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Two novel VHL targets, TGFBI (BIGH3) and its transactivator KLF10, are up-regulated in renal clear cell carcinoma and other tumors

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ABSTRACT

Mutations in the VHL gene are associated with highly vascular tumors of kidney, brain, retina, and adrenal gland. The inability of the mutant VHL protein to destabilize HIF-1 plays a crucial role in malignant angiogenesis. VHL is also associated with ECM assembly but the molecular mechanisms of this activity remain unclear. We used expression arrays and cell lines with different VHL status to identify ECM-associated genes controlled by VHL. One of them, adhesion-associated TGFBI, was repressed by VHL and overexpressed in renal, gastrointestinal, brain, and other tumors. Analyzing the mechanism of TGFBI up-regulation in clear cell carcinoma, we identified a novel VHL target, a Kruppel-like transcriptional factor 10 (KLF10). The TGFBI promoter, which we isolated and studied in Luc-reporter assay, was induced by KLF10 but not hypoxia. These data provide the molecular basis for the observed VHL effect on TGFBI and stimulate further research into the KLF10 and TGFBI roles in cancer.

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The von Hippel-Lindau tumor suppressor (VHL) is the substrate recognition component of the E3 ligase that ubiquitinates HIF-1α (or HIF-2α) and plays a pivotal role in the control of hypoxia response [1,2]. We previously identified several HIF-dependent VHL targets that helped in understanding the VHL function(s) during malignant growth [3–5]. VHL is also involved in HIF-independent regulation of cell–cell interaction, matrix signaling, and adhesion [6–9]. In this study, we asked if novel VHL targets related to ECM deposition can be identified.

We previously reported STRA13 as VHL/HIF-1 target up-regulated in multiple tumors [5]. STRA13 can be also up-regulated by TGF-β [10]. Here we characterize two more targets common for both VHL and TGF-β, such as TGFBI, and KLF10 (TIEG1).

The TGFBI protein (beta-ig, big-h3, keratoepithelin) is a 68 kDa ECM protein with four evolutionary conserved fasciclin-1 (FAS1) domains and a carboxy-terminal Arg-Gly-Asp (RGD) sequence [11,12]. The protein is secreted into extracellular space and may bind to fibronectin and collagen [13] as well as integrins [14,15]. TGFBI was discovered and associated with cancer as a gene induced in the lung adenocarcinoma cell line A549 by TGF-β [12]. The TGFBI function(s) in normal tissues is not fully understood. Mutations in this protein have been implicated in congenital corneal dystrophies [16]. TGFBI was also reported to stimulate adhesion, spreading, migration and proliferation in renal proximal tubular epithelial cells [17]. The RGD peptide of TGFBI can be released from the protein and induce apoptosis [18]. Although TGFBI was associated with cancers in various studies [19–26], the molecular mechanisms of its transcriptional regulation remained unknown.

KLF10, a Kruppel-like transcriptional factor induced by TGF-β, BMP-2, and EGF, mimics the effect of TGF-β in many cells [27,28]. Overexpression of KLF10 was recently reported in clear cell carcinomas, glioblastomas, and head-and-neck carcinomas (www.oncomine.org), but its function in tumorigenesis remains unclear. In this study, we show that both TGFBI and KLF10 are down-regulated by VHL in 786-0 cells, and that KLF10 may serve as a transactivator of the TGFBI promoter.

Materials and methods

Cell lines and hypoxic exposure. Human HEK293T cells were purchased from ATCC; U-87, U-251 (astrocytomas) and LN-229 (glioma) were kindly provided by Dr. D. Zagzag, and MRC-5 cells (normal untransformed human fetal lung fibroblasts) were from Coriell Cell Repositories, Camden, NJ, USA. Immortalized human airway

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epithelial 1HAEo(−) cells were described elsewhere [29]. MEF (HIF-1+/+ and HIF-1−/−) were obtained from Dr. R. Johnson (University of California San Diego). To produce hypoxia cells were exposed to 0.5% O_2, 5% CO_2, and 94.5% N_2 at 37 °C or incubated with 0.5 mM $NiSO_4$ for 20 h.

Gene expression analysis. RNA samples were isolated, ^{32}P-labeled cDNA probes generated and hybridized with GEArrays HS-010 and HS-023 as recommended by the manufacturer (Superarray, Frederick, MD). Human Multiple Tissue Northern blots and cancer arrays were purchased from BD Biosciences (Palo Alto, CA). 786-0 clear cell RCC cells stably transfected with VHL transgenes were described previously [3]. Primers for RT-PCR were generated using GeneFisher server (http://bibiserv.techfak.uni-bielefeld.de/genefisher).

TGFBI promoter and KLF10 construct. The TGFBI promoter was identified using the Genomatix software (http://www.genomatix.de) and isolated via PCR on human DNA with primers 5′-ggtaccTGTGTCTCCCCAGGGCTAG-3′, and 5′-aagcttTG CAGCACCAGCTGGTAG-3′. The promoter was cloned into KpnI/HindIII-digested pGL3-Basic vector and verified by sequencing. The KLF10/TIEG1-expressing construct in pCDNA4/TO was described earlier [28]. *Luc-reporter assay* was used as described previously [30].

Results

Identification of the adhesion-related VHL targets

To assess the VHL effect on adhesion-associated genes we took advantage of the renal clear cell carcinoma cell lines developed by us and successfully used for identification of novel VHL targets [3,5,30]. Original 786-0 cells express VHL devoid of functional domains. These cells were compared to 786-0 stably transfected with wtVHL (786-0/wt2). We used mutVHL (786-0/mut2) that lacks the elongin C-binding alpha domain as a negative control [3]. The results of these experiments and subsequent validation of the VHL targets are shown in Fig. 1, Suppl. Fig. 1, and Table 1.

Five out of nine adhesion-related genes identified by us as VHL-dependent, ITGA5, SERPINE1, IGFBP3, FN1, and TIMP2, turned out to be already associated with VHL (see Refs. in Table 1). STAT1 (number 9 in Fig. 1 and Table 1) that showed up-regulation by wtVHL in this study, was recently characterized by us as a VHL target that is controlled via STRA13 [30]. Identification of all these previously known VHL targets validated our approach as a reliable tool for finding genes transcriptionally modulated by VHL. We then focused on TGFBI (BIGH3, betaig-h3, keratoepithelin) that encodes a secreted matrix protein with apoptotic and adhesion-related growth activities. This gene, as we show by RT-PCR (Suppl. Fig. 1A) and Northern analysis (Suppl. Fig. 1B), was down-regulated by wtVHL but not by mutVHL in 786-0 cells.

Classical TGF-β components are not involved in the transcriptional effects produced by VHL

Expression of TGF-β1 (location f25), -β2 (h25), and -β3 (a26) was barely detectable (Fig. 1 and Suppl. Table 1), and no VHL effect was seen on these genes at a longer exposure (data not shown). The type II activin A receptor (ACVR2), c15; type I activin A receptors ALK-1, ALK-5, endoglin, Smad1-7, and Smad9 were also not affected. In a separate experiment on the same cells, no VHL-dependent changes in the amounts of phosphorylated SMAD1/5/8 or SMAD2 were detected (data not shown).

KLF10 is a VHL target that regulates the TGFBI promoter

Seeking novel possible mediators of the VHL effect on TGFBI we focused on recently characterized transcriptional regulators KLF10 and KLF11 controlled by TGF-β [36,37]. We found that KLF10 but not KLF11 was repressed in 786-0 cells by wtVHL but not mutVHL (Suppl. Fig. 1B and data not shown) suggesting that it may also serve as a VHL target. We then identified the TGFBI promoter, isolated it via PCR, and assessed if KLF10 can transactivate it in a Luc-reporter assay. In this promoter, we identified a potential KLF10 binding site, which is also recognized as an Sp1-binding site and

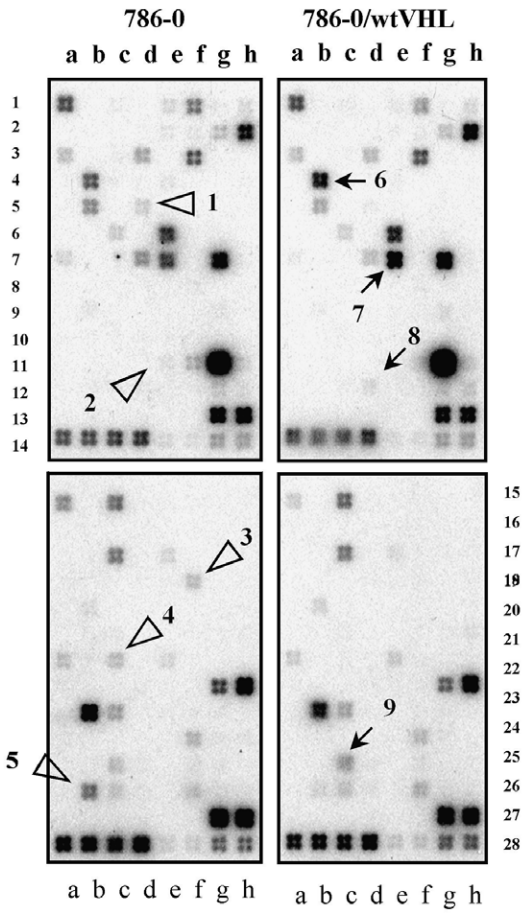

Fig. 1. Identification of adhesion-associated TGF-β targets modulated by VHL. Extracellular matrix and adhesion (rows 1–14) and TGF-β/BMP (rows 15–28) arrays after hybridization with RNA samples extracted from the VHL-positive or VHL-negative cells. Open triangles indicate genes repressed by wtVHL while arrows show wtVHL-stimulated genes. Full gene charts can be obtained from http://www.superarray.com.

is very similar to the one found within the CD11d promoter [38]. This site in the TGFBI promoter is localized 89–80 nucleotides upstream from the transcription initiation site (Suppl. Fig. 2). No HRE motif typical for most of hypoxia-stimulated VHL targets was found in the promoter arguing against its direct activation by HIF-1. Co-expression of TGFBI-Luc-reporter plasmid with KLF10 produced ~1.4- to 7-fold stimulation in two different cell lines (Fig. 2) suggesting that KLF10 may transactivate the TGFBI promoter in a cell type-specific manner. In agreement with the lack of the HRE site, no induction was observed by hypoxic mimetics and atmospheric hypoxia.

TGFBI is commonly overexpressed in cancers

We found that TGFBI is broadly expressed in normal tissues with highest expression levels detected in placenta, leukocytes, and heart. TGFBI expression is moderate in kidney where it is localized predominantly to the epithelial cells of collecting ducts and distal as well as proximal tubules (Suppl. Fig. 3). TGFBI expression in brain was practically undetectable (Fig. 3A). Studying TGFBI expression in cancer we compared between matched tumor/normal RNA samples. As a result, 4 out of 10 renal, 5/7 pancreatic, 4/10 lung, 9/10 colon, 9/10 rectal, and 4/7 small intestine cancers showed TGFBI overexpression (Fig. 3B and C). Our immunohistochemical study showed that in normal kidney TGFBI is expressed in distal and proximal tubular epithelium and in collecting duct epithelium.