Silencing of Epidermal Growth Factor Receptor Suppresses Hypoxia-Inducible Factor-2–Driven VHL −/− Renal Cancer

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Abstract

Inactivating mutations in the von Hippel-Lindau (VHL) tumor suppressor gene are associated with clear cell renal cell carcinoma (VHL −/− RCC), the most frequent malignancy of the human kidney. The VHL protein targets the α subunits of hypoxia-inducible factor (HIF) transcription factor for ubiquitination and degradation. VHL −/− RCC cells fail to degrade HIF resulting in the constitutive activation of its target genes, a process that is required for tumorigenesis. We recently reported that HIF activates the transforming growth factor-α/epidermal growth factor receptor (TGF-α/EGFR) pathway in VHL-defective RCC cells. Here, we show that short hairpin RNA (shRNA)–mediated inhibition of EGFR is sufficient to abolish HIF-dependent tumorigenesis in multiple VHL −/− RCC cell lines. The 2α form of HIF (HIF-2α), but not HIF-1α, drives in vitro and in vivo tumorigenesis of VHL −/− RCC cells by specifically activating the TGF-α/EGFR pathway. Transient incubation of VHL −/− RCC cell lines with small interfering RNA directed against EGFR prevents autonomous growth in two-dimensional culture as well as the ability of these cells to form dense spheroids in a three-dimensional in vitro tumor assay. Stable expression of shRNA against EGFR does not alter characteristics associated with VHL loss including constitutive production of HIF targets and defects in fibronectin deposition. In spite of this, silencing of EGFR efficiently abolishes in vivo tumor growth of VHL loss RCC cells. These data identify EGFR as a critical determinant of HIF-2α-dependent tumorigenesis and show at the molecular level that EGFR remains a credible target for therapeutic strategies against VHL −/− renal carcinoma. (Cancer Res 2005; 65(12): 5221-30)

Introduction

Inactivating mutations of the von Hippel-Lindau (VHL) tumor suppressor genes are associated with inherited VHL syndrome, of which afflicted individuals are at increased risk to develop a wide variety of neoplasms, including central nervous system hemangioblastoma and clear cell renal cell carcinoma (RCC; ref. 1). Mutations in the VHL gene are also found in the vast majority of sporadic clear cell RCC, the most frequent malignancy of the human kidney (2). Surgery remains the mainstay of treatment for kidney cancer as an incomplete understanding of the molecular mechanisms underlying this disease has limited the development of successful nonsurgical therapies (3). Consequently, the prognosis for patients with recurrent or metastatic renal cancer is often bleak with a mortality rate of 20% within 1 year of diagnosis. Hence, novel clinically relevant therapeutic approaches will directly alter the prognosis of patients with metastatic RCC.

Recent studies have yielded important clues as to the function of the VHL protein. Reintroduction of wild-type VHL in VHL-defective RCC cells prevents tumor formation in nude mice confirming the tumor suppressive function of VHL (4). VHL assembles with elongin B, C, rbx1, and cullin-2 to form E3 ubiquitin ligase. VHL recruits the α subunits of hypoxia-inducible factor (HIFα) for cullin-2-mediated ubiquitination and subsequent degradation by the 26s proteasome (5–9). HIF is a transcription factor that activates an array of genes involved in cellular adaptation to hypoxia including the angiogenic vascular endothelial growth factor (VEGF; refs. 10, 11). The interaction between VHL and HIFα is regulated by oxygen tension. In the presence of oxygen (normoxia), HIF prolyl hydroxylases (PHD) hydroxylate key proline residues in the oxygen-dependent degradation domains of HIFα (12–15). This post-translational modification enables recruitment of HIF by VHL to the VBC/Cul-2 complex subsequently leading to ubiquitination and degradation. The HIF PHDs are inhibited by low oxygen tension resulting in the accumulation of HIFα, assembly with the constitutively expressed HIFβ and activation of HIF target genes such as VEGF and glucose transporter-1 (Glut-1). VHL-defective RCC cells fail to degrade HIFα regardless of oxygen tension leading to the constitutive activation of its targets. Silencing of HIFα by short hairpin RNA (shRNA) prevents VHL −/− RCC tumor formation in a nude mice xenograft assay showing that the constitutive activation of HIF targets is required for VHL-defective RCC tumorigenesis (16). VHL −/− RCC cells are also unable to form an extracellular fibronectin matrix, which is thought to play an important role in RCC development (17, 18).

Whereas the role of HIF in promoting the highly vascularized phenotype of VHL loss RCC tumors is well characterized, the mechanisms by which this transcription factor can promote tumorigenesis remain debatable (19, 20). There are two active forms of HIFα called HIF-1α and HIF-2α, which activate common as well as distinct targets (21). Recent reports have led to the suggestion that HIF-2α is the oncogenic form of HIFα, at least in human RCC (22–24). This would imply that HIF-2α differs from HIF-1α in its ability to activate a specific target(s) that is involved in oncogenic growth of RCC cells. We recently reported that HIF activates the transforming growth factor-α/epidermal growth factor (TGF-α/EGFR) pathway in VHL −/− RCC cells (25). We suggested that HIF-mediated constitutive EGFR activation provides permanent self-sufficiency in growth signaling that drives the growth autonomy of VHL-defective RCC cells, a hallmark of cancer (26). In this report, we put forward an explanation for the differential oncogenic potential of HIF-2α in RCC cells by...
identifying TGF-α as a HIF-2α-specific target. We show that HIF-2α, but not HIF-1α, is able to promote in vitro and in vivo tumorogenesis of VHL−/− RCC cells by constitutively activating the TGF-α/EGFR oncogenic pathway. Transient and stable silencing of EGFR is sufficient to prevent HIF-2α-dependent tumorogenesis in multiple VHL−/− RCC cell lines. These data reaffirm EGFR as a crucial therapeutic target for VHL-defective renal cancer treatment.

Materials and Methods

Cell culture and reagents. Normoxic cells were incubated at 37°C under a 5% CO2 environment. Hypoxia was achieved by incubation in a hypoxic chamber at 37°C under 1% O2, 5% CO2, and N2-balanced atmosphere. VHL-deficient, 786-0 and A498 RCC cells, and HCT116 colon carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD). H062, MCF7, SKME1, and PC3 cells were a kind gift from John Bell (Ottawa Regional Cancer Center, Ottawa, Ontario, Canada) and U87MG cells a kind gift from Ian Lorimer (Ottawa Regional Cancer Center, Ottawa, Ontario, Canada). 786-0 and A498 cells stably transfected with HA-VHL (786-0 + VHL and A498 + VHL, respectively) were a kind gift from Dr. W.G. Kaelin (Harvard University, Boston, MA). KTCI140 cell line was a kind gift from Dr. Peter Ratcliffe (University of Oxford, Oxford, United Kingdom). Primary cultures of human renal cortical epithelial cells were purchased from Clonetics (San Diego, CA). Serum containing medium consisted of DMEM or McCoy’s 5A (HCT116) medium with 10% fetal bovine serum (FBS). Serum-free medium consisted of DMEM supplemented with 1% insulin-transferrin-selenium (Invitrogen, Burlington, Ontario, Canada). PD153035 (Calbiochem, San Diego, CA) was used as indicated. Treatment of VHL-deficient cells with antisense targeting TGF-α was carried out as previously described (27).

Adenovirus construction. Adenoviruses encoding the HIF-α mutants, VHL, GFP, and DN-HIF were generated through Cre-lox recombination as described elsewhere (25, 28). Vectors encoding HIF-α subunits mutated at proline hydroxylation sites encoding HA-HIF-2α (P405A and P531A) and HA-HIF-1α (P402A and P564A) were a kind gift from Dr. W.G. Kaelin. cDNA encoding the constitutively active variants of HIFα were transfected by BamHI/NotI restriction digest from their original pcDNA 3.0 vector and subcloned, in-frame, downstream of a Flag-GFP moiety previously cloned into the HindIII and NotI sites of a pAdlox adenoviral vector. DNA sequencing analysis confirmed the alanine substitutions of the targeted proline residues. Experiments used approximately equal multiplicity of infection.

RNA isolation and reverse transcription-PCR analysis. One microgram of total RNA using TRIzol isolation reagent (Roche, Canada). Reverse transcription-PCR (RT-PCR) reactions were done using 5 ng of total RNA using Taqman One-Step RT-PCR master mix reagents (Applied Biosystems, Streetville, Ontario, Canada) and 0.25 μmol/L 5′-VIC TGF-α and 5′-6FAM actin modified probes. Cycling conditions were 30 minutes at 48°C, 10 minutes at 95°C, then 40 cycles of alternating 15 seconds at 95°C and 1 minutes at 60°C. Amplification and analysis of real-time data was done using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The quantity of TGF-α detected in each reaction tube was normalized to the level of coamplified actin endogenous control. Each RNA sample was analyzed in triplicate to obtain an average normalized quantity of TGF-α transcript (arbitrary units). TGF-α forward GACAGTTCGCCGTGATG and TGF-α reverse CAGGTTTCCATGAGAACGAA-GGA TGF-α probe TTTTATAATCGCTCCAGATTCCTCACACTCA; β-actin probe CCGCCGCGGCCTCCACACC CGC. The sequences all other primers used are described elsewhere (25).

Bromodeoxyuridine labeling. Cells were plated at low density on coverslips and incubated overnight in DMEM supplemented with 10% FBS. At the start of the experiment, cells were washed and supplemented with fresh serum-containing or serum-free medium. The cells were infected with adenoviruses as indicated. After the indicated time cells were fixed and stained with an anti-bromodeoxyuridine (BrdUrd) antibody according to instructions of manufacturer (Roche). Nuclei were stained (Hoechst 33258; Sigma, St. Louis, MO) and assessed for BrdUrd incorporation using a Zeiss Axiovert S100TV microscope (Thornwood, NY). Data is presented as proportion of nuclei incorporating BrdUrd.

Transforming growth factor-α ELISA. An equal number of cells wereseed approaching confluence and allowed to settle overnight in DMEM supplemented with 5% FBS. The appropriate cells were infected with adenoviruses after addition of fresh culture medium. Medium and total cell lysates were collected and analyzed for TGF-α protein according to instructions of manufacturer (Oncogene, Boston, MA). Total protein concentration was determined using the bichinchoninic acid protein assay reagent (Pierce, Rockford, IL) to ensure equivalence between samples. Antisense to TGF-α experiments were as described previously (27).

Western blot. Cells were washed with PBS and harvested in 4% SDS in PBS. Samples (20-100 μg of each) were separated on denaturing polyacrylamide gels containing SDS and transferred to membrane-activated polyvinylidene difluoride membrane (NEN, Boston, MA). Membranes were blocked in skimmed milk before incubation with Flag (Sigma) or EGFR (Ab-12; LabVision, Fremont, CA) monoclonal antibodies. Polyclonal antibodies were used to detect py-EGFR (sc-12551; Santa Cruz Biotechnology, Santa Cruz, CA), HIF-2α (Novus, Littleton, CO), Glut-1 (Alpha Diagnostic International, San Antonio, TX), and actin (Sigma). After washing with 0.2% Tween-PBS solution, membranes were blotted with secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) and detected by enhanced chemiluminescence (Pierce).

Immunofluorescence. Cells were grown to confluence for 6 days on glass coverslips, washed thrice with PBS, and fixed/permeabilized in prechilled 95% ethanol at –20°C for 30 minutes. Ethanol was aspirated and residual was allowed to air dry at 4°C. Cells were stained for 1 hour at room temperature with anti-fibronectin antibody as described in (29). Staining was visualized with a Zeiss Axiovert S100TV microscope.

Epidermal growth factor receptor RNA interference. For transient inhibition of EGFR mRNA production, VHL-deficient RCC cells were transfected with commercially available double-stranded 21-nucleotide-long small interfering RNA (siRNA) targeting the EGFR or a control siRNA (Ambion, Austin, TX). VHL-deficient RCC cells were also stably transfected to express one of two different siRNA sequences targeting the EGFR (30). For each sequence, two ssDNA oligonucleotides were synthesized (Invitrogen) and subsequently annealed by incubation for 3 minutes at 90°C followed by 1 hour at 50°C, ssDNA were designed with overhangs encoding restriction sites for BamHI/HindIII, and the annealed products were ligated directly into the pSilencer 3.1-H1 neo vector (Ambion). Sequence 1 (5'-3') shRNA: EGFR-1 forward GATCCCAACCTGAGGAGAGAAGTTCAGAGAACATTTTCTCCACCGATTGGTGGGA and shRNA EGFR-1 reverse AGCTTTCCAAAAAAATCTCGGAGGAAGATTCTCTTGATACGACCTTTTTTTTGGAAA; Sequence 2 (5'-3') shRNA: EGFR-2 forward GATCCCAACCTGAGGAGAGAAGTTCAGAGAACATTTTCTCCACCGATTGGTGGGA and shRNA EGFR-2 reverse AGCTTTCCAAAAAAATCTCGGAGGAAGATTCTCTTGATACGACCTTTTTTTTGGAAA; Sequence 3 (5'-3') shRNA: EGFR-3 forward GATCCCAACCTGAGGAGAGAAGTTCAGAGAACATTTTCTCCACCGATTGGTGGGA and shRNA EGFR-3 reverse AGCTTTCCAAAAAAATCTCGGAGGAAGATTCTCTTGATACGACCTTTTTTTTGGAAA; Sequence 4 (5'-3') shRNA: EGFR-4 forward GATCCCAACCTGAGGAGAGAAGTTCAGAGAACATTTTCTCCACCGATTGGTGGGA and shRNA EGFR-4 reverse AGCTTTCCAAAAAAATCTCGGAGGAAGATTCTCTTGATACGACCTTTTTTTTGGAAA. A pSilencer 3.1-H1 neo vector encoding control shRNA was also purchased from Ambion. Stable clones were selected in neomycin containing medium. All transfections were conducted with Effectene transfection reagent (Qiagen, Valencia, CA). All constructs were verified by standard DNA sequencing.

In vitro tumor spheroid. Multicellular spheroids were prepared by the liquid overlay technique (31–33) and as described by our group elsewhere (34). Briefly, 24-well plates were coated with 250 μL of preheated 1% Seaplaque agarose (Cambrex, Rockland, ME) in serum-free medium; 105 of indicated cells were plated in 1 mL of DMEM per well. To promote cell-cell adhesion, the plates were gently swirled (32 times) 30 minutes after plating. Spheroids were grown for 6 days at 37°C under 5% CO2 in serum-containing medium. Spheroids were harvested in lysis buffer (4% SDS in PBS) before
immunoblotting. Alternatively, spheroids were fixed in 10% formaldehyde, embedded in paraffin, sectioned, mounted on slides, and stained with H&E.

**Nude mouse xenograft assays.** Nude mouse xenograft assays were done as described elsewhere (4). In brief, 10^7 viable cells (trypsin digestion method) were injected s.c. in the flanks of female nude mice (Charles River, Wilmington, MA). Mice injected with both control and EGFR shRNA were sacrificed 9 weeks post-injection according to facility protocol (University of Ottawa). In keeping with the Animal Facility Guidelines to examine latent tumor formation, a subset of mice was only injected with cells expressing shRNA targeting EGFR. These mice were sacrificed 16 weeks post-injection and did not exhibit any latent tumor formation. Tumor size was measured weekly, and at the time of sacrifice tumors were excised and weighed. Experiments were done blinded.

**Results**

**Hypoxia-inducible factor-2α specifically promotes growth autonomy of renal cell carcinoma cells.** VHL loss RCC cells constitutively overproduce HIF-regulated genes as a consequence of the inability of these cells to degrade HIFα in normoxia (8). Constitutive HIF activation is required for serum-independent growth of VHL^-/-^ RCC cells in culture as well as for tumor formation in a xenograft nude mouse tumor assay (16, 25). In addition, constitutive expression of HIF-2α, but not HIF-1α, overrides VHL-mediated RCC tumor suppression (22, 24). To further investigate the role of the two HIFα forms in RCC tumorigenesis, we produced adenoviruses that express HIF-1α and HIF-2α variants that evade VHL recognition in the presence of oxygen (henceforth called HIF-1α and HIF-2α; Fig. 1A schematic). To do so, key proline residues in the ODD domain of HIF were substituted to alanine, which prevents recognition and degradation by VHL in normoxic cells (Fig. 1A; see ref. 22). HIFα variants were expressed to similar levels and were functional as they equally promoted the accumulation of Glut-1 mRNA, a well-characterized HIF-regulated gene, in normoxic VHL-competent cells (Fig. 1A). In the absence of exogenous growth factors or serum, VHL-deficient cells are able to engage in autonomous growth, a hallmark of cellular transformation (26). Reintroduction of VHL in VHL-defective cells enables these cells to respond like primary renal epithelial cells and quiesce, a process measured by a decrease in BrdUrd incorporation (Fig. 1B; ref. 25, 35). As previously shown, dominant-negative HIF (DNHIF) abolished BrdUrd incorporation by VHL-deficient RCC cells in serum-free medium, thereby demonstrating that the observed autonomous proliferation of these RCC cells is a consequence of HIF activation (Fig. 1B; ref. 25). Addition of serum restored BrdUrd incorporation in all conditions showing that RCC cells are equally sensitive to exogenous growth factors, regardless of VHL status or HIF activation (Fig. 1B). We asked whether HIF-1α or HIF-2α activation could promote growth autonomy of RCC cells by overriding the effect of reintroduction of VHL in VHL-defective RCC cells. Expression of HIF-1α had no discernable effect on growth of VHL-competent cells in the presence or absence of serum nor did it promote cell death or dominant cell cycle arrest in the presence of serum, as reported for other cell lines (Fig. 1C; data not shown; see ref. 36). In contrast, expression of HIF-2α was sufficient to promote autonomous growth of RCC cell lines overriding the effect of stable expression of VHL (Fig. 1C). These experiments show that HIF-2α-specific transcriptional activity differs from HIF-1α and enables VHL-competent cells to engage in autonomous growth consistent with its ability to promote tumorigenesis in vivo (22).

**Transforming growth factor-α is a hypoxia-inducible factor-2α-specific target.** Next, we wanted to uncover the
mechanism by which HIF-2α differs from HIF-1α and promotes autonomous proliferation. We recently reported that overproduction of TGF-α, a bona fide epithelial cell mitogen, by RCC cells is triggered by HIF activation (25). Thus, we suspected that TGF-α might play a key oncogenic role in HIF-2α-mediated autonomous growth. As shown in Fig. 2A, TGF-α mRNA is hypoxia-inducible in primary cultures of renal epithelial cells, the cell type from which VHL loss RCC arises (1, 37–39). VHL loss RCC cells overproduce TGF-α mRNA in normoxia, which can be down-regulated by the reintroduction of VHL (Fig. 2B; refs. 27, 40). Whereas HIF-1α and HIF-2α equally promoted Glut-1 transcription in normoxic VHL-competent cells, TGF-α mRNA induction was observed only in cells expressing HIF-2α (Fig. 2B). TGF-α protein was present in cellular lysates of HIF-2α-, but not HIF-1α-infected, VHL-competent cells (Fig. 2C). In addition, quantitative RT-PCR experiments showed that TGF-α is a target of endogenous, wild-type HIF-2α in hypoxic VHL-competent cells or VHL-defective RCC 786-0 cells (Supplementary Fig. S1). An RT-PCR screen revealed that TGF-α is the only known EGFR ligand to be activated by HIF-2α or by VHL loss (data not shown; see ref. 25 for the screen in VHL loss RCC cells).

We next asked if HIF-2α was able to activate EGFR through production of TGF-α ligand. VHL-deficient 786-0 RCC cells
expressing endogenous HIF-2α displayed strong EGFR phosphorylation, in the absence of exogenous growth factors (Fig. 2D). Expression of VHL or DNHIF was essentially as efficient as PD15035 at abolishing EGFR phosphorylation in VHL-deficient RCC cells. In addition, antisense-mediated inhibition of TGF-α production blocks autonomous growth of VHL-deficient cells (27) and abrogates EGFR phosphorylation (Fig. 2F). Expression of HIF-2α resulted in a marked activation of the EGFR in VHL-competent 786-0 (Fig. 2F) and A498 cells (data not shown). In contrast, HIF-1α did not activate the EGFR, further supporting the notion that HIF-1α is unable to drive the production of active EGFR ligands (Fig. 2F). HIF-2α-specific induction of TGF-α mRNA was observed in the majority of tested cancer cell lines maintained in normal oxygen tension (Fig. 2G). No significant TGF-α transcript or protein was detected in HIF-1α-expressing cells, although HIF-1α was as efficient as HIF-2α at promoting Glut-1 mRNA accumulation (Fig. 2G; data not shown). Furthermore, activation of the EGFR was observed only in cancer cell lines where HIF-2α induced TGF-α production. These results support the hypothesis that HIF-2α drives autonomous proliferation by activating EGFR through the specific production of TGF-α. The data also suggest that HIF-1α activation alone is insufficient to promote serum-free growth of VHL-competent RCC cells.

Transient silencing of epidermal growth factor receptor by small interfering RNA prevents formation of dense renal cell carcinoma tumors in vitro. We next asked whether EGFR activation plays a central role in the ability of HIF-2α to drive VHL loss RCC tumorigenesis. To do so, a panel of VHL-defective cancer cell lines was transfected with EGFP-tagged HIF-1α or HIF-2α. Following transient transfection, these cells were serum-starved for 72 hours and then treated with serum. The expression of EGFR was monitored by immunoblotting. The results showed that EGFR expression was significantly reduced in cells transfected with HIF-2α compared to those transfected with HIF-1α.

Figure 3. Transient silencing of EGFR with siRNA prevents serum-free growth and formation of dense tumor spheroids. A, transient incubation with siRNA directed against EGFR mRNA suppresses EGFR protein production in multiple VHL-defective cells. Immunoblots were done to detect EGFR in untreated cells, cells treated with effective, control siRNA, or siRNA against EGFR. B-C, siRNA directed against EGFR prevents serum-independent growth of multiple VHL–/– RCC cells. Cells were incubated in the presence of 10% serum or in the absence of serum for 72 hours followed by addition of serum for 48 hours. BrdUrd labeling was for 3 hours in all conditions. Transient incubation with siRNA was as described in Materials and Methods. PD153035 was added for the duration of the experiments without noticeable toxic effects. Columns, average mean of at least three independent experiments in triplicates; bars, SE. D-E, siRNA directed against EGFR prevents dense spheroid formation. In vitro tumor spheroids were produced as described in Materials and Methods for 6 days in the presence or absence of control or siRNA directed against EGFR. Histology from spheroids is visualized at a magnification of 400×. Immunoblot detection of EGFR shown in (D) was from lysates of spheroids incubated for 6 days to show inhibition of EGFR protein production by siRNA. F, density was measured at 400× magnification in four independent spheroids in at least three sections per spheroid and reported as nuclei/field. Columns, average mean of at least three independent experiments in triplicates; bars, SE.
RCC cell lines was incubated in the presence of a siRNA directed against VHL-defective RCC 786-0 and A498 cell lines. In addition, we used the KTCL140 line reported to harbor VHL inactivating mutations and overexpress HIF-2α and display strong EGFR phosphorylation that can be repressed by reintroduction of VHL or expression of DNHHIF (data not shown, Fig. 5). The siRNA efficiently decreased levels of EGFR protein in VHL −/− RCC cells (Fig. 3A) and, importantly, prevented HIF-2α-mediated autocrine growth as efficiently as the EGFR tyrosine kinase inhibitor PD153035 for 786-0 and A498 (Fig. 3B-C). The effect of silencing EGFR on growth was restricted to serum-free conditions because addition of fresh serum abolished the growth inhibitory effect of the siRNA, as expected. These data show that the ability of HIF-2α to drive growth autonomy of VHL −/− RCC cells requires activation of EGFR.

VHL-deficient RCC cells, like most tumorigenic cell lines, are able to form dense tumor spheroids in vitro (33), an accepted assay that measures the tumorigenic potential of cancer cells (31, 32). This assay has also the advantage of measuring tumorigenicity in the absence of neovascularization, a variable especially important in the case of the highly angiogenic VHL −/− RCC tumors. Tumor density was measured by nuclei/field in several sections of tumor spheroids. As shown in Fig. 3E, VHL-deficient RCC cells form highly dense in vitro tumors. In contrast, VHL-competent RCC cells are characterized by a loose arrangement of cells and a significant decrease of cell density (Fig. 3F). Transient incubation with siRNA against EGFR suppressed EGFR protein levels in the spheroids (Fig. 3D) and prevented formation of dense tumors in VHL −/− RCC 786-0 and KTCL140 cell lines to levels similar to those observed with reintroduction of VHL (Fig. 3E and F). These data show that siRNA to EGFR suppresses autonomous growth and dense tumor spheroid formation as efficiently as reintroduction of VHL.

Stable silencing of epidermal growth factor receptor suppresses tumorigenesis of von Hippel-Lindau–defective renal cell carcinoma. Based on the data obtained in Fig. 3, we decided to engineer VHL −/− RCC cells with stably inactivated EGFR by expression of shRNA. For these experiments, we used the VHL −/− RCC cells 786-0, which has been extensively characterized by several other groups (4, 33, 40, 41). The 786-0 cells form tumors...
in nude mice, which can be suppressed by reintroduction of VHL or shRNA-mediated inhibition of HIF-2α (4, 16). Several different shRNA targeted against different regions of the EGFR mRNA were tested for their ability to efficiently suppress EGFR protein production. After initial screening of several shRNA-expressing stable clones, two different shRNAs, called shRNA1 and shRNA2, were further characterized because of their ability to mediate a stable and efficient decrease in EGFR protein levels (data not shown). VHL−/− RCC 786-0 cells stably expressing shRNA1 or shRNA2 displayed a significant decrease in EGFR protein levels compared with parental cells and cells stably expressing control scrambled shRNA (Fig. 4A). Importantly, loss of phosphorylated EGFR was also observed in shRNA-expressing cells demonstrating a near-complete loss of EGFR function (Fig. 4A). As observed with transient inhibition of EGFR, we did not notice a significant difference in two-dimensional growth in the presence of serum between parental 786-0 cells, or 786-0 cells expressing either VHL, control shRNA or shRNA against EGFR mRNA. However, shRNA-mediated inhibition of EGFR restored the ability of VHL-defective RCC cells to withdraw from the cell cycle upon serum withdrawal, a process that could be rescued by the addition of fresh serum. The ability of the shRNA against EGFR to abolish growth autonomy of VHL-defective RCC cells was similar to that observed upon reintroduction of VHL, expression of DNHIF (Fig. 4B), transient incubation with siRNA against EGFR, treatment with PD153035 (Fig. 3) or incubation with anti-sense oligonucleotides against TGF-α mRNA (27). EGFR protein levels and phosphorylation were also significantly diminished in the KTCL140 line stably expressing shRNA1 (Fig. 4A). The effects of suppressing EGFR expression in KTCL140 cells were very similar to those shown for 786-0 cells (data not shown). Stable silencing of EGFR by shRNA resulted in low-density spheroids formation in 786-0 and KTCL140 cells (Fig. 4C). These experiments confirm that VHL-defective 786-0 clones stably expressing shRNA directed against EGFR mRNA display significant decrease in EGFR protein level, phosphorylation status, and activity without significant dominant effect on the cell cycle in medium supplemented with exogenous growth factors. The data also suggest that observations made in 786-0 can be generalized to other VHL−/− RCC cells.

We next wanted to examine the effect of EGFR knockdown on defects associated with VHL loss. shRNA-mediated silencing of EGFR did not affect HIF-2α protein levels and its ability to activate downstream targets, such as Glut-1 and TGF-α (Fig. 5A–B). In addition, EGFR knockdown failed to restore the ability of VHL−/− RCC 786-0 cells to form a fibronectin extracellular matrix (Fig. 5C; data not shown). These data indicate that RCC cells expressing shRNA against EGFR display all of the cancer-like biochemical characteristics associated with VHL loss including constitutive activation of HIF-2α-regulated genes and loss of fibronectin deposition. Finally, we examined the effect of EGFR knockdown on the tumorigenic potential of VHL-deficient RCC cells by injecting clones into nude mice and monitoring tumor formation for a period of 9 weeks. Parental 786-0 cells as well as 786-0 cells expressing vector alone or control shRNA cells produced large tumors detectable after 4 to 5 weeks. VHL−/− RCC cells expressing reintroduced VHL did not form tumors after 9 weeks of incubation (Fig. 6A–B). RCC 786-0 cells expressing either shRNA1 or shRNA2 directed against EGFR mRNA failed to form tumors after 9 weeks post-injection (Fig. 6A–C). It should be noted that prolong periods of incubation resulted in measurable tumor formation by VHL-competent RCC cells. In contrast, we were unable to detect small tumors in shRNA-expressing cells even after 15 weeks of incubation suggesting that EGFR knockdown abolishes latent tumor formation observed in VHL-competent RCC cells (data not shown). Similar data were obtained with VHL−/− RCC KTCL140 cells expressing shRNA1, although tumor size observed with KTCL140 cells
expressing control shRNA was smaller than that observed with the 786-0 cells (Fig. 6C). These results show that shRNA-mediated silencing of EGFR phenocopies the effect of reintroduction of VHL, or silencing of HIF-2α, suggesting that EGFR is a central downstream target of HIF-dependent tumorigenesis.

**Discussion**

We report that oncogenic EGFR signaling is involved in HIF-2α-dependent tumorigenesis in VHL loss RCC cells because silencing of EGFR prevents tumor formation in vivo, phenocopying the effect of HIF-2α silencing or reintroduction of VHL (4, 16). Kaelin et al. originally proposed that HIF-2α might act as an oncogene based on the ability of this transcription factor to override tumor suppression by VHL and drive tumorigenesis of human renal cancer cells (16, 22). Here, we provide a mechanistic explanation for HIF-2α-dependent tumorigenesis. We show that HIF-2α is able to promote autonomous growth of RCC cells by specifically activating the TGF-α/EGFR pathway. Silencing of EGFR is sufficient to block the oncogenic activity of HIF-2α and to prevent HIF-2α-driven tumor formation of VHL-defective RCC cells in vitro and in a xenograft nude mouse assay. In stark contrast, HIF-1α fails to promote autonomous growth of RCC cells and is unable to activate the TGF-α gene or the EGFR pathway. Our previous observation that overexpression of wild-type HIF-1α induced expression of TGF-α in VHL-competent 786-0 cells is explained by accumulation of endogenous HIF-2α, likely the result of saturation of the β-domain of VHL by overproduced wild-type HIF-1α (23). Our studies provide an explanation for the inability of this particular HIFα form to drive tumorigenesis of renal cancer cells. Inactivating mutations of the VHL gene confer a number of molecular defects to cells including improper assembly of a fibronectin matrix and constitutive HIFα activation. Despite these cellular flaws, silencing of EGFR was sufficient to block tumorigenesis overriding the effect of VHL loss, HIF-2α activation, and failed fibronectin deposition. The observation that transient or stable inactivation of EGFR prevented dense spheroids formation in the avascular in vitro tumor assay argues that suppression of tumorigenesis is not an indirect outcome of failed angiogenesis. Likewise, we showed that silencing of EGFR prevents autonomous growth of VHL-defective RCC cells providing further evidence that HIF-2α acts as an oncogene through activation of the TGF-α/EGFR pathway. It is also noteworthy that TGF-α expression can be induced by hypoxia in primary cultures of human renal epithelial cells, the cell type thought to give rise to RCC. We thus propose that VHL loss and subsequent HIF-2α activation, observed in early multicellular lesions of the distal nephrons, results in aberrant growth by eliciting permanent EGFR signaling by way of TGF-α activation (37). These data also explain why HIF-1α is not tumorigenic in RCC, which is most likely the consequence of the inability of this HIFα subunit to activate TGF-α/EGFR pathway. Immunohistochemical studies have shown expression of HIF-2α, but not HIF-1α, in premalignant multicellular foci in nephrons upon VHL loss, which is consistent with the inability of HIF-1α to drive EGFR activation and proliferation (24, 37). We therefore suggest that the oncogenic activity of HIF-2α is linked to its ability to activate TGF-α/EGFR pathway.

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**Figure 6.** Silencing of EGFR suppresses VHL−/− RCC tumor formation. A, representative nude mice injected with VHL−/− RCC 786-0 cells expressing vector alone, reintroduced VHL (VHL), control shRNA and shRNA-1 directed against EGFR. B, average of tumor mass 9 weeks post-injection of at least four injections per cell line. In all cases, we failed to detect a tumor even after 15 weeks following injection of shRNA to EGFR-expressing VHL−/− RCC cells; thus, no statistical analysis is shown. Small tumors were detected after ~ 12 weeks in the cases of 786-0 cells expressing reintroduced VHL. Notice that the tumor mass is lower in the KTCL140 cells compared with 786-0. Columns, average mean of tumor weight; bars, SE. C, cell line used in this study, number of injections, number of tumors observed after 9 weeks of incubation, and average size of tumors (±SE) when detectable. Experiments were blinded.
Hanahan and Weinberg proposed that the malignant phenotype is the manifestation of six essential alterations in cell physiology called hallmarks of cancer (26). Oncogenic dysregulation or environmental activation of HIF-2α may play a central role in at least two of these hallmarks. First, HIF-2α activation provides an environment of continued production of endogenous growth factor (TGF-α) and permanent EGFR signaling reducing the dependence of cancer cells on exogenous growth factors to maintain proliferation. Therefore, HIF-2α-expressing cells acquire the ability to engage in permanent EGFR signaling, which provides the necessary cellular milieu for growth autonomy. Whereas these data were obtained from renal cancer cells, we suggest that HIF-2α activation and subsequent EGFR signaling may have a broad implication in human malignancies. HIF-2α is induced in the vast majority of human cancer as a consequence of altered microenvironment conditions (e.g., hypoxia and acidosis) found in the core of tumors (34, 42). TGF-α overexpression and oncogenic dysregulation of EGFR activity are common features of human malignancies and are thought to provide permanent self-sufficiency in growth signaling that drives growth autonomy of cancer cells. Whereas still unproven, TGF-α/EGRF activation found in many tumors may be rationalized by HIF-2α activation and may explain why the hypoxic core of tumors is associated with increased probability of metastasis and poor prognosis. This hypothesis is supported by data shown in this report, which suggest that HIF-2α activation drives TGF-α production and EGFR activation in human cancer cell lines from different tissue origins. Second, although the data presented here focuses on the oncogenic potential of HIF-2α, its role in tumor angiogenesis has been previously well documented. HIF-2α, in cooperation with HIF-1α, activates genes such as VEGF and TGF-β to sustain proper tumor vascularization required for growth and metastasis, another hallmark of tumor angiogenesis.

Lastly, the data shown here supports the hypothesis that HIF-2α acts as an oncogene in VHL loss RCC by promoting EGFR signaling. VHL loss is associated with several cancer-like defects and HIF-2α activates an array of genes in RCC, some of which have generated interest as potential therapeutic targets to treat patients afflicted with RCC (43–45). The results shown here argue that the EGFR should remain a prime and bona fide therapeutic target for nonsurgical treatment of VHL-defective RCC. Consistent with our data, preclinical trials testing the effect of EGFR inhibitors in RCC have yielded encouraging results (46, 47). Whereas there have not been many large-scale randomized trials monitoring the effect of multiple EGFR inhibitors specifically targeting VHL loss RCC, a recent phase II trial of Gefitinib (Iressa) in stage IV or recurrent renal carcinoma did not alter outcome in those patients (48). One obvious explanation is that blocking EGFR phosphorylation is insufficient to prevent tumor growth of advance primary or metastatic RCC, which tumor cells may use alternative growth stimulatory pathways. Another possibility is that HIF-2α-mediated activation of the EGFR confers altered biochemical properties to receptor rendering it refractory to the inhibitory effect of these small molecules. Nonetheless, given that all cell lines used in this study were derived from patients with RCC, our findings support the search for effective therapies that target the EGFR pathway in RCC.

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