Bi-allelic-inactivating mutations of the VHL tumor suppressor gene are found in the majority of clear cell renal cell carcinomas (VHL\(^{-/-}\) RCC). VHL\(^{-/-}\) RCC cells overproduce hypoxia-inducible genes as a consequence of constitutive, oxygen-independent activation of hypoxia inducible factor (HIF). While HIF activation explains the highly vascularized nature of VHL loss lesions, the relative role of HIF in oncogenesis and loss of growth control remains unknown. Here, we report that HIF plays a central role in promoting unregulated growth of VHL\(^{-/-}\) RCC cells by activating the transforming growth factor-\(\alpha\) (TGF-\(\alpha\))/epidermal growth factor receptor (EGF-R) pathway. Dominant-negative HIF and enzymatic inhibition of EGF-R were equally efficient at abolishing EGF-R activation and serum-independent growth of VHL\(^{-/-}\) RCC cells. TGF-\(\alpha\) is the only known EGF-R ligand that has a VHL-dependent expression profile and its overexpression by VHL\(^{-/-}\) RCC cells is a direct consequence of HIF activation. In contrast to TGF-\(\alpha\), other HIF targets, including vascular endothelial growth factor (VEGF), were unable to stimulate serum-independent growth of VHL\(^{-/-}\) RCC cells. VHL\(^{-/-}\) RCC cells expressing reintroduced type 2C mutants of VHL, and which retain the ability to degrade HIF, fail to overproduce TGF-\(\alpha\) and proliferate in serum-free media. These data link HIF with the overproduction of a \textit{bona fide} renal cell mitogen leading to activation of a pathway involved in growth of renal cancer cells. Moreover, our results suggest that HIF might be involved in oncogenesis to a much higher extent than previously appreciated.

von Hippel-Lindau (VHL)\(^{1}\) disease is a hereditary cancer syndrome, which predisposes patients to a variety of highly vascularized tumors including retinal angioma, central nervous system hemangioblastoma, pheochromocytoma, endolymphatic sac tumors, and clear cell renal carcinoma (RCC) (1, 2). VHL syndrome is caused by the inheritance of germ-line mutations of the VHL tumor suppressor gene. Although VHL follows an autosomal dominant pattern of inheritance (3), tumor formation arises only when the remaining wild-type allele acquires a somatic inactivating mutation as predicted by Knudson's two-hit hypothesis (4). Bi-allelic-inactivating mutations of the VHL gene are also found in the majority of sporadic clear cell renal cell carcinomas, the most common malignancy of the human kidney (VHL\(^{-/-}\) RCC) (5, 6). Reinforcement of the VHL gene into VHL\(^{-/-}\) RCC cells (hereafter referred to as “VHL(+)+ cells”) abolishes their ability to form tumors in nude mice, suggesting that loss of VHL function is a prerequisite for tumor formation (7). Furthermore, VHL\(^{-/-}\) RCC cells display several key features of transformed cells, including the inability to form an extracellular fibronectin matrix (8) and the loss of dependence on exogenous growth factors for proliferation in culture (9, 10). These features can be corrected by the reintroduction of VHL.

VHL forms a complex with several proteins including elongin B, elongin C, cullin-2, and Rbx1 to form a multiprotein complex referred to as VBC/Cul-2 (11). VBC/Cul-2 displays E3-ubiquitin ligase activity \textit{in vivo} and \textit{in vitro} (12–17). VHL acts as the recognition particle of this complex recruiting the \(\alpha\)-subunit of hypoxia-inducible factor (HIF\(\alpha\)) for cullin-2-mediated ubiquitination and subsequent proteasomal degradation (8, 18–21). The HIF transcription factor assemblies as a heterodimer containing one of three different \(\alpha\)-subunits and a \(\beta\)-subunit, and together, regulates the expression of several genes involved in the cellular response to hypoxia (22). These genes include the glucose transporter-1 (Glut-1), erythropoietin, and the angiogenic factor, vascular endothelial growth factor (VEGF) (22). While the HIF\(\beta\) subunit is constitutively expressed (21), the HIF\(\alpha\) subunits accumulate only under conditions of low oxygen tension. HIF\(\alpha\) regulation is mediated by oxygen-dependent hydroxylation of a conserved proline residue on its oxygen-dependent degradation domain (23,24–28). This post-translational modification increases HIF\(\alpha\) affinity toward VHL, leading to its ubiquitination and subsequent degradation. VHL-deficient RCC cell lines fail to degrade HIF\(\alpha\) irrespective of ambient oxygen tension (21, 29–32). HIF activation is an

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\(^{1}\) The abbreviations used are: VHL, von Hippel-Lindau; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; BrdUrd, bromodeoxyuridine.
HIF Promotes Proliferation of VHL−/− RCC Cells

immediate event upon VHL loss in multicellular foci in the distal nephron associated with expression of its target genes including Glut-1 and VEGF (33). Angiogenesis is considered a key step in tumor progression (34) implying a fundamental role for HIF in the later stages of tumor progression. However, the link between VHL loss, HIF activation, and how this relates to loss of growth control remains a key unanswered question.

Cancer cells are generally capable of engaging in autonomous growth as reflected by their ability to proliferate in serum-free conditions (35–38). We previously reported that VHL−/− RCC cells are also able to proliferate in the absence of exogenous growth factors and that this defect is correctable by the reintroduction of VHL (10). Transforming growth factor-α (TGF-α) was identified as a potential agent responsible for serum-independent growth of VHL−/− RCC cells. Several pieces of evidence point toward an oncogenic role for TGF-α in VHL−/− RCC cells. First, overproduction of TGF-α and its cognate epidermal growth factor receptor (EGF-R) has been described in overt sporadic RCC tumors in vivo (39–44). Second, antisense oligonucleotides directed against TGF-α mRNA abolished serum-independent growth of VHL−/− RCC cells (10). Third, treatment with inhibitors of EGF-R is as efficient as reintroducing VHL at abolishing the ability of RCC cells to form tumors in nude mice (7, 45, 46). However, the mechanism linking VHL loss-of-function to TGF-α overexpression, including the relative contribution of HIF activation to proliferation of VHL−/− RCC cells, remains unknown. In this report, we show that HIF activation is required for serum-independent growth of VHL−/− RCC cells, and this is a consequence of the activation of the EGF-R. We also show that HIF activation is required for overproduction of a TGF-α, an EGF-R ligand and bona fide renal cell mitogen. We thus provide the first direct evidence for a mechanism linking HIF activation with its postulated role in oncogenesis in VHL−/− RCC cells.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The human sporadic RCC cell line 786-o contains a single mutated VHL allele predicted to encode a truncated VHL protein. The 786-o (VHL−/−) renal cell line (i.e. the RCC cells) and the A498 cells were obtained from the AMERICAN Type Culture Collection (Manassas, VA). The WT-7 (VHL+/+ ) cell line is derived from 786-o cells stably transfected with hemagglutinin (HA)-tagged VHL, a kind gift from William Kaelin, Harvard University. The Type 2C VHL cell lines (stable transfecants of 786-o cells with pHVil L189V, pHVil R64P, and pHVil F119S) were a kind gift from Dr. David Park and cultured in DMEM containing 10% FCS. The properties of CRE8 cells are described elsewhere (29, 48). All constructs were authenticated by DNA sequencing.

Expression in Cell Lysates—Cytosolic and nuclear extracts were prepared as described elsewhere (29, 48). Samples were electrophoresed in 1.2% agarose gels and ethidium bromide staining visualized using a Kodak digital science IC440 system. The sequences of the primers used in this study are as follows: TGF-α forward: 5′-TGCCTCTGGATTTTGGTGTG-3′; TGF-α reverse: 5′-GAC-CTGGGAGCACTGTATCA-3′; EGF forward: 5′-CCAGGAAATTTGGA-ATTCTA-3′; EGF reverse: 5′-CTCTTTCTTCTTCTAA-3′; EGF forward: 5′-TGGATTGGACCTCAATGACA-3′; Glut-1 forward: 5′-GGTGTCGTCAGGATGACGAC-3′; Glut-1 reverse: 5′-GGTGGTGCTGAAGCTCTTTC-3′; TGF-β forward: 5′-GACCTGGCTACACAACTTCTC-3′; TGF-β reverse: 5′-GGTGGTGCTGAAGCTCTTTC-3′; Angiopoietin-1 forward: 5′-AGATGGATGACCTGCTCTCT-3′; Angiopoietin-1 reverse: 5′-AGATGGATGACCTGCTCTCT-3′.

Real-time RT-PCR—Cytosolic and nuclear extracts were prepared as described elsewhere (29, 48). Samples were electrophoresed in 1.2% agarose gels and ethidium bromide staining visualized using a Kodak digital science IC440 system. The sequences of the primers used in this study are as follows: TGF-α forward: 5′-TGCCTCTGGATTTTGGTGTG-3′; TGF-α reverse: 5′-GAC-CTGGGAGCACTGTATCA-3′; EGF forward: 5′-CCAGGAAATTTGGA-ATTCTA-3′; EGF reverse: 5′-CTCTTTCTTCTTCTAA-3′; EGF forward: 5′-TGGATTGGACCTCAATGACA-3′; Glut-1 forward: 5′-GGTGTCGTCAGGATGACGAC-3′; Glut-1 reverse: 5′-GGTGGTGCTGAAGCTCTTTC-3′; TGF-β forward: 5′-GACCTGGCTACACAACTTCTC-3′; TGF-β reverse: 5′-AGATGGATGACCTGCTCTCT-3′.

For real time RT-PCR, triplicates of 25–μl multiplex PCR reactions...
were performed on 50 ng of total RNA using TaqMan One-Step RT-PCR master mix reagents (Applied Biosystems) and 0.2 μM 5′-VIC of TGF-α- and 5′-6FAM β-actin-modified probes. Cycling conditions were 30 min at 48 °C, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C, 1 min at 60 °C. Amplification and analysis were performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and data normalized to the β-actin endogenous control. Primers for the real time RT-PCR experiments were designed as follows: Real time TGF-α forward: 5′-GCCAGTCCCCCGTGGATGT-3′; Real time TGF-α probe: 5′-TTTTTAATGACTGCCCAGATTCCCACACTCA-3′; Real time TGF-α reverse: 5′-CAGGTTCCCATGGAAGCACGA-3′; Real time β-actin forward: 5′-AGCTCTGCTTGGGCGGA-3′; Real time β-actin probe: 5′-CCGCCC-ACCGTCCACACCCGCC-3′; Real time β-actin reverse: 5′-CTGGTGCCCTGGGGCGC-3′.

RESULTS

HIF Activation Is Required for EGF-R-dependent Growth of VHL−/− RCC Cells in Serum-free Media—Cancer cells are generally capable of engaging in autonomous growth reflected by their ability to proliferate in serum-free medium (35–37). We recently reported that VHL−/− RCC cells share this defect, which is correctable by the reintroduction of VHL (10). To determine the role of HIF activation in the ability of VHL−/− RCC cells to engage in serum-independent growth, we produced an adenovirus that expresses a truncated version of HIF-2α, which acts as a dominant-negative molecule repressing HIF-mediated gene expression (DNHIF) (54) as well as an adenovirus that expresses a green fluorescent protein (GFP) fusion to HIF-1α (48). We also engineered adenoviruses that express GFP alone and GFP fused to VHL (VHL), and a naturally occurring C-terminal truncation mutant of VHL (Δ157-VHL) that fails to mediate oxygen-dependent degradation of HIFα (Fig. 1A). Infection efficiency was more than 90% in all cell lines used in this study, and production of the recombinant proteins was monitored by Western blots (data not shown and see Refs. 29 and 45). Normoxic VHL−/− RCC cells overproduce Glut-1 as a consequence of HIF activation, which is correctable by the reintroduction of VHL (55). DNHIF was as efficient as VHL at down-regulating Glut-1 levels in normoxic VHL−/− RCC cells incubated in the presence or absence of serum compared with controls (uninfected cells), or cells expressing Δ157-VHL, or GFP alone (Fig. 1A). Furthermore, overproduction of HIF-1α led to Glut-1 accumulation in normoxic VHL−/− cells, regardless of serum conditions (Fig. 1A). These results demonstrate that DNHIF and HIF-1α can be used as tools to inhibit or activate HIF-targets in normoxic VHL−/− RCC or VHL(+ ) cells, respectively.

We then examined whether the serum-independent growth phenotype observed in VHL−/− RCC cells is a consequence of HIF activation. DNHIF was as efficient as VHL at abolishing serum-independent growth of VHL−/− RCC cells, as measured by a marked reduction in the ability of these cells to incorporate BrdUrd (Fig. 1B). In contrast, VHL−/− RCC cells expressing GFP or Δ157-GFP incorporated BrdUrd to levels similar to those of uninfected cells. Addition of 5% serum abolished the growth inhibitory effect of DNHIF and VHL. Likewise, overproduction of HIF-1α was sufficient to enable VHL(+) cells to proliferate in serum-free medium (ITS), in contrast to uninfected VHL(+) cells or cells expressing GFP alone (Fig. 1B). These results indicate that the ability of VHL−/− RCC cells to engage in serum-independent growth is a consequence of HIF activation.

We previously reported that overexpression of TGF-α and activation of EGF-R were involved in serum-independent growth of VHL−/− RCC cells (10). Based on data shown in Fig. 1, we next asked whether HIF activation promoted serum-independent growth of VHL−/− RCC cells through EGF-R-dependent or -independent pathways. To do so, we first compared the effect of DNHIF and reintroducing VHL on EGF-R levels and phosphorylation state in VHL−/− RCC cells. Levels of EGF-R remained essentially unchanged regardless of growth conditions, VHL/HIF activity or incubation with PD153035, a membrane permeable inhibitor of EGF-R tyrosine kinase activity (referred to as EGF-Ri). The specificity of EGF-Ri at abolishing EGF-R activation has been reported elsewhere (51) and controls are shown in Fig. 2B and Fig 5A. DNHIF and VHL did not affect the phosphorylation status of VHL−/− RCC cells in 10% serum (Fig. 2A). In contrast, DNHIF and VHL were essentially as efficient as EGF-Ri in reducing EGF-R phosphorylation in serum-free media. Therefore, abolishing HIF activation as essentially the same effect as reintroducing VHL on EGF-R phosphorylation of VHL−/− RCC cells. The data also shows that EGF-R phosphorylation is dependent upon HIF activation.

We next tested whether serum-independent growth of VHL−/− RCC cells was solely the consequence of HIF-dependent activation of EGF-R that occurs upon VHL loss. We reasoned that, if EGF-R-dependent signaling existed, the EGF-Ri-treated VHL−/− RCC cells would display measurable differences in BrdUrd incorporation relative to VHL(+ ) cells, or VHL−/− RCC cells expressing DNHIF (see Fig. 1B) in serum-free media. EGF-Ri treatment had comparable growth suppressive effects to that of reintroduction of VHL on VHL−/− RCC.
FIG. 2. HIF-mediated activation of the EGF-R is required for serum-independent growth of VHL(−/−) RCC cells. A and B, EGF-R phosphorylation in VHL(−/−) RCC cells incubated in serum-free media requires HIF activation. VHL(−/−) RCC 786-0 cells were not treated (−), treated with MeSO alone, in the presence of 10 μM PD153035 (EGF-Ri), or infected with adGFP, adDNHIF or adVHL. Cells were incubated for 48 h in the presence (A) or absence (B) of 10% serum. Lysates were probed against an anti-phospho-EGF-R antibody (pY-EGF-R) in B, or against an anti-EGF-R antibody (Actin) in A. C, inhibition of the EGF-R has similar effects on proliferation of VHL(−/−) RCC cells as reintroducing wild-type VHL. VHL(−/−) RCC cells were incubated in DMEM supplemented with 5% FCS for 16 h (time 0 h). Cells were transferred to DMEM with ITS and VHL(−/−) RCC cells were treated with either vehicle alone, MeSO (DMSO), or PD153035 (1 μM) for the indicated time. VHL(−/−) RCC cells stably expressing reintroduced VHL (VHL(+) RCC) were incubated in the same growth conditions and in the presence of MeSO. BrdUrd was added for the last 3 h before fixation and staining. Shown is the mean of three independent experiments with S.E. D, EGF-R inhibitor-treated VHL(−/−) RCC cells are in a state of quiescence (G0). VHL(−/−) RCC were incubated in DMEM supplemented with ITS and in the presence of PD153035 (1 μM) for 72 h. VHL(−/−) RCC cells were incubated in the same conditions but with MeSO. The cells were washed and the medium replaced with DMEM supplemented with 5% serum. BrdUrd was added 3 h prior to fixation and staining at the indicated times. E, inhibition of EGF-R activity abolishes serum-free growth of VHL(−/−) RCC cells in hypoxia to the same levels as in normoxia. VHL(−/−) RCC 786-0 cells were plated overnight and then washed, and medium was replaced with DMEM supplemented with ITS. The EGF-R inhibitor (PD153035; 1 μM) with or without bFGF (100 ng/ml) or the PFG-R inhibitor (AG1296; 50 μM) were added, and cells were maintained in normoxia for 24 h. Cells were maintained in normoxia or transferred to hypoxia for another 24 h. BrdUrd was added for the last 3 h prior to analysis. Data shown are the mean of three independent experiments with S.E.

FIG. 3. TGF-α is the only known EGF-R ligand with an expression profile dependent upon VHL status. VHL(−/−) RCC cells were infected with adGFP (VHL status −) or adVHL (VHL status +) and incubated in serum for 48 h prior to isolation of total RNA. RT-PCR was carried out as described under “Experimental Procedures” for 25 cycles (top panel) or 30 cycles (bottom panel). The extreme right of the top panel is a longer exposure of the EGF RT-PCR to show the presence of low amounts of the EGF transcript. Amphi, amphiregulin; Betac, betacellu- lin; Epireg., epiregulin. β-Actin is shown as a reaction and loading control.

Given that HIF activation, EGF-R stimulation and TGF-α overproduction (10) are equally required for serum-independent growth, we hypothesized that TGF-α overproduction by VHL(−/−) RCC cells might be a consequence of HIF activation. Fig. 4A shows that overproduction of TGF-α ligand in VHL(−/−) RCC cell lines correctable
by the reintroduction of VHL. DNHIF was as efficient at reducing TGF-α levels in VHL−/− RCC 786-0 as it was at reducing levels of the well known HIF-target, Glut-1 (Fig. 4, B and C). Maximal DNHIF-mediated inhibition of TGF-α production was attained at 100–200 MOI and was ~6-fold compared with uninfected or GFP-infected cells (Fig. 4D). These results demonstrate that TGF-α overproduction is a general phenomenon observed in many VHL−/− RCC cells and requires HIF activation.

We next decided to further examine the relative role of HIF activation in the overexpression of TGF-α in comparison to that of loss of VHL function. We reasoned that, if HIF activation was the sole cause of TGF-α overexpression, DNHIF would have essentially the same effect at down-regulating TGF-α levels in VHL−/− RCC cells as reintroduction of VHL function. VHL−/− RCC 786-0 cells were infected with DNHIF and VHL, Δ157-VHL, and GFP. DNHIF was as efficient as VHL at down-regulating mRNA levels of Glut-1 and TGF-α in VHL−/− RCC cells (Fig. 5A). Real time RT-PCR (Fig. 5B) and ELISA (Fig. 5C) revealed a similar decrease of TGF-α mRNA and protein levels (about 6-fold) in VHL−/− RCC cells expressing DNHIF or reintroduced VHL. As expected, controls GFP and Δ157-GFP had essentially no effects on Glut-1 and TGF-α mRNA and protein levels (Fig. 5, B and C). Similar data were obtained with VHL−/− RCC A498 cells (data not shown). We next asked if HIF activation was sufficient to promote TGF-α overproduction even in a VHLPositive environment. Accumulation of HIF-α by adenovirus-mediated infection of normoxic VHL (+) cells was sufficient to induce overproduction of TGF-α to levels comparable to those observed in VHL−/− RCC cells (Fig. 5D). Furthermore, VHL (+) cells expressing an ODD mutant of HIF-2α, which fails to assemble to VHL, also displayed increased levels of TGF-α protein compared with VHL (+) transfected with vector alone (Fig. 5E). Therefore, TGF-α overproduction by VHL−/− RCC cells is solely dependent upon HIF activation, as observed with other known HIF targets and does not require VHL loss of function. Furthermore, data shown in Figs. 4 and 5 identify TGF-α as a novel HIF-regulated gene.

TGF-α-mediated Activation of EGF-R Is a Major Mitogenic Event in VHL−/− RCC Cells—The next step consisted of examining the mechanisms involved in TGF-α-mediated proliferation of VHL−/− RCC cells. It is generally thought that TGF-α stimulates proliferation through the activation of the EGF-R although this has yet to be formally shown in renal cancer cells (56, 57). To test this, we analyzed the ability of EGF-Ri to

Fig. 4. Overproduction of TGF-α by VHL−/− RCC cells requires HIF activation. A, TGF-α protein level is dependent on VHL status in RCC cells. Two independent VHL−/− RCC cell lines were either uninfected or infected with VHL or GFP as a control. Cell lysates were prepared 60 h after infection, and Western blots were carried out to detect Glut-1 and actin as a loading control (top panel) or ELISA was performed to measure levels of TGF-α normalized per microgram of total cellular protein with data showing the mean average of at least three independent experiments with S.E. B, Western blot analysis of Glut-1 levels in VHL−/− RCC cells expressing increasing MOI (multiplicity of infection) of DNHIF. VHL−/− RCC cells were infected with increasing amounts of DNHIF, and lysates were prepared for Western blots 60 h post-infection to detect Glut-1 or actin as a loading control. C and D, DNHIF down-regulates TGF-α levels in VHL−/− RCC cells. VHL−/− RCC cells were treated as described in B but lysates were prepared as suggested by the manufacturer. Levels of TGF-α were normalized per microgram of total cellular protein. Data shown in panel D are the mean average of three independent experiments with S.E. at a multiplicity of infection (MOI) of 200.

Fig. 5. HIF activation is sufficient to increase TGF-α mRNA and protein levels in VHL−/− RCC cells. A and B, DNHIF is as efficient as reintroducing VHL at abolishing overexpression of TGF-α mRNA by VHL−/− RCC cells. RT-PCR analysis of TGF-α and Glut-1 mRNA levels in VHL−/− RCC cells infected with adenoviruses expressing DNHNIF or VHL. VHL−/− RCC cells were either uninfected (−) or infected with adenoviruses expressing GFP, VHL, Δ157-VHL, or DNHIF. Total RNA was isolated 60 h post-infection and RT-PCR was performed using primers specific to TGF-α, Glut-1, or β-actin mRNA (A). The same samples were also analyzed by real-time RT-PCR and plotted as fold reduction as compared with uninfected VHL−/− RCC cells (B). Mean average of three independent experiments with S.E. is shown. C, cells were treated as described in A but lysates were prepared to analyze TGF-α protein levels by ELISA. Levels of TGF-α were normalized per μg of total cellular protein assessed by the BCA method. Data shown are the mean average of three independent experiments with S.E. D, effect of HIF-1α overexpression on levels of TGF-α in normoxic VHL (+) cells. VHL (+) cells were either uninfected or infected with adenoviruses expressing GFP or HIF-1α and lysates were prepared 60 h post-infection. Lysates were also prepared from uninfected VHL−/− RCC cells, TGF-α protein levels were assessed by ELISA and normalized per microgram of total cellular protein assessed by the BCA method. Data shown are the mean average of at least three independent experiments with S.E. E, Δ157-VHL, or GFP. DNHIF was as efficient as VHL at down-regulating mRNA levels of Glut-1 and TGF-α in VHL−/− RCC cells (Fig. 5A). Real time RT-PCR (Fig. 5B) and ELISA (Fig. 5C) revealed a similar decrease of TGF-α mRNA and protein levels (about 6-fold) in VHL−/− RCC cells expressing DNHIF or reintroduced VHL. As expected, controls GFP and Δ157-GFP had essentially no effects on Glut-1 and TGF-α mRNA and protein levels (Fig. 5, B and C). Similar data were obtained with VHL−/− RCC A498 cells (data not shown). We next asked if HIF activation was sufficient to promote TGF-α overproduction even in a VHL-positive environment. Accumulation of HIF-α by
abolish exogenous TGF-α growth stimulatory effect on VHL(+) cells grown in serum-free media. VHL(+) cells are abolished by treatment with EGF-Ri. VHL(+) RCC cells were incubated in the absence or presence of PD153035 (1 μM) in ITS alone or ITS with TGF-α (10 ng/ml), or 5% serum for 48 h. Cells were incubated in the presence of BrdUrd 3 h prior to fixing and staining. Shown are the means of at least three independent experiments with S.E. B, growth factors associated with VHL loss-of-function fail to rescue the growth inhibitory effect of an EGF-R inhibitor on VHL(−) RCC cells in culture. VHL(−) RCC cells were incubated in ITS without or with PD153035 (1 μM) or with PD153035 plus TGF-β (10 ng/ml), TGF-β1 (5 ng/ml), VEGF (50 ng/ml), PDGF (25 ng/ml), or bFGF (10 ng/ml). VHL(−) RCC cells were also incubated in DMEM supplemented with ITS and in the presence of the FGF-R inhibitor (AG1296; 50 μM) or the VEGF inhibitor (transfused; 80 μM). Cells were incubated for 48–60 h prior to the addition of BrdUrd for the last 3 h before fixing and staining. Shown is the mean of three independent experiments with S.E.

Fig. 6. TGF-α, but not other cytokines associated with HIF activation, promotes serum-independent growth through the activation of the EGF-R. A, growth stimulatory effect of exogenous TGF-α on VHL(+) cells is abolished by treatment with EGF-Ri. VHL(+) RCC cells were incubated in the absence or presence of PD153035 (1 μM), in ITS alone or ITS with TGF-α (10 ng/ml), or 5% serum for 48 h. Cells were incubated in the presence of BrdUrd 3 h prior to fixing and staining. Shown are the means of at least three independent experiments with S.E. B, growth factors associated with VHL loss-of-function fail to rescue the growth inhibitory effect of an EGF-R inhibitor on VHL(−) RCC cells in culture. VHL(−) RCC cells were incubated in ITS without or with PD153035 (1 μM) or with PD153035 plus TGF-β (10 ng/ml), TGF-β1 (5 ng/ml), VEGF (50 ng/ml), PDGF (25 ng/ml), or bFGF (10 ng/ml). VHL(−) RCC cells were also incubated in DMEM supplemented with ITS and in the presence of the FGF-R inhibitor (AG1296; 50 μM) or the VEGF inhibitor (transfused; 80 μM). Cells were incubated for 48–60 h prior to the addition of BrdUrd for the last 3 h before fixing and staining. Shown is the mean of three independent experiments with S.E.

We had previously demonstrated that other growth factors overproduced by VHL(−) RCC cells, such as VEGF and PDGF, do not incorporate BrdUrd in serum-free media (Fig. 6A). Addition of exogenous TGF-α stimulated BrdUrd incorporation, which was abolished by treatment with EGF-Ri (Fig. 6A). In contrast, addition of 5% serum abolished the growth inhibitory effect of EGF-Ri, a consequence of EGF-R-independent stimulation of other receptors again demonstrating that EGF-R does not block growth nonspecifically (Fig. 6A). These data further confirm that TGF-α promotes proliferation through the activation of EGF-R in our cell culture system and not through a yet unappreciated alternative pathway.

We had previously demonstrated that other growth factors overproduced by VHL(−) RCC cells, such as VEGF and PDGF, do not display mitogenic activity on VHL(+) cells in culture (10). However, it could be argued that mitogenic sensitivity to these cytokines is acquired upon VHL loss. To further investigate this, several factors that are overproduced by VHL(−) RCC cells were tested for their ability to stimulate proliferation of EGF-Ri-treated VHL(−) RCC 786-0 cells. VHL(−) RCC 786-0 cells continued to incorporate BrdUrd in the absence of exogenous growth factors (Fig. 6B). Treatment with EGF-Ri reduced the ability of VHL(−) RCC cells to incorporate BrdUrd by 3–4-fold after 48 h in serum-free media (10) (Fig. 6B). Addition of TGF-α did not stimulate the proliferation of VHL(−) RCC cells in the presence of EGF-Ri, suggesting that TGF-α was not able to stimulate growth through an independent receptor in VHL loss RCC cells (Fig. 6B and see Fig. 6A). Growth factors overproduced by VHL(−) RCC cells and under HIF regulation, such as TGF-β, VEGF, and PDGF, were unable to promote BrdUrd incorporation of EGF-Ri-treated VHL(−) RCC 786-0 cells (Fig. 6B). As expected, addition of FGF stimulated the incorporation of BrdUrd in VHL(−) RCC cells grown in ITS and in the presence of an EGF-Ri though it should be noted that VHL(−) RCC cells do not overproduce FGF. Likewise, treatment with an inhibitor of FGF receptor, as well as a VEGF inhibitor, failed to abolish the ability of VHL(−) RCC 786-0 cells to incorporate BrdUrd in ITS (Fig. 6B). These results indicate that VHL(−) RCC cells are not sensitive to other growth factors overproduced by VHL(−) RCC cells except for TGF-α.

TGF-α can either be retained in cells or secreted in the medium (58–60). We failed to detect TGF-α in the media of VHL(−) RCC 786-0 cells implying that TGF-α is retained within cellular compartments (Fig. 7A). Likewise, neutralizing antibodies to TGF-α had no measurable effect on proliferation on VHL(−) RCC cells in serum-free media (Fig. 7B). In contrast, neutralizing antibodies abolished the mitogenic effect of exogenous TGF-α on VHL(+) in serum free media demonstrating that the antibody could efficiently prevent exogenous TGF-α signaling. Furthermore, conditioned media obtained from VHL(−) RCC cells in serum-free media failed to stimulate proliferation of VHL(+) although the addition of exogenous TGF-α to the conditioned media, or ITS, stimulated proliferation (Fig. 7C). These results demonstrate that TGF-α is retained in VHL(−) RCC cells and not secreted into the media, as observed with VEGF (61).

Type 2C Mutants Negatively Regulate TGF-α and Restore Serum-dependent Growth of VHL(−) RCC Cells—Individuals afflicted with type 2C VHL syndrome develop pheochromocytoma, but not RCC or hemangioblastomas (62, 63). VHL(−) RCC cells expressing reintroduced VHL mutants harboring missense mutations associated with type 2C disease failed to form tumors in nude mice and were able to mediate oxygen-dependent ubiquitination and degradation of HIF (31, 47). Type 2C mutants were able to negatively regulate Glut-1 and TGF-α levels, albeit to a different extent compared with VHL(+) cells (Fig. 8, A and B). Type 2C mutants that were most efficient at down-regulating Glut-1 and TGF-α levels were also able to
abolish the ability of VHL\(^{-/-}\) RCC cells to incorporate BrdUrd in the absence of exogenous growth factors to levels similar to those of VHL\(^{+/-}\) cells (Fig. 8C). This data suggest that VHL\(^{-/-}\) RCC cells expressing reintroduced type 2C mutants of VHL do not engage in an HIF-dependent TGF-\(\alpha\)/EGF-R autonomous growth stimulatory pathway in culture.

**DISCUSSION**

In this report, we link HIF activation to oncogenesis and loss of growth control of VHL\(^{-/-}\) RCC cells in addition to its reported role in adaptation to hypoxia, angiogenesis, and tumor progression. Data presented here link HIF with a *bona fide* renal mitogen and identifies TGF-\(\alpha\) as a novel HIF-regulated gene. We suggest that VHL-null cells engage in an HIF-dependent constitutive activation of the TGF-\(\alpha\)/EGF-R growth stimulatory pathway and that this constitutes the postulated gatekeeper function of VHL in renal epithelial cells (9, 10, 64, 65). This model is founded on experiments focusing on the ability of VHL\(^{-/-}\) RCC cells to proliferate in the absence of exogenous growth factors, whereas primary cultures of renal epithelial cells require addition of exogenous growth factors, or serum, to proliferate in culture (66, 67). The ability of transformed cells to engage in an autonomous growth stimulatory pathway has been appreciated in several types of cancers and is one of the accepted hallmarks of cellular transformation (35–38, 68–70). Our model of HIF-mediated activation of the TGF-\(\alpha\)/EGF-R pathway as a major oncogenic pathway in VHL\(^{-/-}\) RCC cells is supported by the following: First, we show in this report that inhibition of EGF-R phosphorylation and of serum-independent growth of VHL\(^{-/-}\) RCC can be achieved to a similar extent by either reintroducing VHL function, by abolishing HIF activation or by inhibiting EGF-R activity. Second, antisense oligonucleotides to TGF-\(\alpha\) mRNA efficiently abolish serum-independent growth of VHL\(^{-/-}\) RCC cells (10). Third, TGF-\(\alpha\) overproduction is a consequence of HIF activation. While it remains possible that other, as yet unidentified, EGF-R ligands might be overproduced upon HIF activation, the antisense data in addition to results shown here do argue for a crucial role for TGF-\(\alpha\) in activating the EGF-R. Finally, and in agreement with our data, are reports that have shown that the reintroduction of VHL, or treatment with an EGF-R inhibitor, are both equally efficient at suppressing RCC tumor formation in nude mice assays (7, 45, 46, 71, 72). We argue that obvious differences would have been observed in growth inhibitory activity among these very different approaches if VHL\(^{-/-}\) RCC cells had evolved other intrinsic mechanisms of unregulated growth. These finding provide the first evidence linking HIF activation with autonomous and aberrant proliferation of fully transformed VHL\(^{-/-}\) RCC cells. It remains to be determined if other HIF-dependent cytokines, including unidentified EGF-R ligands, are also involved in addition to TGF-\(\alpha\). However, based on our data, we propose that the HIF-mediated activation of the TGF-\(\alpha\)/EGF-R pathway serves as a common oncopgenic event in RCC cells upon the loss of VHL function thereby explaining serum-free growth and tumorigenesis.

Recently, Mandriota *et al.* (33) elegantly showed that VHL loss-of-function and HIF activation are early events in the development of multicellular neoplastic foci of the distal nephron (33). It will obviously be difficult to formally prove that HIF activation, by way of TGF-\(\alpha\) overproduction, provides the initial growth advantage to renal epithelial cells upon the loss of VHL function. However, our interpretation is that HIF-dependent constitutive overproduction of TGF-\(\alpha\) would provide a very strong initial oncogenic signal for renal epithelial cells (70). Consistent with this argument is the observation that transgenic expression of TGF-\(\alpha\) in mice leads to the formation of multiple renal cysts (73) reminiscent of pre-neoplastic lesions of the human kidney (11, 74). This mouse model provides evidence suggesting that TGF-\(\alpha\) overproduction is sufficient to initiate unregulated growth of renal epithelial cells in intact kidneys. Furthermore, renal epithelial cells are particularly sensitive to the growth promoting effect of TGF-\(\alpha\) in culture (75). An autocrine TGF-\(\alpha\) model has also been proposed for other models of epithelial cancers (70, 76). We find that HIF-mediated constitutive activation of the TGF-\(\alpha\)/EGF-R pathway provides an adequate explanation for tumor initiation upon the loss of VHL function in the distal nephron. Nonetheless, data shown in this report establishes an unappreciated link between HIF activation and a potent growth factor of renal epithelial cells suggesting a possible role for HIF in tumorigenesis.

Complex genotype-phenotype correlations have emerged from the study of different families with VHL disease. Patients with type 2C VHL mutations carry an increased risk of pheochromocytoma but not hemangioblastoma or RCC (62, 63, 77–79). Products of type 2C VHL mutations retain the ability to down regulate HIF and HIF responsive genes (47, 80). As
expected, all three type 2C VHL mutants that we tested (L185V, F119S, and R64P) failed to overproduce TGF-α to the same extent as VHL−/− 786-0 cells. We did, however, note different TGF-α levels among the three type 2C mutants, two of which produced higher levels than WT VHL+ + cells. Specifically, F119S exhibited the highest level of TGF-α of all the cell lines tested in correlation to an equivalently increased level of Glut4. Consistent with our hypothesis that TGF-α drives the proliferation of RCC cells, the levels of TGF-α produced in the three Type 2C mutants correlated with their growth profiles in serum-free medium. It is interesting that both F119S and R64P three Type 2C mutants correlated with their growth profiles in lines tested in correlation to an equivalently increased level of phenotype correlation in VHL disease.

Taken together, our study links HIF activation with the aberrant production of a bona fide mitogen of renal epithelial cells and provides evidence for a role of HIF in the initiation of tumorigenesis. We propose that HIF activation-mediated overproduction of TGF-α and subsequent activation of the EGFR is the dominant pathway driving proliferation of fully transformed VHL−/− RCC cells. Our observations suggest that contributions from other putative HIF-dependent or -independent growth pathways that might occur upon VHL loss-of-function are relatively minor, at least in culture and in nude mouse tumor assays. RCC is the most frequent malignant neoplasm arising from the kidney (3). Standard management for stage I or stage II disease is radical nephrectomy, with advanced disease carry a poor prognosis. Since HIF activation is an early event in RCC development, blocking the EGFR pathway has the potential to restore at least some of the normal growth characteristics of fully transformed VHL−/− RCC cells. Based on these data, we suggest that VHL−/− RCC tumors would provide an ideal neoplastic disease for treatment with one of the many inhibitors of EGFR, especially enzymatic inhibitors of tyrosine kinase activity.

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60. Luetteke, N. C., and Lee, D. C. (1990) Semin. Cancer Biol. 1, 265–275
61. Siemeister, G., Weindel, K., Mohrs, K., Barleon, B., Martiny-Baum, G., and Marme, D. (1996) Cancer Res. 56, 2299–2301
62. Ritter, M. M., Frilling, A., Crossey, P. A., Hoppner, W., Maher, E. R., Mulligan, L., Ponder, B. A., and Engelhardt, D. (1996) J. Clin. Endocrinol. Metab. 81, 1035–1037
63. van der Harst, E., de Krijger, R. R., Dinjens, W. N., Weeks, L. E., Bonjer, H. J., Brunning, H. A., Lamberts, S. W., and Koper, J. W. (1998) Int. J. Cancer 77, 337–340
64. Kondo, K., and Kaelin, W. G., Jr. (2001) Exp. Cell Res. 264, 117–125
65. Kinzler, K. W., and Vogelstein, B. (1997) Nature 386, 761–763
66. Humes, H. D., Bea, T. F., Cieslinski, D. A., Sanchez, I. O., and Page, T. P. (1991) Lab. Invest. 64, 538–545
67. Gomella, L. G., Sargent, E. R., Wade, T. P., Anglard, P., Linehan, W. M., and Kasid, A. (1999) Cancer Res. 59, 6972–6975
68. Rosenthal, A., Lindquist, P. B., Bringman, T. S., Goeddel, D. V., and Derynck, R. (1996) Cell 85, 301–309
69. Watanabe, S., Lazar, E., and Sporn, M. B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1258–1262
70. Sporn, M. B., and Todaro, G. J. (1980) N. Engl. J. Med. 303, 878–880
71. Bos, M., Mendelsohn, J., Kim, Y. M., Albanell, J., Fry, D. W., and Baserga, J. (1997) Clin. Cancer Res. 3, 2099–2106
72. Weber, K. L., Douet, M., Price, J. E., Baker, C., Kim, S. J., and Fidler, I. J. (2003) Cancer Res. 63, 2940–2947
73. Lowden, D. A., Lindemann, G. W., Merlino, G., Barash, B. D., Calvert, J. P., and Gattone, V. H., 2nd. (1994) J. Lab. Clin. Med. 124, 386–394
74. Everett, J. J., Walker, C. L., Goldsworthy, T. W., and Wolf, D. C. (1997) Mol. Carcinog. 19, 213–219
75. Neufeld, T. K., Douglass, D., Grant, M., Ye, M., Silva, F., Nadasy, T., and Grantham, J. J. (1992) Kidney Int. 41, 1222–1226
76. Smith, J. J., Derynck, R., and Korc, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7567–7570
77. Neumann, H. P., Lips, C. J., Hsia, Y. E., and Zbar, B. (1995) Brain Pathol. 5, 181–193
78. Crossey, P. A., Eng, C., Ginalska-Malinowska, M., Lennard, T. W., Wheeler, D. C., Ponder, B. A., and Maher, E. R. (1995) J. Med. Genet. 32, 885–886
79. Eng, C., Crossry, P. A., Mulligan, L. M., Healey, C. S., Houghton, C., Prowse, A., Chew, S. L., Dahia, P. L., O’Riordan, J. L., and Toledo, S. P. (1995) J. Med. Genet. 32, 934–937
80. Clifford, S. C., Astuti, D., Hooper, L., Maxwell, P. H., Ratcliffe, P. J., and Maher, E. R. (2001) Oncogene 20, 5667–5674