as a feature of cellular transformation (25). Fibronectin has the ability to decrease the metastatic behavior of malignant cells by increasing the interaction between tumor cells and their micro-

![Image](image_url)

**Figure 3: Secretion of IGFBP3 and fibronectin are inversely regulated by VHL.**

(A) Intracellular protein levels of IGFBP3, fibronectin and VHL determined by western blot analyses. Left and right lanes contain protein lysates derived from 786-0-RC cell line (RC) and 786-0-VHL (VHL) cell lines, respectively. Upper panel: fibronectin levels increase in the presence of VHL. Middle panel: less IGFBP3 is detected in the presence of VHL. Lower panel: VHL levels. (B) Levels of secreted IGFBP3 and fibronectin (FN) depicted left and right, respectively, in conditioned medium from 786-0-RC (RC; black bars) and 786-0-VHL (VHL; gray bars) cell lines. Detection of secreted protein by ELISA was performed in at least three independent experiments. Fibronectin is an extracellular glycoprotein that binds to and signals through heterodimeric cell surface receptors known as integrins (24). Loss of fibronectin matrix assembly has been recognized as a feature of cellular transformation (25). Fibronectin has the ability to decrease the metastatic behavior of malignant cells by increasing the interaction between tumor cells and their micro-environment via the integrin receptor family (26). Furthermore, fibronectin can revert some aspects of the malignant phenotype of tumor cells, including proliferation and migration (27). Previous reports have described a physical interaction between VHL and intracellular fibronectin in vivo; this interaction affected the ability of cells to assemble an extracellular fibronectin matrix (7). Wereporttheree--usingthesamecellsystems used to determine VHL-fibronectin binding—that fibronectin mRNA levels are regulated by VHL. The difference in fibronectin secretion in our experiments could not be directly accounted for by mRNA
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increase (15-fold vs. 3.7-fold). Our data thus does not exclude the notion that VHL binding to fibronectin regulates the amount of fibronectin deposition in a post-transcriptional manner (7). However, we propose an additional way that VHL regulates fibronectin: at the transcriptional level.

Effect of hypoxia on the VHL-target genes FN1 and IGFBP3

Because many of known VHL target genes (including VEGF) are regulated by the HIF transcription factors, we next determined the effect of hypoxia on the expression of FN1 and IGFBP3. Therefore, we cultured 786-0-VHL or 786-0-pRC cells under normoxic (20% O₂) or hypoxic (1% O₂) conditions for 18 hours. Total RNA was extracted from these cells and subjected to quantitative real-time PCR to follow the expression of GLUT-1 (which is a known hypoxia-inducible gene) (4), IGFBP3 and FN1. The expression of 18S (which is not affected by hypoxia) was taken as an internal control and used to normalize expression of GLUT-1, IGFBP3 and FN1.

As shown in Figure 4A, hypoxia significantly induces the expression of GLUT-1 by 6.7-fold in 786-0-VHL cells after 18 hours of hypoxic treatment (p = 0.025; Fig. 4A). In 786-0-pRC cells the expression of GLUT-1 mRNA was not significantly changed. Similar results were also shown for VEGF expression under these conditions (data not shown). As expected, hypoxia also induced IGFBP3 mRNA expression (approximately 5-fold; p = 0.029) in 786-0-VHL cells, whereas in the absence of functional VHL (i.e. 786-0-pRC), IGFBP3 expression did not significantly differ (Fig. 4B). This implicated a role of HIF in the regulation of the IGFBP3 gene by VHL. Although the expression of both GLUT-1 and IGFBP3 in 786-0-pRC was not significantly altered by hypoxia, a trend towards induction of transcription was present (Fig. 4A and B). In contrast, FN1 expression was not affected by hypoxia in 786-0-VHL cells as well as in 786-0-pRC cells, and suggested a HIF-independent mechanism of regulation by VHL. To chemically mimic the effect of hypoxia we administered cobalt chloride to these cell lines, thereby artificially stabilizing HIF and stimulating HIF-mediated transcription (28). This method was able to reproduce hypoxia

Figure 4: Fibronectin is a hypoxia-independent target of VHL transcriptional regulation.

Cells were either cultured under normoxia (20% O₂; light bars) or hypoxia (1% O₂; dark bars) for 20 hours. The left two columns represent cDNA derived from the 786-0-VHL cell line (VHL) and the right two columns represent cDNA derived from the 786-0-RC (RC) cell line. These results represent the mean of six independent experiments in triplicate ± standard error of the mean. P-values were determined by paired, two-tailed t-test. (n.s., not significant). (A) GLUT-1 mRNA levels were measured by real-time PCR as a positive control for hypoxia-induction. As expected, GLUT-1 expression increases in response to hypoxia in the 786-0-VHL cell line. (B) Like GLUT-1, IGFBP3 mRNA levels are significantly regulated by hypoxia. (C) FN1 mRNA expression does not respond to hypoxia in either the 786-0-RC or in the 786-0-VHL cell lines.
effects on GLUT-1, VEGF, IGFBP3 and FN1 expression (data not shown).

Our data demonstrate positive regulation of FN1 by VHL thereby suggesting the existence of a HIF-independent pathway of transcriptional control by VHL. Our findings support the hypothesis that VHL is positioned in both HIF-independent and HIF-dependent pathways that are involved in extracellular matrix deposition (14, 29). HIF-independent VHL transcriptional targets provide a possible link with the tumor suppressor mechanism of VHL. It is tempting to speculate that the induction of genes in the presence of VHL and the downregulation of these genes in the absence of VHL both play a role in the development of the malignant properties of RCCs.

Acknowledgments

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Reference List: