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

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Abstract 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 it is not affected by hypoxia. Thus regulation of fibronectin deposition by VHL occurs at the transcriptional level, irrespective of oxygen levels.

Key words: VHL; Fibronectin; Microarray; Renal cell carcinoma; Hypoxia

1. 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, which 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 O_2 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 (ECM) 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 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 ECM 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 a 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 earlier reports using differential hybridization [11,13–16]. Interestingly, we identified the gene encoding fibronectin, *FN1*, as a gene positively regulated by VHL, though its 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.

2. Materials and methods

2.1. Cell lines

Stable transfectants of 786-0 cells expressing vector backbone alone (786-0-RC) or wildtype VHL (786-0-VHL) (obtained from W. Kaelin, Dana Farber Cancer Institute, USA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen), L-glutamine (2 μM), penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml) (Invitrogen).

2.2. RNA isolation

Total RNA was isolated using RNAzol B as described by the man-

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ufacturer (Campro Scientific, Veenendaal, The Netherlands) and dissolved in DepC-treated Milli-Q H_2O. Poly(A)$^+$ RNA was extracted and purified using the Oligotex mRNA Mini kit (Qiagen, Valencia, CA, USA).

2.3. Microarray analysis

Poly(A)$^+$ RNA (1 μg) of each sample was reverse transcribed into cDNA using a reverse transcription kit (Invitrogen) in combination with an oligo-dT15 primer (Invitrogen) 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 1.7 K human microarray (obtained from the Microarray Facility of University of Toronto; http://www.uhnres.utoronto.ca/services/microarray). Hybridization buffer contained 25% formamide, 2.5×sodium saline citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 100 μg/ml herring sperm DNA and 200 μg/ml *Escherichia coli* tRNA. A coverslip (25×25 mm) was applied to the microarray after which 30 μl of hybridization mix was applied to hybridize in a hybridization chamber (Corning, New York, NY, USA) overnight in a 42°C water bath. After hybridization, microarrays were washed at room temperature in 1×SSC with 0.2% SDS for 4 min, 0.1×SSC with 0.2% SDS for 4 min, and 0.1×SSC for 4 min, successively. Subsequently, microarrays were dried by centrifugation (5 min at 124×*g*) and scanned using a confocal laser scanner (Packard Scanarray 4000XL from Perkin Elmer, Boston, MA, USA). Data were analyzed using Imagene software (Biodiscovery, El Segundo, CA, USA).

2.4. RT-PCR analysis

Total RNA (1.5 μg) was reverse transcribed to obtain cDNA for reverse transcription polymerase chain reaction (RT-PCR) using SuperScript II RNase H$^-$ Reverse Transcriptase (according to the manufacturer, Invitrogen). 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′-cgaaaccatgaactttctctgc, reverse primer: 5′-ccactcgtgatgattctgc, glucose transporter-1 (*GLUT-1*) forward primer: 5′-ttcaatgctgatgatgaacc, reverse primer: 5′-gtacacaccgatgatgaagc, insulin-like growth factor binding protein-3 (*IGFBP3*) forward primer: 5′-gaacttctcctccgagtcc, reverse primer: 5′-ccttcttgtcacagttggga, *FN1* forward primer: 5′-caagccagatgtcagaagc, reverse primer: 5′-ggatggtgcatcaatggca, *18S* forward primer: 5′-agttggtggagcgatttgtc, reverse primer: 5′-tattgctcaatctcgggtgg.

2.5. Western analysis

Approximately 1×10^6 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% bromophenol 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 was analyzed by SDS–polyacrylamide gel electrophoresis 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, CA, USA), respectively. Antibodies were diluted in phosphate-buffered saline containing 5% non-fat dried milk and 0.1% Tween 20. Swine anti-rabbit or rabbit anti-mouse horseradish peroxidase (1:10 000; Pierce, Rockford, IL, USA) was used as a secondary antibody and enhanced chemiluminescence (Perkin Elmer) was used for detection.

2.6. 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 immunosorbent assay (ELISA) [18] using a polyclonal antibody specific for fibronectin and a monoclonal antibody against fibronectin (respectively M010 and FN30-8, TaKaRa Biomedicals). Rabbit anti-mouse peroxidase was used as a third antibody for detection and quantification.

2.7. Real-time PCR

Real-time PCR was performed using the iCycler 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) were used for PCR amplification. The final reaction mixture of 15 μl consisted of diluted cDNA, 1×PCR buffer (Amersham Biotech), 0.05 mM dNTPs (Amersham Biotech), 100 μM forward primer, 100 μM reverse primer and 0.375 U Taq polymerase (Amersham Biotech). For detection of the PCR product in real time, the SYBR green I fluorophore (Roche) was used in a final 10 000-fold dilution. The cDNA was further diluted six-fold or 2000-fold prior to PCR amplification of *GLUT-1*, *IGFBP3* 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 s at 94°C, 30 s at 58°C and 30 s at 72°C. For each assay, specific targets were amplified in triplicate with their respective standard curves. 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.

Table 1
VHL target genes

Accession number	Gene ID		Cy3/Cy5 log2 ratio	Biological function
Upregulated				
AA099195	GDIB	(GDP dissociation inhibitor B)	1.2	Intracellular vesicle transport
AA136799	GADD45	(Gene activated by DNA damage)	0.7	Stress response; cell cycle arrest; cell death
AA195589	FN1	(Fibronectin)	1.6	Cellular adhesion
H07071	VCAM1	(Vascular cell adhesion molecule 1)	0.8	Cellular adhesion
R81846	FRIL	(Ferritin light chain)	0.8	Metal transport; RNA stability
W69515	MKK2	(Mitogen-activated protein kinase kinase 2)	0.8	Stress response; nuclear export; cell growth
H52752	GR75	(Heat shock 70 kDa protein)	0.7	Stress response; cell growth; cellular aging
AA023029	PPP5	(Serine/threonine protein phosphatase 5)	0.7	Mitosis; RNA biogenesis, transcription
AA151568	TEGT	(Testis enhanced gene transcript)	0.7	Cell death
H02641	VHL	(Von Hippel–Lindau)	0.9	
Downregulated				
AA037034	COXA	(Cytochrome *c* oxidase	−1.0	Metabolic response; electron transfer
H86642	CERU	(Ceruloplasmin)	−1.0	Metabolic response; copper transport
N31417	IGFBP3	(Insulin-like growth factor binding protein 3)	−2.3	Cell growth
N40420	CGD1	(Cyclin D1)	−0.8	Cell growth; cell cycle regulator
N91060	VEGF	(Vascular endothelial growth factor)	−0.9	Angiogenesis; cell growth
N31209	NUP153	(Nucleoporin 153)	−0.8	Nuclear-cytoplasmic transport
R92231	TLE1	(Transducin-like enhancer protein 1)	−0.7	Cell differentiation; cell fate

3. Results and discussion

3.1. 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-defective 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 nine genes, with an average log2 ratio above 0.7, were considered positively regulated by VHL, whereas seven genes that had an average log2 ratio of −0.7 or below, were considered negatively regulated (Table 1). 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 Fig. 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.

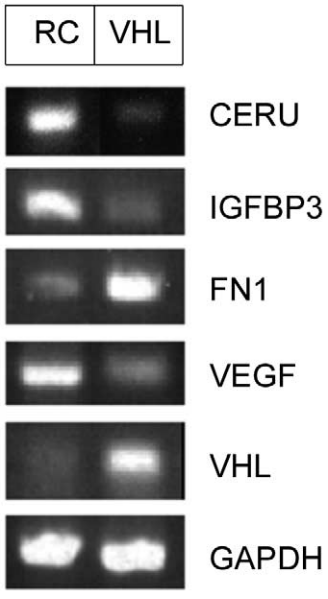

Fig. 1. *FN1* gene expression is positively regulated by VHL. Gene expression levels of *CERU*, *IGFBP3*, *FN1*, *VEGF*, *VHL*, and *GAPDH* were determined by RT-PCR analyses. All panels show specific products for the indicated genes. Left lanes represent VHL mutant cell line 786-0-RC (RC); right lanes 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.

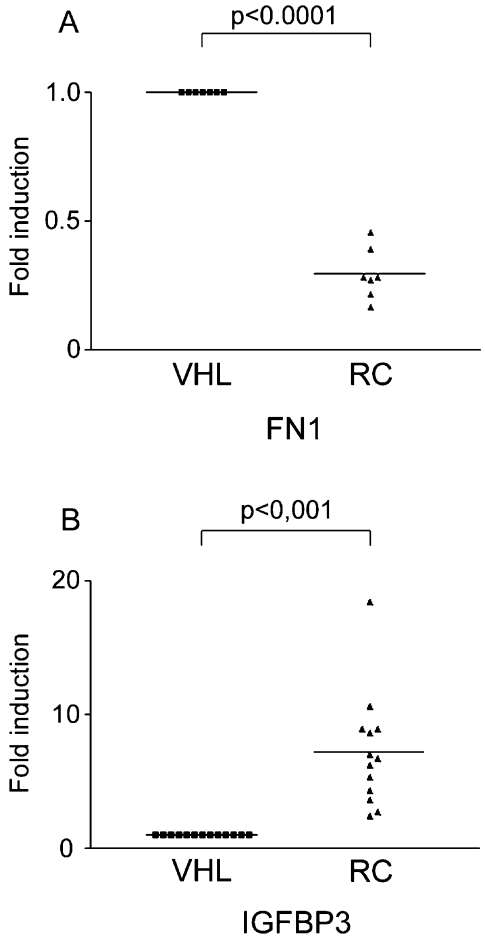

Fig. 2. Quantification of *IGFBP3* and *FN1* gene expression. A: Levels of fibronectin mRNA expression were determined by real-time PCR. The amount of mRNA measured in the 786-0-VHL cell line (VHL) was normalized to 1 and the amount of mRNA in the 786-0-RC cell line (RC) is relative to this amount. Each individual point in the plot is an independent measurement. There is a statistically significant upregulation of fibronectin in the presence of VHL. B: Quantification of IGFBP3 mRNA expression performed as described above shows a statistically significant decrease of mRNA levels in the presence of VHL.

Using the Gene Ontology Consortium classification of biological processes, these target genes could be classified according to their biological function (Table 1). In this way, VHL was shown to be a negative regulator of genes involved in cell proliferation such as *IGFBP3* and *CGD1*. 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*, *CGD1*, *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 tran-

scriptional regulation of *FN1* by VHL has not been previously reported.

3.2. 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 downregulated by VHL. Protein expression levels were determined in whole cell lysates of 786-0-VHL versus 786-0-pRC cells by Western analysis. Fig. 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 worth noting that fibronectin mRNA levels are remarkably less sensitive to re-expression of VHL than detectable secreted protein levels (approximately four-fold, discussion below).

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 microenvironment 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]. We report here – using the same cell system 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 increase (15-fold vs. 3.7-fold). Our data thus do 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.

3.3. Effect of hypoxia on the VHL target genes FN1 and IGFBP3

Because many of the 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% O_2) or hypoxic (1% O_2) conditions for 18 h. 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 Fig. 4A, hypoxia significantly induces the expression of *GLUT-1* by 6.7-fold in 786-0-VHL cells after 18 h 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 five-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 differ significantly (Fig. 4B). This implied 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,B). In contrast,

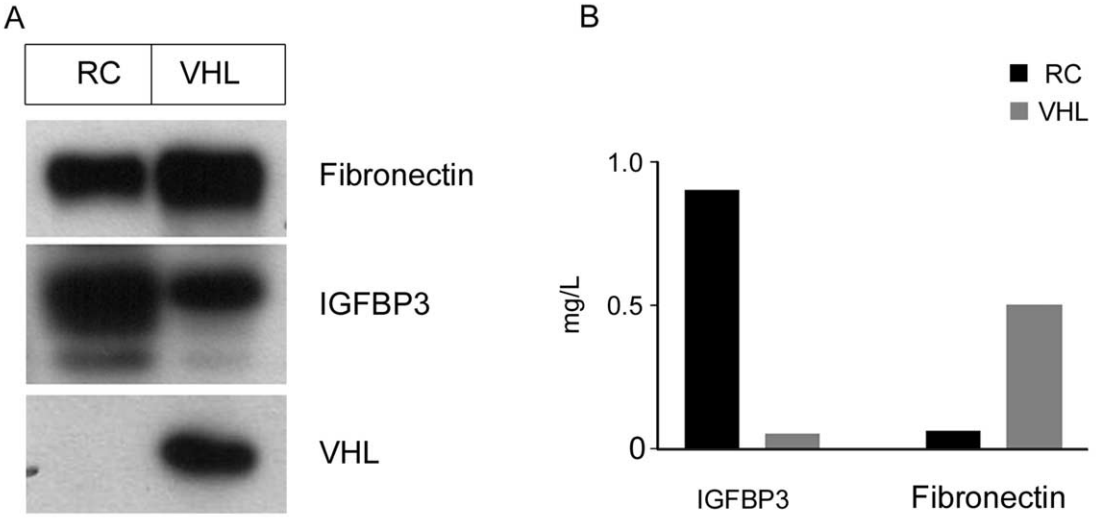

Fig. 3. Secretion of IGFBP3 and fibronectin is inversely regulated by VHL. A: Intracellular protein levels of IGFBP3, fibronectin and VHL determined by Western blot analyses. Left and right lanes contain protein lysates derived from 786-0-RC cell line (RC) and 786-0-VHL (VHL) cell lines, respectively. 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.

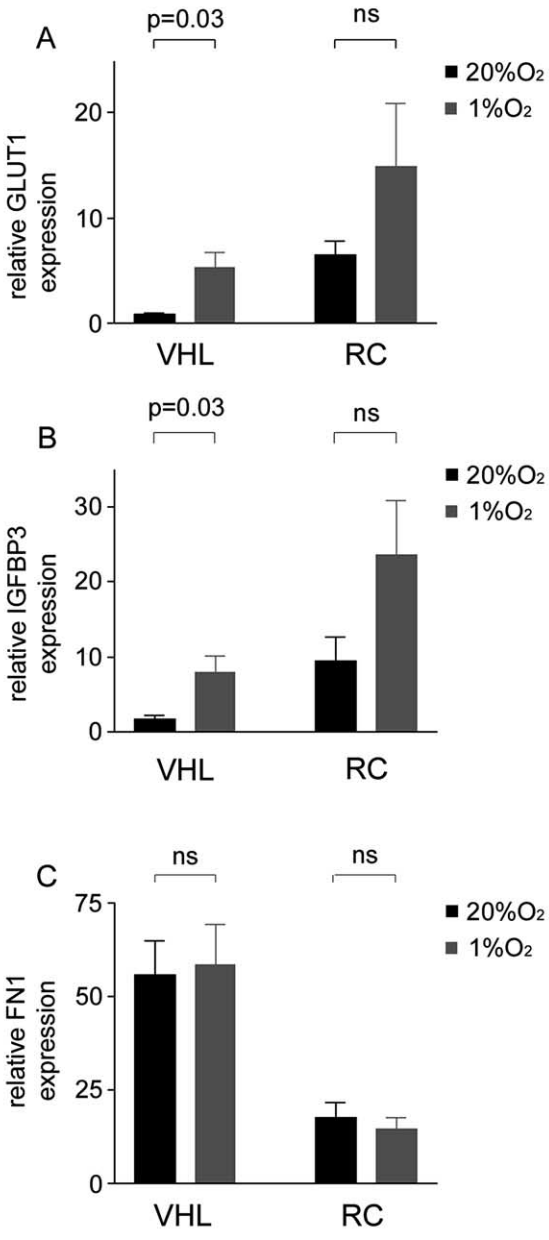

Fig. 4. Fibronectin is a hypoxia-independent target of VHL transcriptional regulation. Cells were cultured either under normoxia (20% O_2; light bars) or under hypoxia (1% O_2; dark bars) for 20 h. The two left columns represent cDNA derived from the 786-0-VHL cell line (VHL) and the two right columns represent cDNA derived from the 786-0-RC (RC) cell line. These results represent the mean of six independent experiments in triplicate ± S.E.M. *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: Fibronectin mRNA expression does not respond to hypoxia in either the 786-0-RC or the 786-0-VHL cell lines.

FN1 expression was not affected by hypoxia in 786-0-VHL cells or 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 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 ECM 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.

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