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The Role of the EGFR in HIF-2-driven VHL$^{-/-}$RCC Tumorigenesis

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The Role of the EGFR in HIF-2-driven $VHL^{-/-}$ RCC Tumorigenesis

By

Karlene Smith

This thesis is submitted as a partial fulfillment of
The M.Sc. program in Cellular and Molecular Medicine

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Statement of Contribution

A manuscript titled "Silencing of Epidermal Growth Factor Receptor Suppresses Hypoxia-Inducible Factor-2-Driven *VHL*$^{-/-}$ Renal Cancer", of which I am co-first author and that includes data reported here, is published in the June 15th issue of *Cancer Research* [Jun 15, 2005: 65(12)]. Experiments detailed in Figures 9A and 10 were performed by M. Morley. The experiments presented in Figure 9B replicate previous experiments in Gunaratnam *et al.* (2003), with additional experimental variants.

ABSTRACT

Inactivating mutations in the von Hippel-Lindau tumor suppressor gene are associated with renal cell carcinoma ($VHL^{-/-}$ RCC). The VHL protein targets the alpha subunits of hypoxia inducible factor (HIF) transcription factor for ubiquitination and degradation. $VHL^{-/-}$ RCC cells thus fail to degrade HIF-α, resulting in constitutive activation of HIF target genes and RCC tumorigenesis. Different HIF-α isoforms exist, however, in RCC tumor formation is associated with HIF-2α. We previously demonstrated that HIF-dependent transforming growth factor alpha (TGF-α) production and subsequent epidermal growth factor receptor (EGFR) activation drives autonomous growth of VHL-loss RCC cells. Here we show that TGF-α is a HIF-2 specific target, and that the EGFR is required for HIF-2-dependent RCC tumor formation. RNA interference-mediated silencing of EGFR expression was sufficient to abrogate the growth autonomy, *in vitro* tumor spheroid formation, and *in* vivo tumorigenesis of $VHL^{-/-}$ RCC cells. These data identify EGFR as a critical determinant of HIF-2-dependent tumorigenesis and confirm EGFR as a central therapeutic target in VHL-loss kidney cancer.

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LIST OF ABBREVIATIONS

BrdU	Bromodeoxyuridine
CTAD	carboxy-terminal transactivation domain
DMEM	Dulbecco's modified Eagle's medium
DNHIF	dominant-negative hypoxia inducible factor
dsRNA	double-stranded RNA
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbant assay
EPO	erythropoietin
FBS	Fetal Bovine Serum
FGF-β	fibroblast growth factor beta
GFP	green fluorescent protein
Glut-1	glucose transporter 1
H&E	hematoxylin and eosin
HIF	hypoxia inducible factor
HIF-α	hypoxia inducible factor alpha
HIF-β	hypoxia inducible factor beta
HRE	hypoxia-response element
ITS	insulin-transferrin-selenium
LOH	loss of heterozygosity
MEFs	mouse embryonic fibroblasts
MOI	multiplicity of infection
NCIC	National Cancer Institute of Canada
NTAD	amino-terminal transactivation domain
PBS	phosphate buffered saline
PDGF-β	platelet derived growth factor beta
PFK	Phosphofructokinase
PHD	prolyl hydroxylase
Py	phosphotyrosine

Rb	retinoblastoma
RCC	clear cell renal cell carcinoma
RISC	RNA-induced silencing complex
RTK	receptor tyrosine kinase
RT PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
shRNA	short hairpin RNA
siRNA	small interfering RNA
STAT	signal transducers and activators of transcription
TACE	tumor necrosis factor α converting enzyme
TGF-α	transforming growth factor alpha
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
Tsc-2	Tuberous Sclerosis
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

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INTRODUCTION

1.1 Cancer

1.1.1 The genetic basis of cancer

Cancer is a multifaceted disease with increasing prevalence in Western Society. According to the National Cancer Institute of Canada (NCIC), the rising incidence of cancer in Canada is primarily due to an aging population. During their lifetime, 38% of Canadian women and 43% of men will develop cancer, and approximately one out of every four Canadians will die of this disease (National Cancer Institute of Canada: Canadian Cancer Statistics 2004). Cancer is a disease involving dynamic changes in the genome. It is a multi-step process in which successive genetic changes drive the progressive conversion of normal human cells into malignant cells. These genetic alterations yield cells with defects in the cellular regulatory circuits that govern normal cell proliferation and that maintain tissue homeostasis, and thus yield cells with acquired selective growth advantages (Hanahan and Weinberg 2000). Elucidating the genetic defects and molecular processes that result in malignant transformation is of critical importance for successfully treating and/or curing this disease.

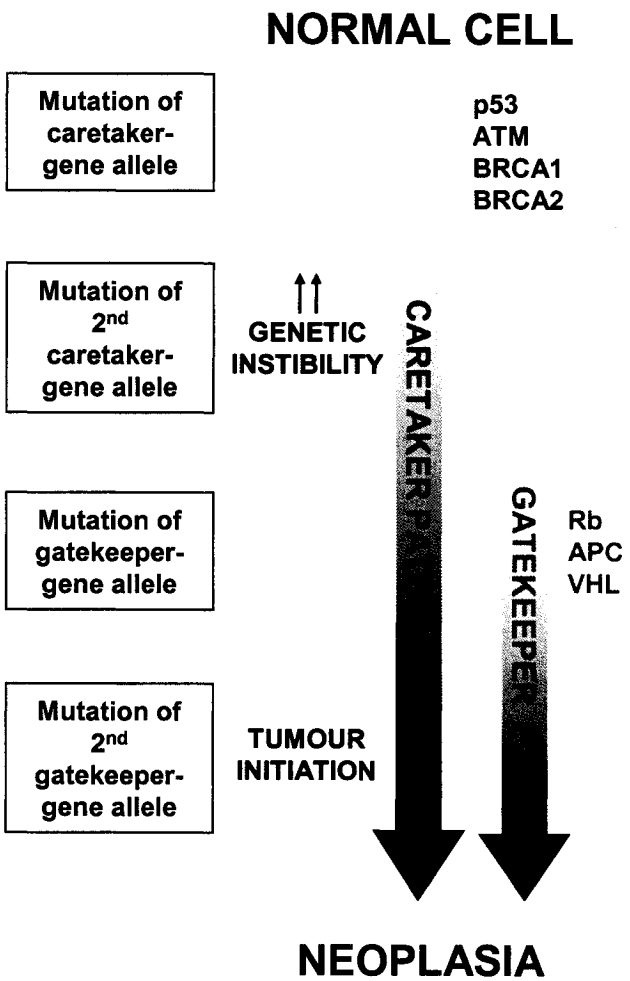
1.1.2 Oncogenes and tumor suppressor genes

Tumor promoting genetic alterations can be classed in two broad groups: mutations that convert normal genes into oncogenes, and mutations that inactivate the function of tumor suppressor genes. Oncogenes, such as myc and ras, promote deregulated cellular growth through a dominant gain of function mutation at a single allele of their normal cellular counterpart, the proto-oncogene. Conversely, mutations that inactivate tumor suppressor genes result in a recessive loss of function. Tumor suppressor genes can be further categorized as either gatekeepers or caretakers depending

on their cellular role (Kinzler and Vogelstein 1997). The product of gatekeeper tumor suppressor genes normally functions to inhibit cell growth or promote cell death. Biallelic loss of a gatekeeper tumor suppressor gene, such as retinoblastoma (Rb), can directly initiate tumor development (Figure 1) and leads to a very tissue specific distribution of cancer. Caretaker tumor suppressor genes, such as BRCA1 and BRCA2, function to maintain the integrity of the genome. The prototypes for this class of tumor suppressor genes encode proteins involved in DNA repair or chromosomal segregation (Levitt and Hickson 2002). Caretakers, sometimes referred to as stability genes, indirectly suppress neoplasia since their inactivation increases the probability of acquiring additional cancer-causing mutations of all genes, including gatekeepers (Figure 1; Kinzler and Vogelstein 1997).

According to Knudson's two-hit hypothesis (Knudson 1971), malignant transformation is initiated following loss of function mutations of both alleles within the same cell. In familial cancer syndromes, this can arise in an autosomal dominant hereditary fashion. When an individual inherits a germline mutation in a single copy of a tumor suppressor gene (the 'first hit'), only one somatic inactivation event in the remaining wild type copy of the gene is required to promote malignancy (the 'second hit'). Conversely, in a nonhereditary setting, an individual may acquire somatic inactivating mutations in each allele of a tumor suppressor gene of a single cell. According to this model, sporadic tumors arise in the same cell type as the hereditary cancer following somatic inactivating mutations of both alleles of the gene responsible for the hereditary cancer (Knudson 1985).

Figure 1. Tumor suppressor genes – pathways to neoplasia. Inherited mutations in either caretaker or gatekeeper tumor suppressor genes can predispose a person to neoplasia. However, in order to convert a susceptible predisposed cell to a malignant cell, additional genetic mutations are required. In the caretaker pathway this usually involves three additional mutations; though following inactivation of the second caretaker allele genetic instability hastens the amassing of the latter mutations. In the gatekeeper pathway only one additional inactivation event – in the second gatekeeper allele – is needed for tumor formation (modified from Kinzler and Vogelstein 1997).

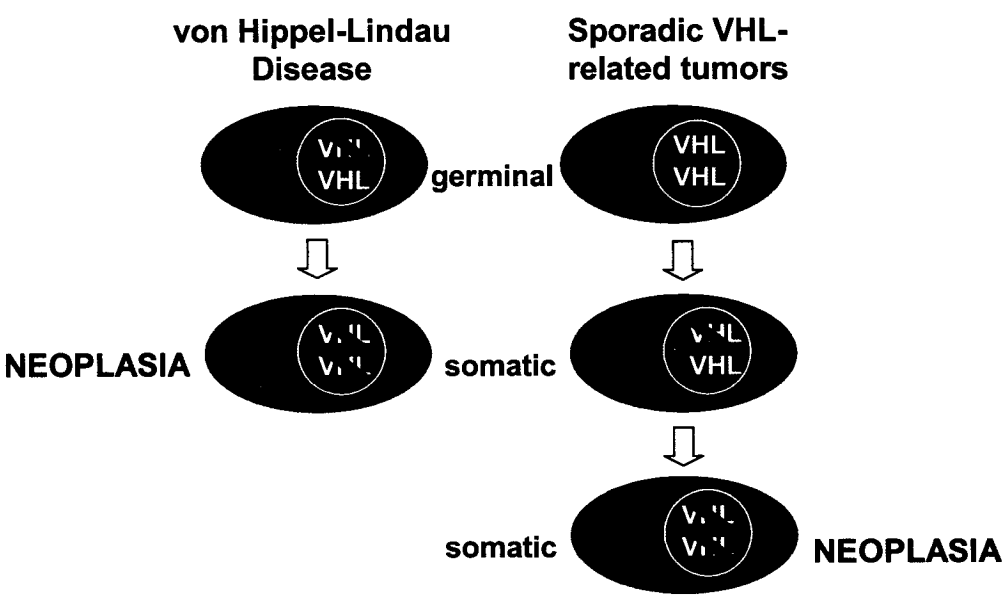

NORMAL CELL
Mutation of caretaker-gene allele
p53
ATM
BRCA1
BRCA2
Mutation of 2nd caretaker-gene allele
GENETIC INSTIBILITY
CARETAKER PATHWAY
Mutation of gatekeeper-gene allele
GATEKEEPER PATHWAY
Rb
APC
VHL
Mutation of 2nd gatekeeper-gene allele
TUMOUR INITIATION
NEOPLASIA

1.2 The VHL tumor suppressor gene - Genetics of VHL disease and sporadic RCC

Von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome that was first described in the medical literature over a hundred years ago by, amongst others, Eugene von Hippel and Arvind Lindau. People afflicted with VHL disease develop a variety of highly vascularized tumors. These include cerebellar haemangioblastoma, retinal angioma, pheochromocytoma, and clear cell renal cell carcinoma (RCC). VHL disease is a relatively rare disorder with an incidence of roughly one in thirty-six thousand births. The age at which clinical symptoms present is variable, although most patients exhibit tumor development between the ages of four to eight years, and die in mid-life due to unresectable hemangioblastomas or complications arising from primary renal tumors (Maher and Kaelin 1997).

The VHL gene resides on the short arm of chromosome 3 (3p25) (Tory *et al.,* 1989; Latif *et al.,* 1993). Early studies examining genetics associated with familial VHL disease and sporadic RCC found a consistent loss of heterozygosity (LOH) at chromosome 3p, and either mutations or epigenetic inactivation by hypermethylation of the remaining VHL allele (Latif *et al.,* 1993; Gnarra *et al.,* 1994; Herman *et al.,* 1994). Clinically, VHL disease is inherited in an autosomal dominant fashion (Maher *et al.,* 1991), and genetically VHL behaves as a typical tumor suppressor, as defined by Knudson's two hit recessive mechanism (Figure 2). Patients who have inherited a germ-line mutation of the VHL gene are predisposed to the development of the above-mentioned tumors. In these tissues, tumor formation arises from cells following somatic loss of function of the remaining wild-type copy of the VHL gene. Biallelic loss of VHL

Figure 2. Genetics of VHL-related tumors. According to Knudson's two-hit hypothesis, malignant transformation is initiated following recessive loss of function mutations of both alleles within the same cell. Individuals predisposed to the development of VHL disease inherit a germline mutation in one copy of the VHL tumor suppressor gene. To initiate neoplasia they need only one additional somatic mutation. Sporadic tumors form in individuals in a non-hereditary setting when both copies of the relevant gatekeeper gene are somatically mutated in the same cell.


von Hippel-Lindau Disease
Sporadic VHL-related tumors
VHL
VHL
germinal
VHL
VHL
NEOPLASIA
somatic
VHL
somatic
NEOPLASIA

function is also found in the majority of sporadic RCCs (Gnarra *et al.,* 1994; Motzer *et al.,* 1996). The VHL gene is categorized as a gatekeeper tumor suppressor gene, as its biallelic inactivation ($VHL^{-/-}$) is associated with a significant increased probability of tumorigenesis (Kaelin and Maher 1998). Consistent with this classification, reintroduction of wild type VHL into VHL-deficient RCC cells is sufficient to prevent tumor formation in nude mice (Iliopoulos *et al.,* 1995).

1.3 Clinical treatment for kidney cancer

RCC is the most common malignancy of the human kidney, accounting for about 75% of all renal neoplasms (Linehan *et al.,* 2003). According to the NCIC, approximately 1 in 80 Canadians will develop kidney cancer during his/her lifetime, and 1 in 90 Canadians will die from this disease. Surgery remains the mainstay treatment for kidney cancer, as an incomplete understanding of the molecular mechanisms underlying the disease has limited the development of successful non-surgical therapies. In fact, there are no effective treatments for the majority of patients diagnosed with advanced metastatic disease, which is the case of most patients (Bukowski and Novick 2000). Systemic treatment of RCC patients with interferon-α or interleukin-2 is the most common therapeutic approach following surgery. However, interferon-α treatment only improves the median time of disease progression from 4 months (historical control data) to 4.7 months. As a consequence, the prognosis for patients with renal cancer is often bleak, with a five-year survival rate of less than 10% (Motzer *et al.,* 1996).

1.4 VHL loss is associated with several cancer like properties

Hanahan and Weinberg (2000) proposed that the malignant phenotype is the manifestation of six essential alterations in cell physiology, referred to as hallmarks of cancer. These acquired capabilities, which are common to most human tumors and distinguish cancer cells from their non-cancerous counterparts, include self-sufficiency in growth signals, inappropriate cellular proliferation, lack of differentiation, limitless replicative potential, sustained angiogenesis, and propensity toward tissue invasion and metastasis. RCC tumors display several of these neoplastic characteristics, and re-introduction of VHL into VHL-deficient RCC cells is sufficient to correct these acquired traits.

1.4.1 Autonomous growth

Normal cells are dependent on exogenous growth signals for proliferation. They need to be supplied with diffusible mitogenic factors to maintain an active proliferative state when cultured outside of the body. Upon serum withdrawal, normal cells will exit the cell cycle and quiesce (Herlyn *et al.,* 1990), whereas one of the key characteristics of cancer cells is their ability to grow in the absence of exogenous growth factors – a capability referred to as autonomous growth (Hanahan and Weinberg 2000). Cancer cells can often produce and release the polypeptide growth factors they require for their own proliferation and become independent of the need for exogenous growth factor stimulation for growth. A similar end result can occur when cells express mutant growth factor receptors, which signal constitutively even when no ligand is bound, or through unregulated or constitutive activation of molecules involved in intracellular signal transduction cascades (Hanahan and Weinberg 2000; Blume-Jensen and Hunter 2001).

RCC cells behave like most other cultured cancer cells and are able to proliferate in serum-free conditions. VHL-deficient RCC cells grow in the absence of exogenous growth factors, whereas the re-introduction of VHL results in cell cycle arrest (Pause *et al.,* 1998). The means by which $VHL^{-/-}$ RCC cells are able to engage in autonomous growth will be further discussed and examined in this thesis.

1.4.2 Angiogenesis

Another hallmark of cancer cells is the ability to sustain proper tumor vascularization, required for growth and metastasis. Angiogenesis is the process by which new blood vessels are formed and grow from existing vascular endothelium. Like untransformed cells, tumor cells require oxygen and nutrients for their survival, and the ability of tumor cells to induce and sustain angiogenesis is a feature of malignancy (Hanahan and Weinberg 2000). VHL-associated tumors are highly vascularized, and VHL-deficient RCC cells overproduce angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor beta (FGF-β). Overproduction of these factors, involved in vascular development, is also correctable by the re-introduction of VHL (Gnarra *et al.,* 1996b; Iliopoulos *et al.,* 1996).

1.4.3 Defects in extracellular matrix deposition and cellular differentiation

VHL-deficient RCC cells fail to deposit an extracellular fibronectin matrix, which is thought to play a role in RCC development. VHL-competent RCC cells are able to deposit an extracellular fibronectin matrix, and this is associated with the differentiated phenotype of these cells (Ohh *et al.,* 1998; Stickle *et al.,* 2004). Fibronectin is an extracellular glycoprotein that signals through integrin cell surface receptors. Loss of fibronectin matrix assembly is another characteristic that has long been recognized as a

feature of cellular transformation (Ruoslahti 1984), as is the ability of tumor cells to avoid cellular differentiation (Hanahan and Weinberg 2000). In a three-dimensional *in vitro* growth assay that mimics tumor formation *in vivo*, VHL-deficient cells form very dense tumor spheroids comprised of undifferentiated cells packed together with no apparent organization. This contrasts with VHL-competent RCC cells, which form cellular aggregates with tubular and trabecular like structures and proper fibronectin deposition (Lieubeau-Teillet *et al.*, 1998). In fact, Davidowitz *et al.* (2001) found that VHL mediates both the morphological and biochemical differentiation of renal proximal tubule epithelial cells. VHL can induce renal cell differentiation and mediate growth arrest through the integration of cell-cell and cell-extracellular matrix signals. Combined, these findings suggest that the only genetic alteration required for tumor formation and progression in human RCC is loss of VHL function.

1.5 Function of the VHL protein

1.5.1 The VHL protein

The VHL protein is encoded by three exons within a 20kb region of chromosome 3p25. VHL mRNA is widely expressed during embryogenesis and ubiquitously in adult tissues (Kessler *et al.*, 1995; Richards *et al.*, 1996). Widespread expression of VHL at the protein level has also been observed in adult tissues (Corless *et al.*, 1997). Two variants of the VHL protein exist. The full-length VHL protein is 213 amino acids and migrates with a molecular weight of about 30kDa (Iliopoulos *et al.*, 1995). A second form of the VHL protein is generated by translation initiation at an internal methionine, at residue 54, and this 160 amino acid protein migrates with a molecular weigh of about

19kDa. Both variants appear to retain similar biochemical and functional properties (Schoenfeld *et al.,* 1998), and will henceforth be generically referred to as VHL. Protein crystallography studies have identified two major domains within VHL: a β-domain spanning amino acids 63 to 154, and a smaller α-helical domain spanning residues 155 to 192 (Stebbins *et al.,* 1999). The α- and β-domains are 'hot spots' for cancer causing mutations observed in patients with VHL disease and sporadic RCC (Figure 3; Gnarra *et al.,* 1996a).

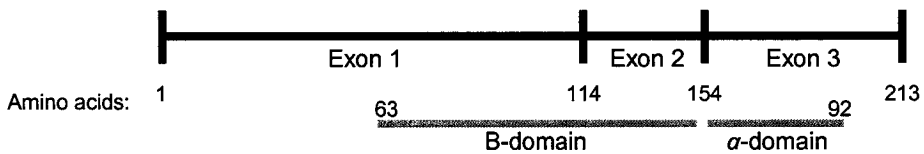
1.5.2 VHL is part of a multi-protein E3 ubiquitin ligase complex

The human VHL protein has no sequence homology to other known proteins (Latif *et al.,* 1993). Therefore, the cellular function of VHL was elucidated through studies examining VHL interacting proteins. Via its alpha domain, VHL binds to elongin C, which in turn nucleates a complex containing elongin B, cullin-2, and Rbx1 (Duan *et al.,* 1995; Kibel *et al.,* 1995; Pause *et al.,* 1997; Lonergan *et al.,* 1998; Kamura *et al.,* 1999; Stebbins *et al.,* 1999). This complex, referred to as the VBC/Cul2 (Figure 4A), shares sequence and structural similarities to the well characterized yeast SCF (Skp-1-Cdc53/Cullin-F-box) E3 ubiquitin ligase. This observation led to the initial suggestion that the VBC/Cul2 complex might similarly be involved in ubiquitin-mediated protein degradation, and that VHL serves as the substrate recognition particle of this complex (Pause *et al.,* 1999; Stebbins *et al.,* 1999). In agreement, VBC/Cul2 complex immunoprecipitates were subsequently found to exhibit ubiquitin ligase activity *in vitro*, when supplemented with an exogenous ubiquitin conjugating enzyme (Iwai *et al.,* 1999).

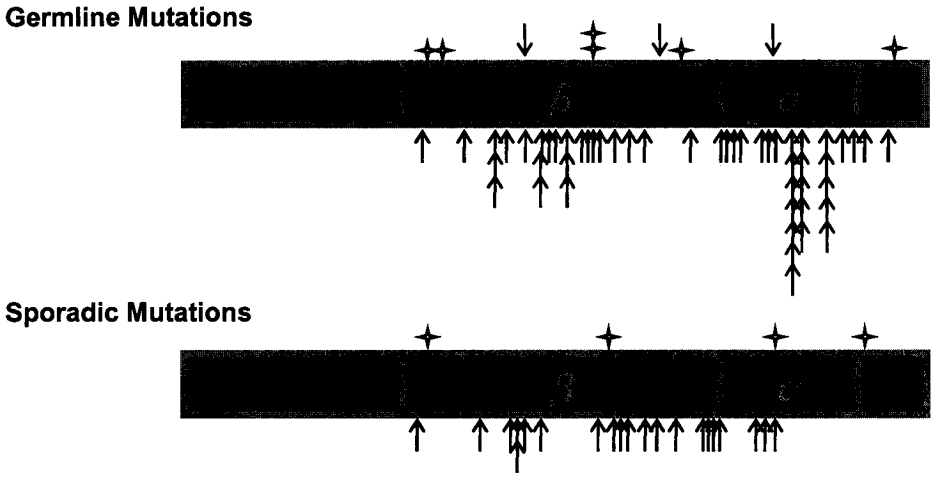
In ubiquitin-mediated proteolysis, specific proteins are targeted for rapid and irreversible degradation via the 26S proteosomal pathway. Three elements are involved

Figure 3. VHL mutations associated with inherited VHL disease and sporadic RCC. **A)** The full length VHL protein is 213 amino acids and is encoded by three exons. The VHL protein has two major domains: a β-domain spanning amino acids 63 to 154, and a α-helical domain spanning residues 155 to 192. **B)** Both the α- and β-domain are hotspots for mutations causing inherited VHL disease (top) and sporadic $VHL^{-/-}$ RCC (modified from Gnarra *et al.*, 1996a).

A.


Exon 1
Exon 2
Exon 3
Amino acids:
1
63
114
154
92
213
B-domain
α-domain

B.


Germline Mutations
Sporadic Mutations
Nucleotide substitution
Nucleotide insertion
Nonsense mutation
Nucleotide deletion

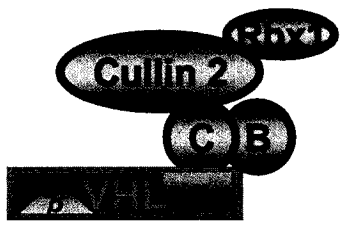
in the cascade of ubiquitin transfer reactions to target proteins for degradation: an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase (Figure 4B). The E3 ubiquitin ligase acts as the specificity factor in this reaction, functioning in substrate recognition, and facilitating the transfer of activated ubiquitin molecules, from E2, to lysine residues within the target protein or to a growing polyubiquitin chain (Ciechanover *et al.,* 2000).

1.5.3 The VBC/Cul2 E3 ubiquitin ligase targets hypoxia inducible factor-α for degradation

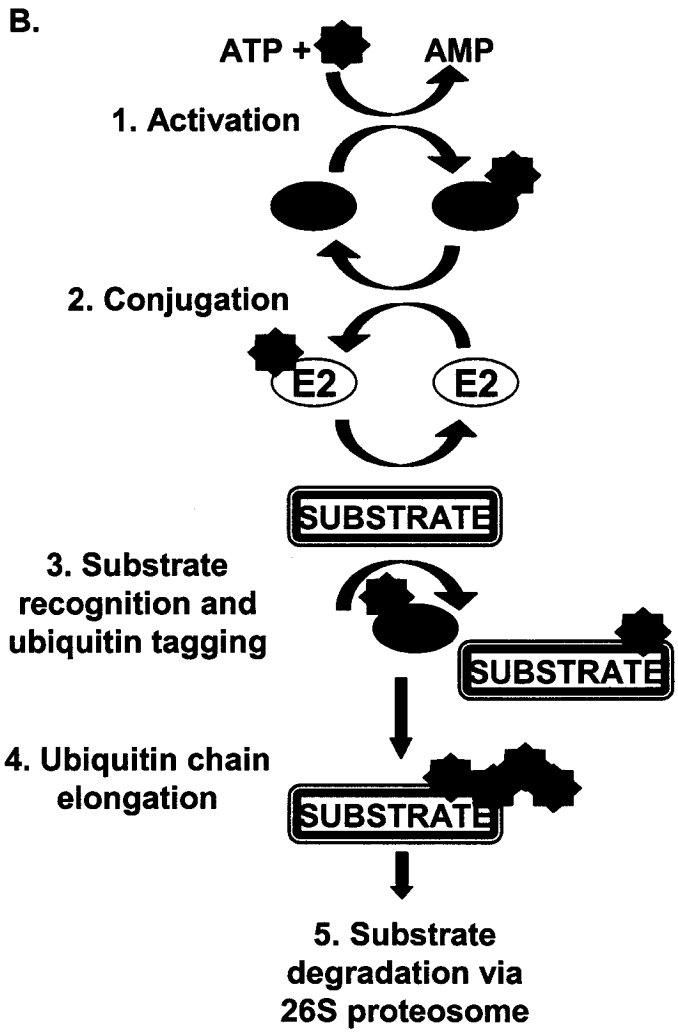
VHL-associated tumors are typically highly vascularized and overproduce angiogenic peptides such as VEGF and erythropoietin (EPO) (Maher and Kaelin 1997). Furthermore, in RCC cell lines, the absence of wild type VHL results in the deregulated expression of a set of genes. These include VEGF, glucose transporter 1 (Glut-1) and platelet derived growth factor beta (PDGF-β), which, like EPO, are all well characterized genes that are activated by oxygen deprivation. These hypoxia inducible genes are constitutively upregulated in VHL-deficient RCC cell lines, irrespective of changes in ambient oxygen, and this defect is correctable by restoration of VHL function (Gnarra *et al.,* 1996b; Iliopoulos *et al.,* 1996). Such observations led to the early hypothesis that VHL may be involved in sensing changes in oxygen tension and may have a hypoxia-associated protein as a target. Maxwell and colleagues examined the relationship between VHL expression and the transcription factor hypoxia inducible factor (HIF), which regulates the expression of the above mentioned hypoxia inducible genes

Figure 4. The VBC/Cul2 complex is an E3 ubiquitin ligase. A) The VHL protein is part of an E3 ubiquitin ligase complex. Via its alpha domain, VHL forms a stable complex with elongin B, elongin C, Cullin-2, and Rbx1. VHL serves as the substrate-recognition particle of this complex. **B)** Three enzymes are involved in the cascade of ubiquitin transfer reactions that target specific proteins for degradation via the 26S proteosomal pathway. ATP is required to activate ubiquitin molecules, and this is accomplished via an E1 ubiquitin activating enzyme. Next, ubiquitin is transferred from E1, via a thioester linkage, to an E2 ubiquitin conjugating enzyme. An E3 ubiquitin ligase facilitates the positioning and transfer of ubiquitin from E2 directly onto the target substrate. Chain elongation follows by conjugation of activated ubiquitin molecules (modified from Burger and Seth 2004).

A.


Rbx1
Cullin 2
C
B
VHL

B.


ATP +
AMP
1. Activation
2. Conjugation
E2
E2
SUBSTRATE
3. Substrate recognition and ubiquitin tagging
SUBSTRATE
4. Ubiquitin chain elongation
SUBSTRATE
5. Substrate degradation via 26S proteosome

(Wenger 2002), and found that VHL targets HIF's alpha subunits (HIF-α) for oxygen-dependent proteolysis (Maxwell *et al.,* 1999).

VHL functions as the particle recognition component of the VBC/Cul2 E3 ubiquitin ligase, binding HIF-α proteins through its β-domain docking site (Ohh *et al.,* 2000). In cells treated with a proteasome inhibitor the only proteins to newly co-immunoprecipitated with VHL are the HIF-α proteins, suggesting that these proteins might be the exclusive targets of the VBC/Cul2 E3 ubiquitin ligase (Cockman *et al.,* 2000). Moreover, the majority of mutations associated with VHL-loss RCC disrupt either VBC/Cul2 complex formation (α-domain mutations) or VHL HIF-α binding (β-domain mutations). This suggests that VHL-mediated HIF-α recognition, ubiquitylation, and subsequent proteosomal degradation serve as the primary function of this tumor suppressor in the kidney (Iwai *et al.,* 1999).

1.6 Hypoxia Inducible Factor (HIF)

1.6.1 HIF is a key regulator of cellular oxygen homeostasis

Under conditions of low oxygen tension (hypoxia) cells engage in an array of adaptive biological responses to activate signaling pathways that regulate proliferation, angiogenesis, cellular metabolism and cell death. The transcription factor HIF is one of the major regulators of the hypoxic response (Wenger 2000). HIF activates transcription of genes whose protein products function to maintaining tissue homeostasis under conditions of low oxygen tension and stress (Table 1). For example, in order to maintain an adequate ATP supply in the absence of oxidative phosphorylation, HIF activates glycolytic enzymes, such as aldolase, enolase, lactate dehydrogenase A, and

Table 1. Identified HIF-target genes*

Oxygen Transport: Erythropoiesis and Iron Metabolism	
Erythropoietin (EPO)	Erythropoiesis
Transferrin	Iron transport
Transferrin receptor	Iron uptake
Ceruloplasmin	Iron oxidation
Oxygen Transport: Vascular Regulation	
Vascular endothelial growth factor (VEGF)	Angiogenesis
Flt-1	VEGF receptor 1
Endocrine-gland-derived (EG)-VEGF	Angiogenesis
Plamsminogen activator inhibitor-1 (PAI-1)	Angiogenesis
Nitric oxide synthase (NOS)	NO production
Heme oxygenase 1	CO production
Adrenomedullin	Vascular tone
α_{1B}-adrenergic receptor	Vascular tone
Endothelin-1	Vascular tone
Anaerobic Energy: Glucose Uptake and Glycolysis	
Glucose transporters 1 and 3 (Glut-1 and -3)	Glucose uptake
PFKFB3	Glycolysis regulation
Phosphofructokinase L (PFKL)	Glycolysis
Aldolase A and C	Glycolysis
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Glycolysis
Phosphoglycerate kinase 1 (PGK1)	Glycolysis
Enolase 1	Glycolysis
Lactate dehydrogenase A (LDHA)	Glycolysis
Various	
p35srj	HIF-1 feedback regulation
Collagen prolyl-4-hydroxylase α_1	Collagen matrix formation
Intestinal trefoil factor	Growth factor
ETS-1	Transcription factor
Insulin-like growth factor 2 (IGF-2)	Growth factor
Insulin-like growth factor binding proteins (IGFBP) 1, 2 and 3	Transcription factors
Platelet derived growth factor (PDGF)-β	Growth factors/Angiogenesis
Transforming growth factor (TGF)-β_3	Growth factors/Angiogenesis

*Modified from Wenger, 2002 and Maynard and Ohh, 2004

phosphoglycerate kinase (PGK1). Other HIF target genes increase glucose uptake (e.g. Glut-1) and enhance oxygen transport to hypoxic tissues by promoting red blood cell production (e.g. EPO) and angiogenesis (e.g. VEGF) (Wenger 2002; Kewley *et al.*, 2004).

1.6.2 HIF-mediated gene activation

HIF is a heterodimeric transcription factor composed of a HIF-α subunit complexed with a HIF-β subunit (Wang *et al.*, 1995). Both HIF-α and HIF-β are members of the basic helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family of transcription factors. PAS is an acronym for first 3 proteins observed to contain this motif. PAS domains appear to cooperate with the HLH motif to facilitate heterodimerization between the bHLH-PAS family members, and are also necessary for basic domain-mediated DNA binding (Jiang *et al.*, 1996). HIF was first identified as a regulator of hypoxia-induced EPO expression (Wang and Semenza 1993), and the HIF binding site was later identified in the 3'promoter or enhancer region of a wide range of genes upregulated by hypoxia (Levy *et al.*, 1995; Norris and Millhorn 1995; Semenza *et al.*, 1996). The HIF binding sites of these genes, referred to as hypoxia-response elements (HREs) were found to contain the minimal consensus sequence 5'-RCGTG-3' (Levy *et al.*, 1995; Norris and Millhorn 1995; Semenza *et al.*, 1996).

Hypoxia inducible nuclear import and the recruitment of cAMP-response element-binding (CREB) binding protein (CBP)/p300 histone acetyltransferase co-activators are required for HIF-target gene expression (Arany *et al.*, 1996; Kallio *et al.*, 1998; Ema *et al.*, 1999). The interaction between HIF and the transcriptional co-activator CBP/p300 is reliant on a carboxy-terminal transactivation domain (CTAD) within the

HIF-α protein (Jiang *et al.,* 1997; O'Rourke *et al.,* 1999). Competitive peptide binding assays have revealed that blocking the interaction between HIF and CBP/p300 transcriptional co-activators attenuates the hypoxic induction of an HRE(EPO)-luciferase reporter construct by 80-90% (Kung *et al.,* 2000).

1.6.3 HIF-α isoforms

To date three different HIF-α subunits have been identified, all of which are regulated by VHL (Maxwell *et al.,* 1999; Maynard *et al.,* 2003). HIF-1α and HIF-2α are the two active HIF-α subunits. HIF-3α, the most recently identified and least well-characterized HIF-α isoform, consists of multiple splice variants, one of which has been suggested to act as a dominant negative HIF protein (Maynard *et al.,* 2003). HIF-1α and HIF-2α share 48% amino acid sequence identity, and are very similar in terms of genomic organization, protein structure, hypoxic protein stabilization, HIF-β heterodimerization, DNA binding, and transactivation of reporter genes (Ema *et al.,* 1997; Tian *et al.,* 1997; Wiesener *et al.,* 1998; Ema *et al.,* 1999).

Although HIF-1α and HIF-2α share similar biochemical properties, studies of knockout mice have shown that they are not redundant proteins. The knockout of either one is embryonically lethal but yields mice with different developmental defects. The knockout of HIF1-α generates mice with severe defects in vascularization (Iyer *et al.,* 1998; Kotch *et al.,* 1999), whereas HIF-2$\alpha^{-/-}$ mice exhibit abnormal lung maturation, bradycardia, and diminished catecholamine levels, on top of blood vessel defects (Tian *et al.,* 1998). Furthermore, studies have reported differences in the patterns of tissue expression for the HIF-α subunits: HIF-1α mRNA is ubiquitously expressed at low levels. HIF-2α is also widely expressed, but it is more abundantly produced in highly

vascularized tissues such as the heart, lung and liver (Ema *et al.,* 1997). As well, increasing evidence indicates that the different HIF-α isoforms may have their own unique transcriptional targets. HIF-1α is strongly implicated in the transcription of glycolytic enzymes (Hu *et al.,* 2003), whereas HIF-2α may be exclusive in its ability to transactivate the endothelial specific tyrosine kinase tie-2 gene (Tian *et al.,* 1997) and the VEGF receptor flk-1 gene (Kappel *et al.,* 1999). The different transcriptional activity of the different HIF-α isoforms may be due to differential recruitment of co-factors. Recently, Bracken *et al.* (2005) reported a novel and specific interaction of HIF-2α, but not HIF-1α, with the NF-κB Essential Modulator (NEMO). This interaction enhances normoxic HIF-2α transcriptional activity by aiding in the recruitment of the CBP/p300 transcriptional co-activator. The above-mentioned findings exemplify a growing body of evidence demonstrating distinct differences between HIF-1 and HIF-2 function.

1.7 The role of VHL in oxygen-dependent HIF-α regulation

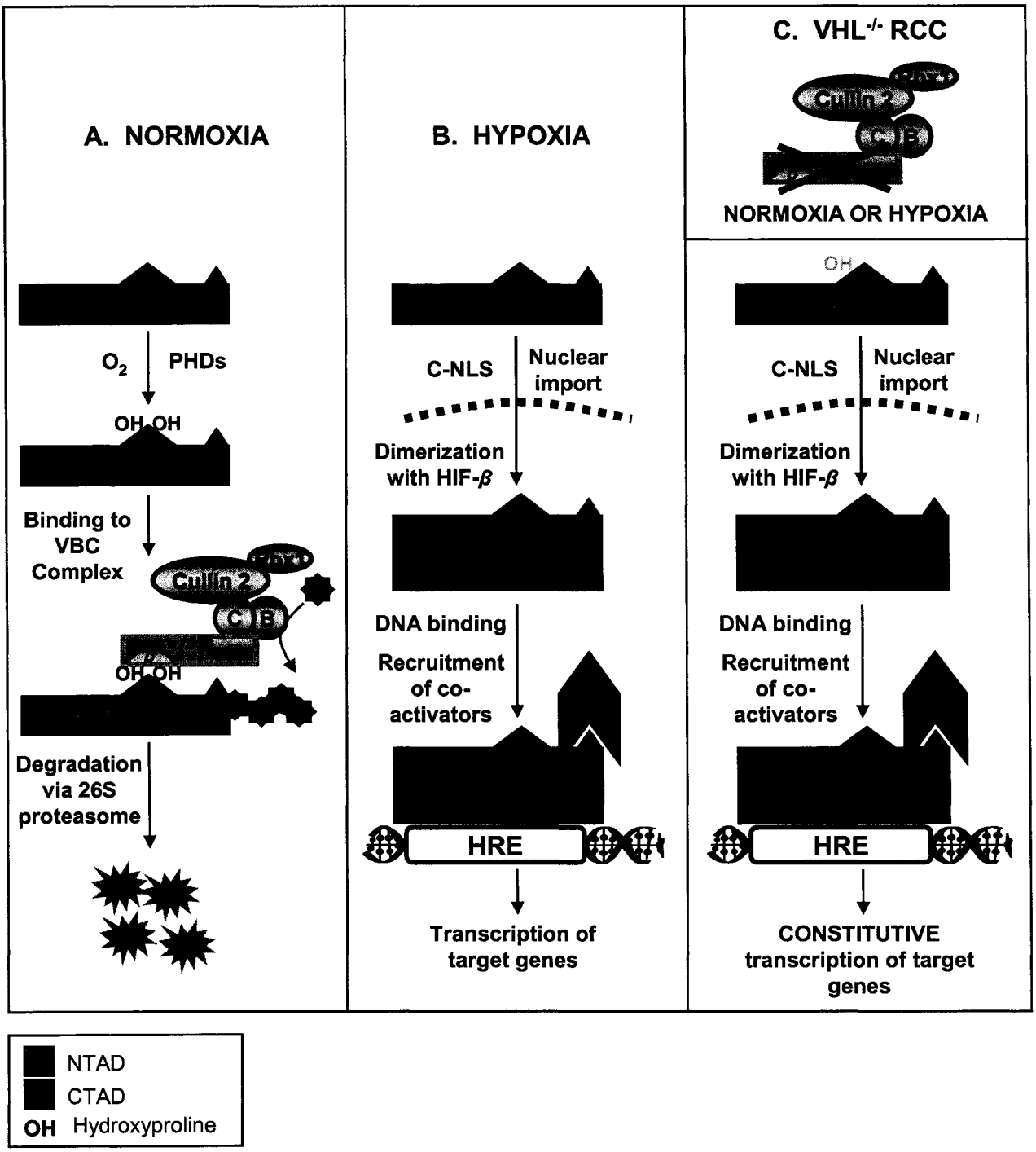
Activation of the heterodimeric HIF transcription factor involves oxygen-dependent regulation of HIF-α protein. HIF-α is unstable under normoxic conditions, while hypoxia significantly prolongs its half-life, allowing it to accumulate and couple with HIF-β, which is constitutively expressed (Huang *et al.,* 1996). Under normoxic conditions HIF-α subunits are rapidly degraded by the proteosome (Salceda and Caro 1997) in a VHL-dependent ubiquitylation process (Maxwell *et al.,* 1999). The interaction between VHL and HIF-α is regulated by oxygen tension. VHL-mediated HIF-α degradation is governed by hydroxylation of key proline residues within the oxygen dependent degradation domain (ODD) in HIF-α's VHL-binding domain. The VHL-

binding domain and ODD reside in an amino-terminal transactivation domain (NTAD) within the HIF-α protein. (Huang *et al.,* 1998; Sutter *et al.,* 2000; Ivan *et al.,* 2001 ; Jaakkola *et al.,* 2001; Masson *et al.,* 2001; Chan *et al.,* 2002). In the presence of oxygen, HIF prolyl hydroxylases (PHDs) hydroxylate the key proline residues (Epstein *et al.,* 2001; Ivan *et al.,* 2001). This post-translational modification enables VHL to recognize and recruit HIF-α to the VBC/Cul-2 complex, resulting in HIF-α ubiquitination and 26S proteosomal degradation (Figure 5A). In settings of low oxygen tension, the PHDs are inhibited, resulting in the accumulation of HIF-α, assembly with the constitutively expressed HIF-β, and activation of HIF target genes (Figure 5B). Cells lacking VHL are unable to degrade HIF-α, regardless of oxygen tension, resulting in the accumulation of HIF-α and the constitutive overexpression of genes transcribed by HIF (Figure 5C; Maxwell *et al.,* 1999).

1.8 HIF activation in cancer

HIF activation in the hypoxic tumor microenvironment induces proteomic changes that allow tumor cells to adapt to and overcome oxygen and nutrient deprivation and survive. This is accomplished by hypoxia-stimulated upregulation of proteins involved in glycolysis, tumor growth, inhibition of apoptosis, angiogenesis, and tumor invasiveness (Harris 2002; Vaupel 2004). Of clinical relevance, tumor hypoxia is associated with increased probability of metastasis, poor prognosis, and resistance to conventional therapies (Shannon *et al.,* 2003; Subarsky and Hill 2003). Upregulation of HIF-1α and HIF-2α protein expression has been observed in many cell types and tumors from various tissue origins, including bladder, brain, breast, colon, ovarian,

Figure 5. The VBC/Cul2 complex targets HIF-α for oxygen-dependent degradation. **A)** In the presence of cellular oxygen, prolyl hydroxylases (PHDs) catalyze the hydroxylation of two key proline residues within HIF-α's oxygen-dependent degradation domain (ODD) - the ODD is found within the amino-terminal transactivation domain (NTAD). This post-translational modification facilitates recognition by VHL and binding of the VBC/Cul2 complex. In concert with E1 ubiquitin-activating enzymes and E2 ubiquitin-conjugating enzymes, the VBC/Cul2 complex polyubiquitinates HIF-α, and targets it for degradation via the 26S proteasome. **B)** In hypoxic conditions, HIF-α subunits are stabilized and translocate to the nucleus via a carboxy-terminal nuclear localization signal (C-NLS). In the nucleus, HIF-α dimerizes with HIF-*β*, and the heterodimer binds to the hypoxia-responsive elements (HRE) of target genes. The CBP/p300 transcriptional co-activators bind to HIF-α's carboxy-terminal transactivation domain (CTAD) to activate transcription of HIF-regulated genes. **C)** In VHL-loss RCC, cells are unable to degrade HIF-α, regardless of oxygen tension, resulting in the accumulation of HIF-α and the constitutive expression of HIF target genes (modified from Kaelin, 2002 and Maynard and Ohh, 2004).


A. NORMOXIA
B. HYPOXIA
C. VHL-/- RCC
NORMOXIA OR HYPOXIA
O2
PHDs
OH OH
Binding to VBC Complex
Cullin 2
C
B
VHL
OH OH
Degradation via 26S proteasome
C-NLS
Nuclear import
Dimerization with HIF-β
DNA binding
Recruitment of co-activators
HRE
Transcription of target genes
OH
C-NLS
Nuclear import
Dimerization with HIF-β
DNA binding
Recruitment of co-activators
HRE
CONSTITUTIVE transcription of target genes
NTAD
CTAD
OH Hydroxyproline

pancreatic, prostate, and renal carcinomas (Zhong *et al.,* 1999; Talks *et al.,* 2000). In xenograft models inhibition of HIF function in different human cancer cells has had potent anti-tumor effects (Kung *et al.,* 2000; Sun *et al.,* 2001; Buchler *et al.,* 2004; Rapisarda *et al.,* 2004).

1.8.1 In human RCC oncogenesis is associated with HIF-2α

Recent reports have led to the suggestion that HIF-2α is the oncogenic form of HIF-α in human $VHL^{-/-}$ RCC. Using small interfering RNAs (siRNAS) to compare specific inactivation of HIF-1α or HIF-2α in human breast cancer, RCC, and endothelial cell lines, HIF-mediated gene expression was dependent on HIF-1α in both endothelial and breast cancer cells, but on HIF-2α in RCC (Sowter *et al.,* 2003). Additionally, in VHL-loss RCC cells, shRNA-mediated silencing of HIF-2α is as efficient as re-introduction of VHL at abolishing the ability of these cells to form tumors in nude mice (Iliopoulos *et al.,* 1995; Kondo *et al.,* 2003). Constitutive expression of HIF-2α, but not HIF-1α, is sufficient to override VHL tumor suppressive activity and promote *in vivo* tumorigenesis of VHL-competent RCC cells (Kondo *et al.,* 2002; Maranchie *et al.,* 2002). Consistent with these findings is the observation that while HIF-1α is found solely in single cells, pre-neoplastic multi-cellular foci in the kidneys of patients with VHL disease express HIF-2α (Mandriota *et al.,* 2002). $VHL^{-/-}$ RCC cell lines almost always express the HIF-2α isoform, either alone or co-expressed with HIF-1α (Maxwell *et al.,* 1999; Turner *et al.,* 2002). Together, these data suggest that activation of HIF-2α-specific genes confers the oncogenic potential that drives RCC tumorigenesis. As both HIF-α isoforms are able to trans-activate angiogenic genes, the unique ability of HIF-2 to promote tumorigenesis is likely an additional characteristic of this transcription factor.

1.9 The growth autonomy of RCC cells

As previously mentioned, the ability of cells to engage in autonomous growth is a hallmark of cancer (Hanahan and Weinberg 2000). VHL-deficient RCC cells are able to grow in the absence of exogenous growth factors, and this malignant phenotype can be reversed through re-introduction of wild-type VHL (Pause *et al.,* 1998). In view of the primary role for VHL in mediating HIF-α degradation, our laboratory decided to investigate the function of HIF in promoting the growth self-sufficiency of VHL-loss RCC cells. By inhibiting HIF transcriptional activity, through expression of a dominant-negative form of the protein (DNHIF), we established that the growth autonomy of VHL-deficient RCC cells is dependent on the activity of the HIF transcription factor (Gunaratnam *et al.,* 2003). This data suggested a previously unappreciated role for HIF in promoting tumor progression, by directly inducing cellular growth as well as angiogenesis.

Upon examination of growth factors regulated by VHL expression and HIF activity, we determined that the transforming growth factor alpha (TGF-α) oncogene was the chief candidate responsible for the growth self-sufficiency of VHL-loss RCC cells. TGF-α is a hypoxia-inducible growth factor that is negatively regulated by VHL. In normoxic conditions, VHL-deficient RCC cells overexpress TGF-α mRNA and protein relative to their VHL-competent counterparts, and in hypoxic growth conditions, TGF-α expression is induced in VHL-positive cells (Knebelmann *et al.,* 1998; de Paulsen *et al.,* 2001). TGF-α is mitogenic to renal proximal tubule epithelial cells, the cells thought to give rise to $VHL^{-/-}$ RCC (Petrides *et al.,* 1990; Humes *et al.,* 1991; de Paulsen *et al.,* 2001). We have shown that ectopic expression of this growth factor stimulates the

proliferation of quiescent VHL-positive RCC cells in culture, whereas other growth factors overproduced by VHL-deficient RCC cells and associated with HIF activation, such as VEGF, PDGF-β, insulin-like growth factor, and transforming growth factor beta, do not display such mitogenic activity (de Paulsen *et al.,* 2001). Furthermore, antisense oligonucleotide-mediated inhibition of TGF-α protein expression in $VHL^{-/-}$ RCC cells abrogates their ability to proliferate in serum-free conditions (de Paulsen *et al.,* 2001). TGF-α signals through the epidermal growth factor receptor (EGFR), and inhibition of EGFR activity in $VHL^{-/-}$ RCC cells has similar growth-inhibitory effects (Gunaratnam *et al.,* 2003). Combined, these data suggest that VHL inactivation, and resulting HIF-mediated TGF-overproduction and EGFR signaling, confer a growth advantage to RCC cells.

1.9.1 Transforming growth factor alpha

TGF-α is a growth factor belonging to the epidermal growth factor (EGF) family of peptides. Growth factors in this family are synthesized as transmembrane proteins and have a characteristic 6-cysteine motif in their extracellular domain. This family of growth factors includes EGF, TGF-α, epiregulin, amphiregulin, betacellulin, heparin-binding EGF, and epigen. TGF-α is translated from a 4.8kb transcript into a 160 amino acid protein, which is processed and glycosylated in the endoplasmic reticulum (Derynck *et al.,* 1984; Lee *et al.,* 1995). This is followed by maturation of sugar chains in the Golgi compartment and transport to the plasma membrane (Teixido *et al.,* 1987). Membrane bond TGF-α (pro-TGF-α) then undergoes a two-step cleavage process to release a mature, soluble, 6kDa peptide (Teixido *et al.,* 1990). This process, referred to as ectodomain shedding, is mediated by the enzyme TACE (tumor necrosis factor α

converting enzyme); and cleavage regulates the availability of soluble TGF-α that can act in a autocrine and paracrine fashion, as well as the availability of membrane-bound TGF-α that can act on neighboring cells in a juxtacrine fashion (Peschon *et al.,* 1998; Sunnarborg *et al.,* 2002). Both forms of TGF-α impart their biological activities through interactions with the EGFR, the only known receptor for TGF-α (Brachmann *et al.,* 1989).

TGF-α is important in fetal development, and is produced by epithelial cells in regenerating adult tissues such as the skin, liver, and gut (Lee *et al.,* 1985; Derynck 1992). This growth factor was originally identified in the media of several virally, oncogenically, and chemically transformed cells, by virtue of its ability to confer a transformed phenotype to immortalized fibroblasts. Overexpression of TGF-α enabled normal anchorage-dependent cells to grow in soft agar (de Larco and Todaro 1978; Todaro *et al.,* 1980; Anzano *et al.,* 1983; Derynck 1988). In this regard, TGF-α expression has been observed in many human tumors and tumor derived cell lines (Derynck *et al.,* 1987), and has been implicated in the pathogenesis of cancer in a number of different tissues (Massague 1990; Salomon *et al.,* 1995).

1.9.2 The epidermal growth factor receptor

According to the autocrine hypothesis, cells may become malignant by the endogenous overproduction of polypeptide growth factors that may signal through cognate receptors on their own cell surfaces (Sporn and Todaro 1980). As such, while the overexpression of TGF-α alone does not confer a transformed phenotype to NIH 3T3 murine fibroblasts (Finzi *et al.,* 1987), overexpression of the EGFR in combination with ligand does (Di Fiore *et al.,* 1987). The EGFR is a 170 kDa transmembrane glycoprotein.

The gene for this protein is localized on chromosome 7p12-13. The EGFR is a receptor tyrosine kinase (RTK) whose activity is important in normal developmental processes. The EGFR has a broad expression pattern on epithelial, mesenchymal, and neuronal cells, and signaling through this receptor plays a critical developmental role. Aberrant activation of this receptor has also been implicated in malignant transformation (Salomon *et al.,* 1995). In fact, the EGFR was the first cell-surface receptor to be directly linked to cancer, and is the most common receptor to be upregulated in a wide variety of human tumors (Aaronson 1991).

The EGFR (erbB1) is one of four proteins belonging to the EGFR family. Other members of the EGFR family are erbB2 (hER2/neu), which has no known high affinity ligands and thus must rely on heterodimerization with other family members for activation, erbB3, which lacks tyrosine kinase (TK) activity, and erbB4 (Rowinsky 2004). Receptors belonging to this family have a common structure comprising an extracellular ligand-binding region, a hydrophobic transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity, which is imperative for signal transduction. Binding of a ligand, such as TGF-α, to the EGFR results in dimerization of monomeric receptors and tyrosine kinase mediated auto- and trans-phosphorylation of key tyrosine residues. The phosphotyrosines serve to recruit numerous signaling molecules that initiate cascades of intra-cellular signal transduction. As a variety of signaling molecules associate with activated EGFR, multiple signaling pathways are simultaneously activated. These include the Ras/MAP kinase, STAT, phospholipase C, and PI3 kinase/Akt pathways. Amongst others, the downstream effects of EGFR activation include increased proliferation and angiogenesis, cell migration and invasion,

and inhibition of apoptosis (Figure 6; Salomon *et al.,* 1995; Arteaga 2002; Marmor *et al.,* 2004). Unregulated EGFR signaling can thus confer a malignant phenotype to cancer cells (Martin 2003). High levels of receptor expression, activating receptor mutations, heterodimerization with other members of the EGFR receptor family, overexpression of ligands, and alteration in other molecules that mediate ligand and EGFR processing are various mechanisms that can lead to EGFR signal amplification (Yarden and Sliwkowski 2001; Arteaga 2002).

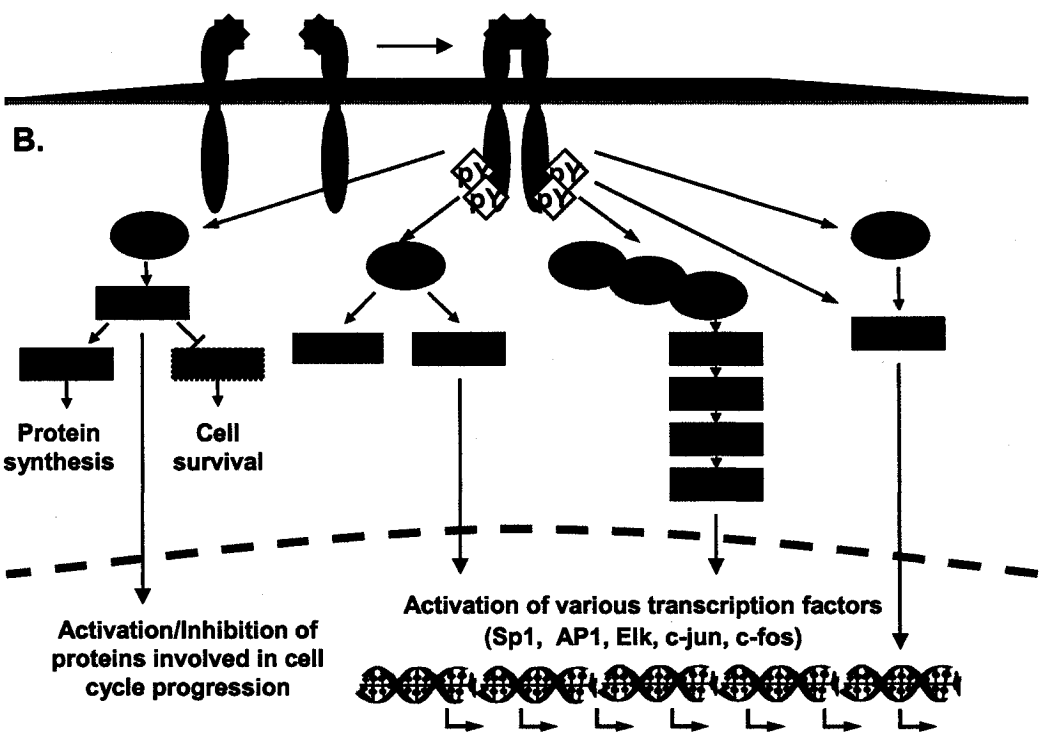
1.10 TGF-α and the EGFR are involved in $VHL^{-/-}$ RCC

Of all of the EGFR ligands, TGF-α has most often been identified as a key player in human cancers. TGF-α and EGFR are co-expressed in a large number of human cancers, including non-small-cell lung cancer (NSCLC), breast cancer, head and neck cancer, gastric carcinoma, prostate cancer, bladder, ovarian, and colorectal carcinomas, glioblastomas, and renal cell carcinomas (Salomon *et al.,* 1995). In a study of 13 clear cell renal cell carcinoma cell lines, TGF-α mRNA was observed in 12 cell lines and detectable levels of protein in 10, and EGFR expression was observed in all 13 RCC cell lines (Ramp *et al.,* 1997). Similarly, numerous studies have reported concurrent overexpression of TGF-α and EGFR mRNA in RCC tissue specimens, as compared to adjacent normal renal tissue, and southern blot analysis of genomic DNA revealed that the accumulation of TGF-α and EGFR was not a consequence of gene amplification or rearrangement (Derynck *et al.,* 1987; Gomella *et al.,* 1989; Mydlo *et al.,* 1989; Petrides *et al.,*1990). The altered expression of TGF-α appears to be an early event in the

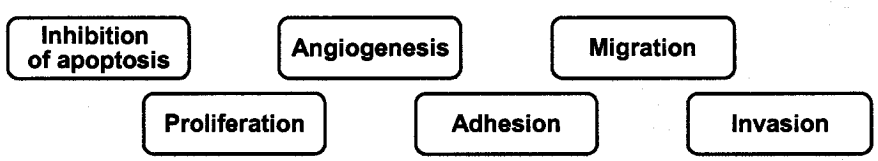
Figure 6. EGFR signaling and oncogenesis. **A)** The EGFR belongs to a family of receptors that have a common structure comprising an extracellular ligand-binding region, a hydrophobic transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity. Binding of ligands results in dimerization of monomeric receptors and tyrosine kinase mediated auto- and trans-phosphorylation. **B)** The phosphotyrosines (pY) serve to recruit numerous signaling molecules that initiate cascades of intra-cellular signal transduction. A variety of signaling molecules associate with activated EGFR to simultaneously activate an array of signaling pathways. Only a few of these pathways are shown here, and have been greatly simplified. **C)** The downstream effects of EGFR activation translate to a variety of cellular responses that promote malignant behaviors (modified from Arteaga 2002).

A.

EGF, TGF-α, epiregulin, amphiregulin, betacellulin, heparin-binding EGF, or epigen



C.



development of renal cell tumors. In a rat model of hereditary RCC, strong TGF-α immunoreactivity was present at all stages of renal cell tumor development, including the earliest detectable dysplasias, but non-neoplastic epithelium did not stain for TGF-α (Everitt *et al.,* 1997). Furthermore, TGF-α is the only know EGFR ligand that has a VHL-dependent expression profile in RCC cells (Gunaratnam *et al.,* 2003).

1.11 Rationale

Renal cell carcinoma is a devastating disease that will result in the death of one in ninety Canadians this year alone (National Cancer Institute of Canada: Canadian Cancer Statistics 2004). Treatment for RCC is limited, and for any therapeutic modality very few objective responses are achieved and long-term patient survival is extremely rare (Amato 2005). In order to develop clinically relevant and effective therapeutic approaches to treat this disease, the molecular mechanisms and pathways driving RCC tumorigenesis must be clearly understood. In previous studies, we established that the growth autonomy of VHL-loss RCC cells can be abolished through reintroduction of wild-type VHL (Pause *et al.,* 1998), by inhibiting HIF transcriptional activity (Gunaratnam *et al.,* 2003), through the use of antisense oligonucleotides against TGF-α mRNA (de Paulsen *et al.,* 2001), or through the inhibition of EGFR activity (Gunaratnam *et al.,* 2003). Hence, we elucidated a pathway in which HIF-mediated TGF-α expression and constitutive EGFR activation provide permanent self-sufficiency in signaling that drives the growth autonomy of VHL-deficient RCC cells (Figure 7A). Recent data suggests that activation of HIF-2α-specific genes confers the oncogenic potential that drives RCC tumorigenesis. In the current study, we decided to investigate the link

between HIF-mediated induction of the TGF-α/EGFR growth stimulatory pathway and HIF-2α-dependent RCC tumorigenesis.

In vivo data supports the involvement of TGF-α and the EGFR in RCC tumorigenesis, as the co-overexpression of these proteins is a common feature of this disease. Several studies have been undertaken to test the effects of anti-EGFR therapies in RCC, though the clinical results have not been very impressive (Dancey 2004). The EGFR may yet be a valid target for the treatment of RCC, but, due to pharmacokinetic issues or the development of drug resistance in tumor cells, therapeutics designed to inhibit EGFR activity have not been able to achieve their desired effects in patients. Conversely, the importance of the EGFR may be overestimated in *in vitro* studies. Here, we decided to firmly address the role of the EGFR in RCC tumor formation *in vivo* by targeting the receptor at the molecular level.

1.12 Statement of hypothesis and objectives

Based on the oncogenic potential associated with HIF-2, but not HIF-1, in VHL-loss RCC, we hypothesize that TGF-α is a HIF-2 specific target, and that HIF-2-dependent EGFR signaling drives $VHL^{-/-}$ RCC tumorigenesis (Figure 7B). We propose to test this hypothesis with the following objectives:

Objective #1. To determine which of the HIF-α isoforms can induce TGF-α expression and recruit the TGF-α/EGFR growth stimulatory pathway.

Equivalent amounts of constitutively stable HIF-1α and HIF-2α protein will be expressed in VHL-competent RCC cells, and the ability of HIF-1 and HIF-2 to induce TGF-α

mRNA and protein, and promote EGFR signaling and serum-independent growth will be tested.

Objective #2. To verify that the EGFR is required for growth autonomy of VHL-loss RCC cells.

RNA interference technology will be employed to silence EGFR expression in a panel of VHL-deficient RCC cell lines derived from patients, and the requirement of EGFR activation for the serum-independent growth of these cells will be evaluated.

Objective #3. To investigate the requirement of EGFR signaling for avascular tumor formation *in vitro*.

An *in vitro* tumor spheroid assay will be employed to examine the tumorigenic potential of VHL-defective RCC cells expressing siRNA or shRNA against the EGFR. This assay has the advantage of measuring tumorigenicity in the absence of neovascularization, a variable that is especially important given that angiogenesis is critical for tumor progression.

Objective #4. To discern the role of the EGFR in VHL-loss HIF-2-dependent RCC tumorigenesis *in vivo*.

VHL-deficient RCC cells stably expressing shRNA targeting the EGFR will be generated to investigate the requirement of EGFR activity for tumor formation by RCC cells in nude mice.

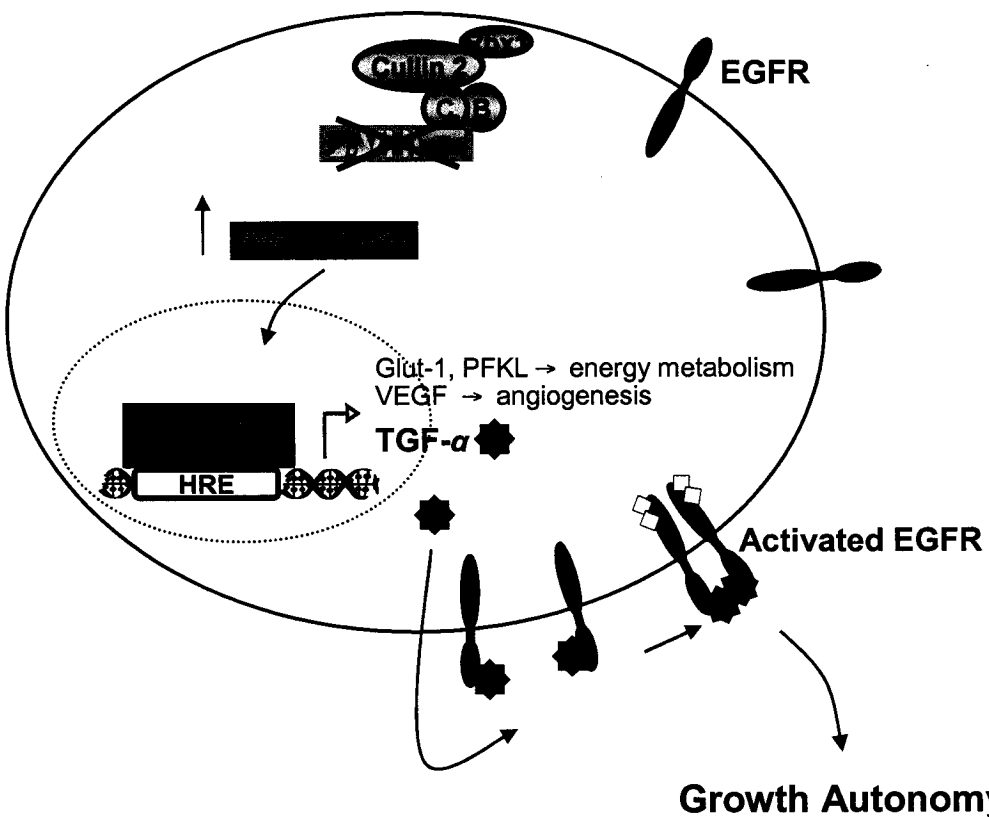
Objective #5. To determine if EGFR silencing is sufficient to override the effects of other neoplastic characteristics associated with VHL-loss RCC.

Inactivating mutations of the VHL gene confer a number of molecular defects to cells that are implicated in tumor progression. In order to address the centrality of EGFR

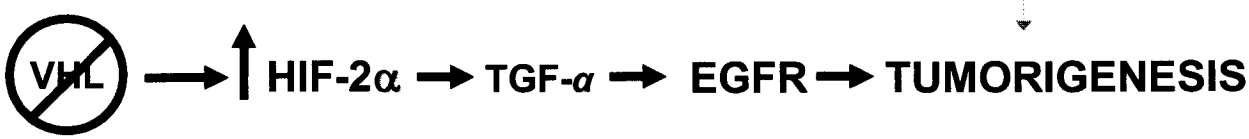
activation in mediating HIF-2-dependent RCC tumor formation we will examine if silencing of the EGFR will block tumorigenesis despite these cellular flaws. Fibronectin deposition and expression of HIF-2α and HIF-2 target genes will be examined in VHL-loss RCC cells stably expressing shRNA targeting the EGFR.

Figure 7. HIF activates a TGF-α/EGFR growth stimulatory pathway in VHL-loss RCC cells. A) VHL-deficient RCC cells are able to grow in the absence of exogenous growth factors. In these cells HIF-α protein is stabilized - regardless of oxygen tension - accumulates, and heterodimerizes with HIF-β. This results in the constitutive expression of HIF-target genes. While the aberrant expression of HIF-induced angiogenic factors such as VEGF might account for the highly vascularized nature of $VHL^{-/-}$ RCC tumors, HIF-mediated TGF-α expression and constitutive EGFR activation provide self-sufficiency in signaling that drives growth autonomy of VHL-deficient RCC cells. **B)** VHL-loss RCC tumorigenesis is associated with the expression of HIF-2α, but not HIF-1α. This suggests that activation of HIF-2-specific genes confers the oncogenic potential that drives RCC tumorigenesis. **We suspect that TGF-α is a HIF-2 specific target, and that TGF-α-driven EGFR signaling is the key oncogenic event mediating HIF-2-dependent RCC tumor formation.**

A.


EGFR
Glut-1, PFKL → energy metabolism
VEGF → angiogenesis
TGF-α
HRE
Activated EGFR
Growth Autonomy

B.


VHL
HIF-2α
TGF-α
EGFR
TUMORIGENESIS

METHODS

2.1 Cell culture and reagents

VHL-deficient 786-O and A498 RCC cells were purchased from the American Type Culture Collection (Rockville, MD). 786-O and A498 cells stably transfected with hemagglutinin (HA)-tagged VHL (786-O+VHL and A498+VHL, respectively) were a kind gift from Dr. W.G. Kaelin (Harvard University, Boston, MA, USA). VHL-deficient KTCL140 RCC cells were a kind gift from Dr. Peter Ratcliffe (University of Oxford, UK). 786-0 and A498 RCC cells contain frameshift mutations at codons 104 and 142 of the VHL protein, respectively. KTCL140 cells contain a His-to-Tyr amino acid substitution at codon 115 of the VHL protein. All three of these VHL-deficient RCC cell lines express HIF-2α but not HIF-1α protein, and overexpress HIF-2 target genes (Maxwell *et al.,* 1999).

Serum containing medium consisted of Dulbecco's modified Eagle's medium (DMEM; Wisent Incorporated, St. Bruno, QC) supplemented with 10% Fetal Bovine Serum (FBS; Wisent) and 1% Penicillin and Streptomycin (Pen/Strep; Wisent). Serum-free medium consisted of DMEM supplemented with 1% insulin-transferrin-selenium (ITS; Invitrogen, Burlington, ON). Cells were maintained in serum-containing medium and were incubated at 37°C under a 5% CO_2 environment. For EGFR inhibition experiments the EGFR tyrosine kinase inhibitor (TKI), PD153035, (Calbiochem, San Diego, CA) was employed. PD153035 was re-suspended in DMSO and added to cells to achieve a concentration of 1μM in the medium.

2.2 Adenoviruses

Where indicated, cells were infected with adenoviruses to express different proteins. The adenoviruses encoding GFP, wild type VHL, and DNHIF have been previously characterized and described (Groulx *et al.,* 2000; Groulx and Lee 2002; Gunaratnam *et al.,* 2003). Adenoviruses encoding constitutively stable variants of HIF-1α and HIF-2α (referred to as vHIF-1α and vHIF-2α) were recently generated and characterized by M. Morley (Smith *et al.,* 2005). These viruses encode HIF-α subunits with proline to alanine substitutions at ODD proline hydroxylation sites: HIF-1α (P402A, P564A) and HIF-2α (P405A, P531A). The cDNAs encoding the constitutively active HIF-α variants were a kind gift from Dr. W.G. Kaelin (Harvard University, Boston, MA, USA). All adenovirus constructs include a GFP moiety, and for the various viruses multiplicity of infections (MOIs) of 20-100 were used to achieve equal expression of the different proteins.

2.3 RNA isolation and reverse transcriptase polymerase chain reaction (RT PCR) analysis

Total RNA was collected using TRIPURE isolation reagent (Roche Diagnostics Co., Indianapolis, IN). RT PCR was performed on 1μg of RNA using the One Step Superscript RT Platinum Taq RT PCR kit (Invitrogen) and 0.6μM of each primer. The cycling conditions used were as follows: a first strand synthesis of 30min at 50°C, denaturation for 2min at 94°C, 30 amplification cycles of 40s at 94°C, 20s at 60°C, and 20s at 72°C, then a final elongation step of 10min at 72°C. The sequences (5' to 3') of the RT PCR primers are as follows: TGF-α forward TCGCTCTGGGTATTGTGTTG,

TGF-α reverse GACCTGGCAGCAGTGTATCA; Glut-1 forward CTGTCTGGCATC AACGCTGTCTTC, Glut-1 reverse TCCTCGGGTGTCTTATCACTTTGG; and β-actin forward CGTACCACTGGCATCGTGAT, β-actin reverse GTGTTGGCGTACAG GTCTTTG. RT PCR products were analyzed with gel electrophoresis and ethidium bromide staining, and visualized using a Kodak Digital Science IC440 system.

2.4 BrdU 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 media. After the indicated times, cells were incubated in the presence of 10μM BrdU for 3hr prior to fixation, for 30min at -20°C, with 70% ethanol in 50mM glycine (pH 2). Following fixation, cells were washed and stained with an anti-BrdU antibody (1:20, Roche, Indianapolis, IN) for 1hr at 37°C. After removal of excess primary antibody, cells were exposed to a Texas Red conjugated anti-mouse secondary antibody (1:200, Jackson ImmunoResearch) for 30min at 37°C. Nuclei were counterstained with Hoechst reagent (Hoechst 33258; Sigma, St. Louis, MO) and assessed for BrdU incorporation using a Ziess Axiovert S100TV microscope (Thornwood, NY). Data is presented as the proportion of nuclei incorporating BrdU, relative to the total number of nuclei identified by Hoechst staining.

2.5 TGF-α enzyme-linked immunosorbant assay (ELISA)

An equal number of cells were seeded approaching confluence and allowed to settle overnight in DMEM supplemented with 10% FBS. Cells were then washed with

PBS and maintained in fresh serum-containing or serum-free media, and were infected with the indicated adenoviruses. Forty-eight hours later, media and total cell lysates were collected and analyzed for TGF-α protein, according to the manufacturer's instructions (Oncogene, Boston, MA). Total protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL) to ensure equivalence between samples.

2.6 Western blot

Cells were washed with phosphate buffered saline (PBS) and lysed in PBS containing 4% sodium dodecyl sulphate (SDS). The DNA was sheared with a 21-guage needle. Total protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Equal amounts of protein from each sample were resolved by electrophoresis on denaturing polyacrylamide gels containing SDS, and transferred to methanol-permeabilized polyvinylidene difluoride membranes (PVDF; NEN, Boston, MA). Membranes were blocked in 5% (w/v) non-fat dried milk (Carnation, Glendale, CA) in 0.2% Tween 20-PBS for 1hr at room temperature before incubation overnight at 4°C with the indicated primary antibody. Monoclonal antibodies were used to detect Flag (1:5000, Sigma, St. Louis, MO) and total EGFR (Ab-12; 1:300, LabVision, Fremont, CA). Polyclonal antibodies were used to detect activated Py-EGFR(Tyr 1173) (sc-12351; 1:1000, Santa Cruz Biotech, Santa Cruz, CA), HIF-2α (1:500, Novus, Littleton, CO), Glut-1 (1:1000, Alpha Diagnostic International, San Antonio, TX) and actin (1:5000, Sigma, St. Louis, MO). After washing with 0.2% Tween 20-PBS solution, membranes were blotted for 1h at room temperature with secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch,

West Grove, PA) and labeled proteins were detected by enhanced chemiluminescence (Pierce, Rockford, IL).

2.7 Fibronectin deposition

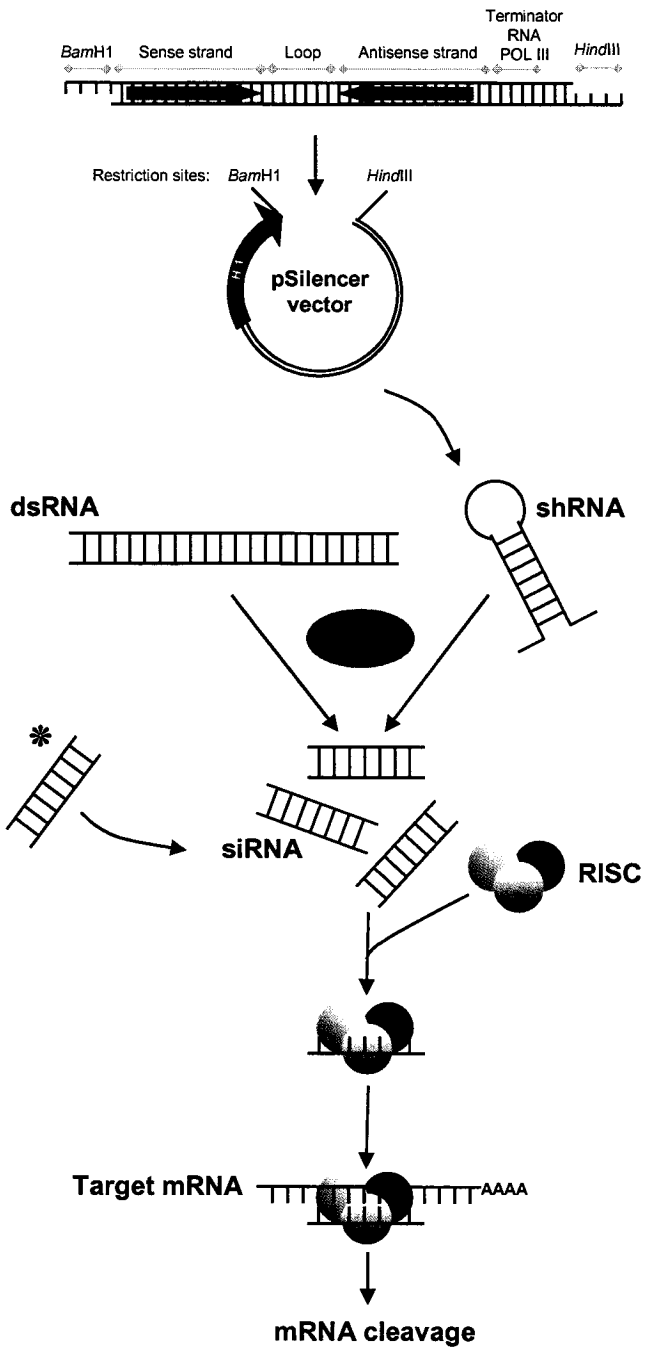
Cells were grown to confluence for six days on glass coverslips, washed three times with PBS, and fixed/permeabilized in pre-chilled 95% ethanol at –20°C for 30min. Ethanol was aspirated and residual was allowed to air dry at 4°C. Cells were stained for 1hr at room temperature with anti-fibronectin antibody (1:500, DAKO Diagnostics Canada, Mississauga ON). After washing with PBS, coverslips were incubated with Texas Red conjugated secondary antibody (Jackson ImmunoResearch). Coverslips were counterstained with Hoescht to identify all nuclei, and fibronectin deposition was assessed with a Ziess Axiovert S100TV microscope (Thornwood, NY).

2.8 EGFR RNA interference

For transient inhibition of EGFR production, VHL-deficient 786-0, A498, and KTCL140 cells were transfected with commercially available double-stranded 21-nucleotide-long small interfering RNA (siRNA) targeting the EGFR (Figure 8, see *; Ambion, Austin, TX). A set of cells were transfected with a non-silencing siRNA as a control (Ambion). Transfections were conducted with Effectene transfection reagent (Qiagen, Valencia, CA). Briefly, cells plated at approximately 70% confluence in 6-well plates were transfected with 50nM siRNA. At the time of transfection cells were washed in PBS and supplemented with fresh medium.

VHL-deficient 7860 and KTCL140 cells were also stably transfected to express one of two different short hairpin RNA (shRNA) sequences targeting the EGFR (Nagy *et al.,* 2003). The EGFR shRNA was designed using software provided on the Ambion website (www.ambion.com). For each sequence, two complementary single stranded DNA oligonucleotides were synthesized (Qiagen): Sequence 1 (5' to 3'): shRNA EGFR-1 forward GATCCAACTCTGGAGGAAAAGAAAGTTTCAAGAGAACTTTCTTTTC CTCCAGAGTTTTTTTTGGAAA and shRNA EGFR-1 reverse AGCTTTTCCAAA AAAAACTCTGGAGGAAAAGAAAGTTCTCTTGAAACTTTCTTTTCCTCCAGAGT T. Sequence 2 (5' to 3'): shRNA EGFR-2 forward GATCCAACACAGTGGAG CGAATTCCTTTCAAGAGAAGGAATTCGCTCCACTGTGTTTTTTTTGGAAA and shRNA EGFR-2 reverse AGCTTTTCCAAAAAAAACACAGTGGAGCGAATTCCT TCTCTTGAAAGGAATTCGCTCCACTGTGTTG. By inclusion of a central loop sequence separating two complementary domains, the single stranded DNA oligonucleotides were designed to form a double stranded RNA hairpin structure when transcribed. The single stranded DNA oligonucleotides were also designed with overhangs encoding restriction sites for *Bam*HI/*Hind*III. The single stranded DNA were annealed by incubation for 3min at 90°C followed by 1hr at 50°C, and the annealed products were ligated directly into a linearlized pSilencer 3.1-H1 neo vector with corresponding restrictions sites. The pSilencer 3.1-H1 neo expression vector contains the human HI RNA polymerse III promoter (Figure 8, top; Ambion). A pSilencer 3.1-H1 neo vector encoding random control shRNA was also purchased from Ambion. Constructs were verified by standard DNA sequencing.

Figure 8. RNA interference-mediated gene silencing. RNA-mediated gene silencing triggers may be presented in the cell as synthetic RNAs, replicating viruses, or may be transcribed from nuclear genes. Long double-stranded RNAs (dsRNAs) are recognized by the enzyme Dicer and processed into small interfering RNAs (siRNAs). In this study cells were either directly transfected with commercially available double-stranded 21-nucleotide-long siRNA (*, Ambion) or with expression vectors encoding short hairpin RNA (shRNA). In the case of the latter, expression from the H1 RNA polymerase III promoter of the pSilencer vector (Ambion) yields a dsRNA structure consisting of a central hairpin loop and complementary RNA sequences corresponding to the target transcript (top of Figure). Dicer then processes this shRNA into smaller siRNA fragments. The duplex siRNAs assemble with RISC (RNA-induced silencing complex), and the complex becomes activated by unwinding of the dsRNA fragment. The antisense strand of the siRNA is used by RISC to guide mRNA cleavage and target degradation (modified from Meister and Tuschl 2004).


Terminator
RNA
POL III
BamH1
Sense strand
Loop
Antisense strand
HindIII
Restriction sites: BamH1
HindIII
H1
pSilencer
vector
dsRNA
shRNA
siRNA
RISC
Target mRNA
AAAA
mRNA cleavage

Transfections were conducted with Effectene transfection reagent (Qiagen, Valencia, CA). To generate stable cell lines, cells plated at approximately 70% confluence in 10cm plates were transfected with 2ug of plasmid DNA. At the time of transfection cells were washed in PBS and supplemented with fresh serum-containing medium. The following day the cells were split into two 15cm dishes and incubated in G418-containing medium. The selective medium was replaced every three to four days to remove dying cells not transfected with the vector conferring antibiotic resistance. After approximately two weeks, when the majority of the non-transfected cells had been killed and distinct colonies were evident to the naked eye, the individual colonies were trypsinized and transferred to a 24-well plate for maintenance in selective medium. Cells were gradually passaged to larger dishes and maintained in G418-free medium. Western blot analysis to detect a decrease in EGFR protein in lysates from transiently and stably transfected cells was employed to detect EGFR silencing.

2.9 *In vitro* tumor spheroid

Multicellular spheroids were prepared by the liquid overlay technique (Sutherland 1988; Kunz-Schughart *et al.*, 1998; Lieubeau-Teillet *et al.*, 1998). Briefly, 24-well plates were coated with 250µl of pre-heated 1% Seaplaque agarose (Cambrex, Rockland, ME) in serum-free medium. 10^5 of the indicated cells were plated per 1ml of DMEM media per well. To promote cell–cell adhesion, the plates were gently swirled (32 times) 30min after plating. Where indicated, cells were transfected 24hr prior to plating spheroids. Spheroids were grown for six days at 37 °C under a 5% CO_2 environment. Spheroids were harvested in lysis buffer (4% SDS in PBS) prior to immunoblotting. Alternatively,

spheroids were fixed in 10% formaldehyde, processed, embedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin and eosin (H&E).

2.10 Nude mouse xenograft assays

Nude mouse xenograft assays were done as described elsewhere (Iliopoulos *et al.*, 1995). In brief, 10^7 viable cells (trypan dye exclusion method) were injected subcutaneously in the flanks of female nude mice (Charles River, FA). Mice were injected with the following cell lines: $VHL^{-/-}$ 786-0 cells, 786-0 cells stably transfected with pcDNA 3.1 vector alone, 786-0 cells stably expressing pcDNA 3.1-VHL (VHL-competent RCC cells), 786-0 cells expressing control shRNA, 786-0 cells expressing EGFR silencing shRNA, KTCL cell expressing control shRNA, and KTCL cells expressing EGFR silencing shRNA. Mice were sacrificed nine 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 VHL or EGFR targeting shRNA. These mice were sacrificed 16 weeks post-injection. A technician blind to the injection pattern measured tumor sizes weekly, and at the time of sacrifice tumors were excised and weighed.

RESULTS

3.1 HIF-2α specifically promotes the growth autonomy of RCC cells

VHL loss RCC cells constitutively overproduce HIF-regulated genes as a consequence of the inability of these cells to degrade HIF-α in normoxia (Maxwell *et al.*, 1999). Constitutive HIF activation is required for serum-independent growth of $VHL^{-/-}$ RCC cells in culture, as well as for tumor formation in a xenograph nude mice tumor assay (Kondo *et al.*, 2002; Gunaratnam *et al.*, 2003). In addition, constitutive expression of HIF-2α, but not HIF-1α, overrides VHL-mediated RCC tumor suppression (Kondo *et al.*, 2003). To specifically investigate the roles of the two HIF-α proteins in RCC tumorigenesis, we infected VHL-competent RCC cells with adenoviruses encoding Flag-tagged HIF-1α and HIF-2α variants that evade VHL recognition in the presence of oxygen (hence forth referred to as vHIF-1α and vHIF-2α). In these constructs, key proline residues in the ODD domain of HIF-α were substituted to alanine, which prevents recognition and degradation by VHL in normoxic cells (Figure 9A schematic; Kondo *et al.*, 2002). Western blot analysis to detect Flag protein revealed that vHIF-1α and vHIF-2α were expressed to similar levels (Figure 9A). RT PCR analysis revealed that both proteins were functional, as they equally promoted the accumulation of Glut-1 mRNA, a well-characterized HIF-regulated gene, in normoxic VHL-competent cells (Figure 9A).

In the absence of exogenous growth factors, or serum, VHL-deficient cells are able to engage in autonomous growth, a hallmark of cellular transformation (Hanahan and Weinberg 2000). 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 BrdU incorporation (Figure 9B; Pause *et al.*, 1998; Gunaratnam *et al.*, 2003). In order to reiterate the involvement of HIF in driving the growth autonomy of VHL-

deficient RCC cells, we infected cells to express DNHIF to inhibit HIF transcriptional activity. The DNHIF adenovirus was engineered to express a truncated version of HIF-2α, which retains the ability to heterodimerize with HIF-β and bind to DNA hypoxia-response elements, but lacks the HIF-α transactivation domain. This results in the inhibition of both HIF-1 and HIF-2 transcriptional activity (Maemura *et al.,* 1999). As previously demonstrated, expression of DNHIF abolished BrdU incorporation by VHL-deficient RCC cells in serum-free media, thereby confirming that the observed autonomous proliferation of these cells is a consequence of HIF activation (Figure 9B; Gunaratnam *et al.,* 2003). HIF activation was indeed attenuated since the expression of DNHIF abolished the expression of Glut-1 as efficiently as reintroduction of VHL (Figure 9B). Addition of serum restored BrdU incorporation in all conditions, demonstrating that RCC cells are equally sensitive to exogenous growth factors, regardless of VHL status or HIF activation (Figure 9B). Infecting cells with a control adenovirus, encoding GFP, had no effect on BrdU incorporation, thereby alleviating concerns of a non-specific dominant viral effect on the sensitivity of cells to exogenous growth factors (Figure 9B).

VHL-deficient 786-0 RCC cells express HIF-2α, but not HIF-1α. Therefore, DNHIF-mediated inhibition of the growth autonomy of these cells (Figure 9B; Gunaratnam *et al.,* 2003) provided us with evidence that HIF-2 activity is required to promote the aberrant growth of RCC cells, yet did not address the ability of HIF-1 to do likewise. To specifically address the roles of the HIF-α isoforms in RCC tumorigenesis, we asked whether HIF-1α or HIF-2α activation could promote growth autonomy of RCC cells by overriding the effect of reintroduction of VHL. Expression of vHIF-1α had no

discernable effect on the growth of VHL-competent cells, in the presence or absence of serum (Figure 9C). In contrast, expression of vHIF-2α was sufficient to promote autonomous growth of VHL-competent RCC cells, overriding the effect of reintroduction of VHL (Figure 9C). These experiments demonstrate that HIF-2-specific transcriptional activity differs from HIF-1, and enables VHL-competent cells to engage in autonomous growth.

3.2 TGF-α is a HIF-2-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 (Gunaratnam *et al.,* 2003). Thus, we suspected that TGF-α might play a key oncogenic role in HIF-2α-mediated autonomous growth. VHL-loss RCC cells overproduce TGF-α mRNA in normoxia, which can be downregulated by the reintroduction of VHL (Figure 10A; Knebelmann *et al.,* 1998; de Paulsen *et al.,* 2001). Whereas the expression of vHIF-1α and vHIF-2α equally promoted Glut-1 transcription in normoxic VHL-competent cells, TGF-α mRNA induction was observed only in cells expressing vHIF-2α (Figure 10A). Concomitant with RT PCR results, TGF-α protein was present in cellular lysates of vHIF-2α-infected, but not vHIF-1α-infected, VHL-competent cells (Figure 10B). Thus, it would appear that TGF-α is a HIF-2-specific transcription target.

Figure 9. HIF-2α promotes autonomous growth of RCC cells. A) HIF-1α and HIF-2α variants carrying P-to-A mutations at prolyl hydroxylation sites (schematic) are both able to activate HIF-target genes. VHL-deficient 786-O cells, stably transfected to express VHL (786-0+VHL) were infected with adenoviruses expressing flag-tagged vHIF-1α, vHIF-2α, or GFP. 48hr post infection immunoblotting or RT-PCR was performed to detect Flag protein or Glut-1mRNA, respectively. **B)** HIF activation is required for serum-independent growth of VHL-deficient renal cancer cells. 786-O cells infected to express GFP, VHL, or dominant-negative HIF were incubated for 72hr in the presence or absence of serum and assessed for BrdU incorporation. Columns represent the average mean of at least three independent experiments in triplicates; bars represent the standard error of the mean (SEM). Immunoblotting was performed to detect Glut-1. **C)** HIF-2α but not HIF-1α is able to promote serum-independent growth in VHL-competent cells. Growth was measured in 786-O or A498 RCC cells deficient or competent for VHL expression (786-0+VHL or A498+VHL, respectively). VHL-competent RCC cells were infected to express GFP, vHIF-1α, or vHIF-2α. Cells were incubated in the presence or absence of serum for 72hr prior to BrdU labeling. Columns represent the average mean of at least three independent experiments in triplicates; bars represent the SEM.

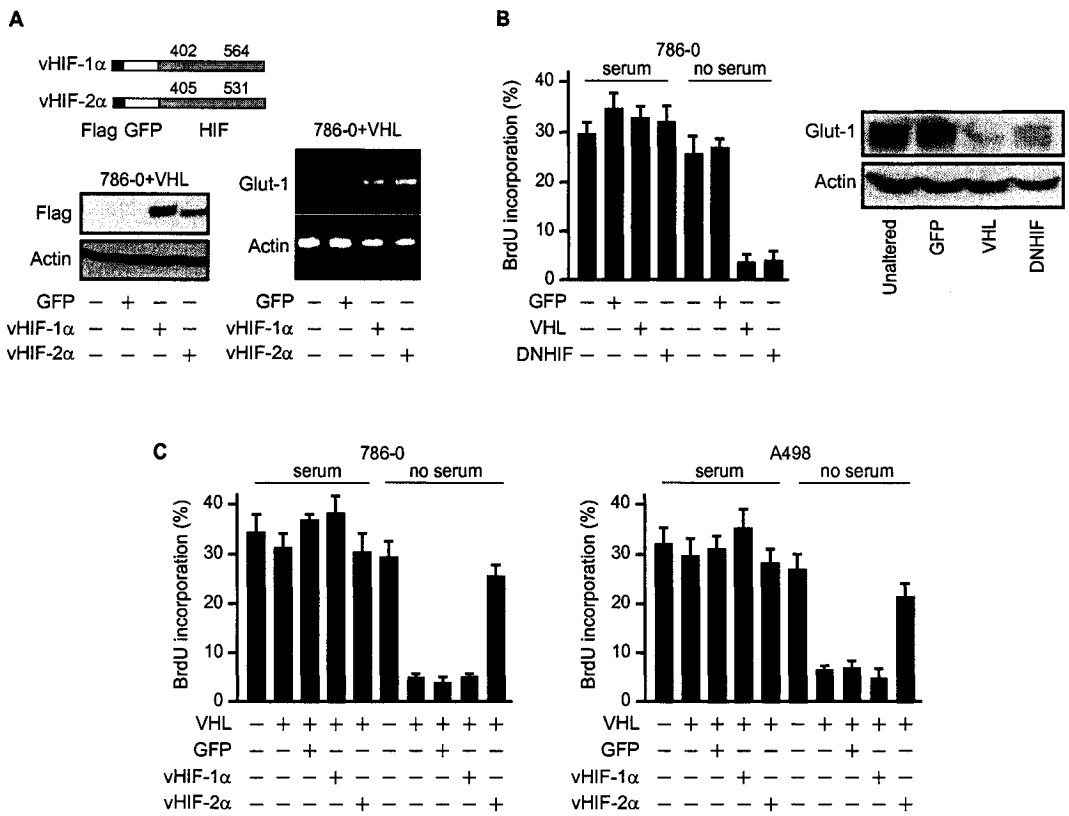
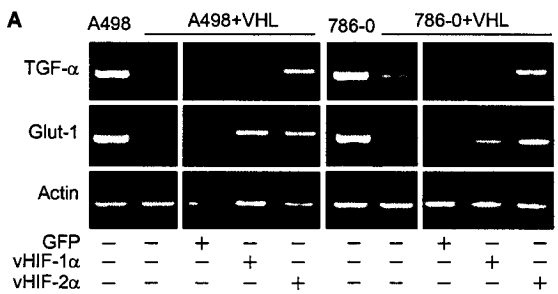
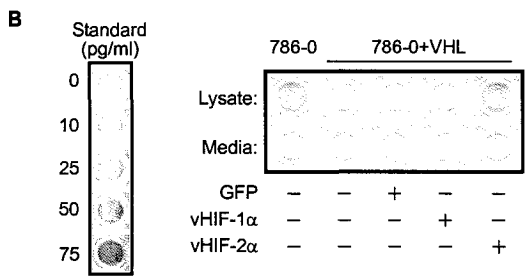

A
vHIF-1α
402
564
vHIF-2α
405
531
Flag GFP
HIF
786-0+VHL
786-0+VHL
Flag
Actin
Glut-1
Actin
GFP
vHIF-1α
vHIF-2α
B
786-0
serum
no serum
BrdU incorporation (%)
40
30
20
10
0
GFP
VHL
DNHIF
Glut-1
Actin
Unaltered
GFP
VHL
DNHIF
C
786-0
A498
serum
no serum
BrdU incorporation (%)
VHL
GFP
vHIF-1α
vHIF-2α

Figure 10. TGF-α is a HIF-2-specific target. A) HIF-2α but not HIF-1α induces TGF-α mRNAs synthesis in VHL-competent cells. RT-PCR was used to detect TGF-α and Glut-1 transcripts in VHL-deficient A498 and 786-0 cells and in their VHL-competent counterparts (A498+VHL; 786-0+VHL). VHL-competent RCC cells were infected to express GFP, vHIF-1α, or vHIF-2α. A parallel RT PCR for β-actin demonstrated equivalence between samples. **B)** HIF-2α but not HIF-1α induces TGF-α protein in VHL-competent cells. ELISA was performed to detect TGF-α protein in the cellular lysates and media from VHL-deficient and VHL-competent 786-0 cells. VHL-competent cells were infected to express GFP, vHIF-1α, or vHIF-2α.

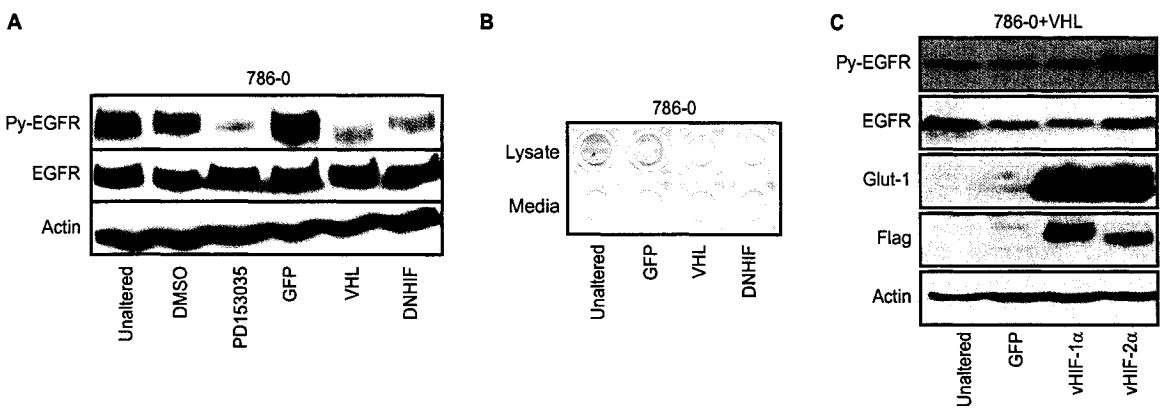

A
A498
A498+VHL
786-0
786-0+VHL
TGF-α
Glut-1
Actin
GFP – – + – – – – + – –
vHIF-1α – – – + – – – – + –
vHIF-2α – – – – + – – – – +


B
Standard
(pg/ml)
0
10
25
50
75
786-0
786-0+VHL
Lysate:
Media:
GFP – – + – –
vHIF-1α – – – + –
vHIF-2α – – – – +

3.3 HIF-2 activates the EGFR through TGF-α

We next asked if HIF-2α was able to activate the 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 (Figure 11A). Expression of VHL or DNHIF was essentially as efficient as the EGFR tyrosine kinase inhibitor PD15035 at abolishing EGFR phosphorylation in these VHL-deficient RCC cells (Figure 11A). In addition, ELISA results correlate this loss of EGFR activity with an inhibition in TGF-α expression (Figure 11B). These results are consistent with previous experiments showing that antisense-mediated inhibition of TGF-α production blocks the autonomous growth of VHL-deficient cells and abrogates EGFR phosphorylation (de Paulsen *et al.,* 2001). Importantly, expression of vHIF-2α resulted in a marked activation of the EGFR in VHL-competent 786-0 cells (Figure 11C). This finding supports the hypothesis that HIF-2 drives the autonomous proliferation of $VHL^{-/-}$ RCC cells by activating the EGFR through the specific production of TGF-α (Figure 10), and provides an explanation for the oncogenesis associated with HIF-2α in RCC (Kondo *et al.,* 2003). In contrast, vHIF-1α did not activate the EGFR in VHL-competent 786-0 cells, though detection of Flag protein and Glut-1 transcript confirmed that vHIF-1α was being expressed and was functional (Figure 11C). Data presented here further support the notion that HIF-1α is unable to drive the production of active EGFR ligands, and exclude HIF-1α activation as a growth-promoting event in kidney cancer cells. These findings also provide an explanation for the inability of this form of HIF to promote RCC tumorigenesis (Kondo *et al.,* 2002; Maranchie *et al.,* 2002).

Figure 11. Induction of TGF-α by HIF-2α activates the EGFR. **A)** Immunoblot detecting total EGFR and phosphorylated activated EGFR (Py-EGFR) in 786-O cells treated with PD153035, or infected to express GFP, VHL or DNHIF. Cells were incubated for 48hr in serum-free conditions prior to collection of lysates. **B)** 786-O cells were infected to express GFP, VHL, or DNHIF. ELISA was used to detect TGF-α protein in cellular lysates and media. Cells were incubated for 48hr in serum-free conditions prior to collection of lysates and media. **C)** HIF-2α but not HIF-1α induces EGFR activation in VHL-competent 786-0 cells. VHL-competent 786-0 cells were infected to express GFP, vHIF-1α, or vHIF-2α. Cells were incubated for 48hr in serum-free conditions prior to collection of lysates. Lysates were submitted to immunoblotting to detect the indicated proteins.

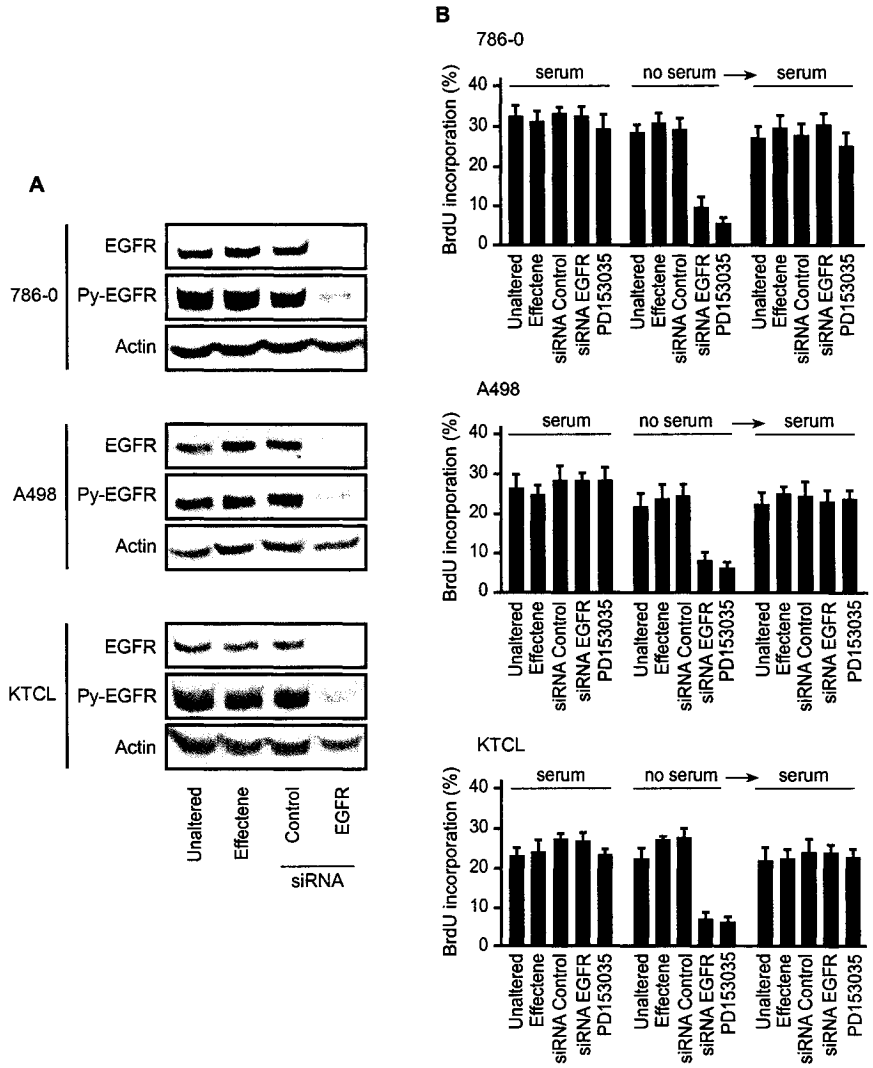

A
786-0
Py-EGFR
EGFR
Actin
Unaltered
DMSO
PD153035
GFP
VHL
DNHIF
B
786-0
Lysate
Media
Unaltered
GFP
VHL
DNHIF
C
786-0+VHL
Py-EGFR
EGFR
Glut-1
Flag
Actin
Unaltered
GFP
vHIF-1α
vHIF-2α

3.4 Transient siRNA-mediated EGFR silencing inhibits the growth autonomy and spheroid forming capacity of VHL-deficient RCC cells

To verify the involvement of EGFR signaling in promoting the aberrant growth of $VHL^{-/-}$ RCC cells, and to examine the centrality of EGFR activation in the ability of HIF-2α to drive VHL-loss RCC tumorigenesis, we decided to employ RNA interference technology. For transient inhibition of EGFR mRNA production, 786-0, A498, and KTCL VHL-deficient RCC cells were transfected with small interfering RNA (siRNA) targeting the EGFR. Western blot analysis revealed a considerable reduction of total EGFR protein by cells expressing siRNA directed against the EGFR compared to untransfected cells or cells transfected with control non-silencing siRNA or the transfection reagent alone (Figure 12A). Importantly, a reduction in EGFR phosphorylation was also observed, demonstrating a near complete impairment in EGFR function (Figure 12A). Silencing EGFR expression suppressed the ability of VHL-deficient cells to engage in HIF-2α-mediated autonomous growth as efficiently as treatment with PD153035 (Figure 12B). The effect of EGFR silencing on growth was restricted to serum-free conditions since addition of fresh serum abolished the growth inhibitory effect of the siRNA (Figure 12B, arrows). This argues against indiscriminate or non-specific effects of the siRNA on the cell cycle. These data suggest that the ability of HIF-2α to drive growth autonomy of $VHL^{-/-}$ RCC cells requires activation of the EGFR.

Next we examined whether inhibiting the HIF-2/TGF-α/EGFR pathway, by siRNA-mediated silencing of receptor function, would further translate to impaired tumor formation. To first address this question a three-dimensional *in vitro* tumor spheroid

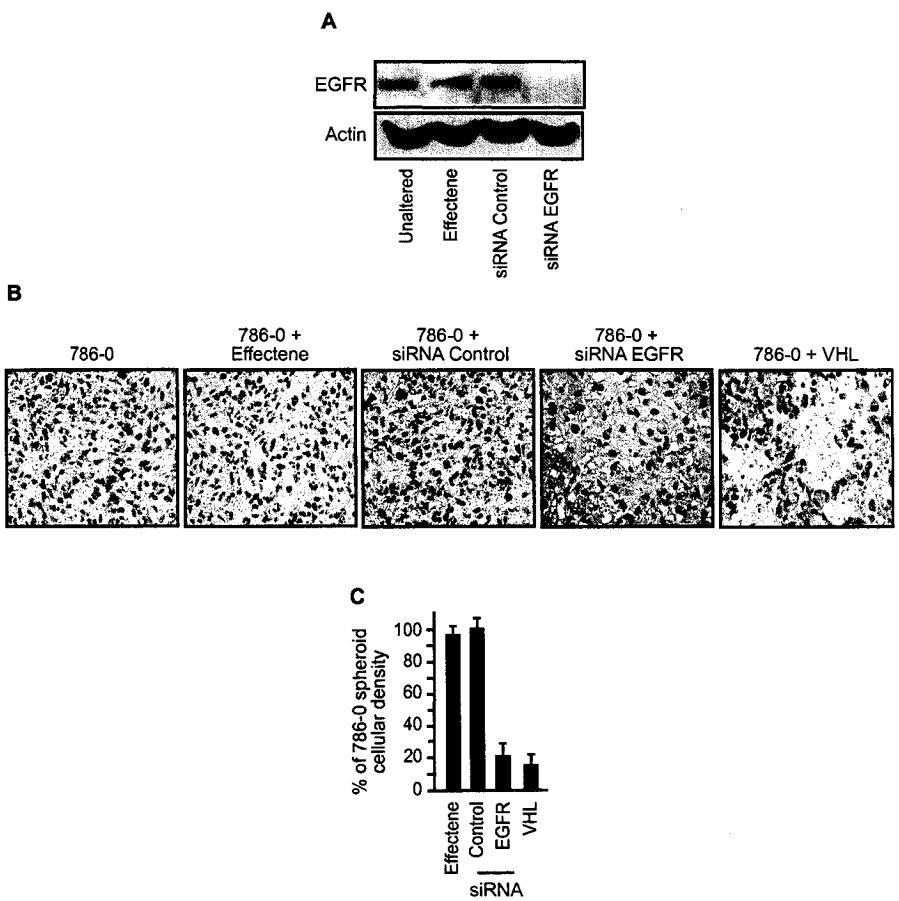
Figure 12. Transient silencing of EGFR with siRNA inhibits the growth self-sufficiency of $VHL^{-/-}$ RCC cells. A) Transient incubation with siRNA directed against EGFR mRNA suppresses EGFR protein production in multiple VHL-defective cells. Immunoblotting was performed to detect total EGFR and activated EGFR in untreated cells, cells treated with the transfection reagent alone (Effectene), control siRNA, or siRNA targeting the EGFR. **B)** EGFR silencing prevents the serum-independent growth of multiple $VHL^{-/-}$ RCC cells. VHL-deficient cells were untransfected, treated with Effectene, or transfected with control siRNA or siRNA against the EGFR. Cells were incubated in the presence or absence of serum for 72hr prior to labeling with BrdU. A growth rescue experiment (→) was performed after 72hr of serum-starvation by the addition of fresh serum-containing medium for another 48hr. PD153035 (1μM) was added for the duration of the experiments without noticeable toxic effects. Columns represent the average mean of at least three independent experiments in triplicates; bars represent the SEM.


A
786-0
EGFR
Py-EGFR
Actin
A498
EGFR
Py-EGFR
Actin
KTCL
EGFR
Py-EGFR
Actin
Unaltered
Effectene
Control
EGFR
siRNA
B
786-0
serum
no serum
serum
BrdU incorporation (%)
0
10
20
30
40
Unaltered
Effectene
siRNA Control
siRNA EGFR
PD153035
A498
KTCL

assay was employed. The *in vitro* tumor spheroid assay mimics tumor formation *in vivo*, and is an accepted assay that measures the tumorigenic potential of cancer cells (Sutherland 1988; Mueller-Klieser 1997; Kunz-Schughart *et al.,* 1998). Multicellular growth conditions mimic the basic nature of solid tumors. Avascular tumors *in vivo* typically do not grow beyond a diameter of one or two millimeters. As such, multilayered spheroids will form structures around one millimeter in diameter, the size being constrained by the availability of nutrients and oxygen (Bates *et al.,* 2000). An advantage to using the spheroid assay is that tumorigenicity can be measured in the absence of neovascularization, a variable especially important in the case of the highly angiogenic $VHL^{-/-}$ RCC tumors. With this assay we were able to examine the role of EGFR signaling in RCC tumor formation in a setting that ruled out the contribution of angiogenic factors to the initial growth stage of malignancy.

VHL-deficient RCC cells, like most tumorigenic cell lines, are able to form dense tumor spheroids *in vitro* (Lieubeau-Teillet *et al.,* 1998). Transient incubation with siRNA against the EGFR suppressed EGFR protein expression in the spheroids (Figure 13A) and prevented the formation of dense tumor spheroids (Figure 13B). Spheroid density was measured as nuclei/field of view in several spheroid sections, and for each experimental condition presented as a proportion of the cellular density of 7860 spheroids (Figure 13C). Cells treated with the transfection reagent alone or expressing non-silencing control siRNA formed dense tumor spheroids, similar to those formed by VHL-deficient RCC cells. However, the expression of EGFR-silencing siRNA in VHL-deficient RCC cells resulted in the formation of spheroids with low cellular density, similar to levels observed with reintroduction of VHL (Figure 13B, C). These data

Figure 13. SiRNA-mediated EGFR silencing attenuates the ability of VHL-loss RCC cells to form compact tumor spheroids. **A)** VHL-deficient RCC cells were untransfected, treated with Effectene, or transfected with control siRNA or siRNA against the EGFR. 24hr later, cells were plated for the *in vitro* tumor spheroid assay. On day six spheroids were harvested, total cell lysates were collected, and western blot analysis was performed to demonstrated inhibition of EGFR protein production by siRNA. **B)** EGFR silencing inhibits dense spheroid formation. Histology from spheroids generated from VHL-deficient 786-0 cells treated as described in A). VHL-competent 786-0 cells were plated as a control. Histology from spheroids is visualized at a magnification of 400X. **C)** Spheroid density was determined from H&E sections and measured at a magnification of 400X as nuclei/field of view. For each spheroid, density was measured in at least three different areas. For each treatment group, density was compared to the cellular density of 786-0 spheroids, and is presented as a proportion of this cellular density. Columns represent the average mean of at least four independent spheroids; bars represent the SEM.


A
EGFR
Actin
Unaltered
Effectene
siRNA Control
siRNA EGFR
B
786-0
786-0 + Effectene
786-0 + siRNA Control
786-0 + siRNA EGFR
786-0 + VHL
C
% of 786-0 spheroid cellular density
100
80
60
40
20
0
Effectene
Control
EGFR
VHL
siRNA

demonstrate that expression of siRNA targeting the EGFR suppresses the tumorigenic potential of $VHL^{-/-}$ RCC cells, as measured by growth autonomy and the spheroid assay, as efficiently as reintroduction of VHL.

3.5 Stable silencing of EGFR activity in VHL-deficient RCC cells inhibits their ability to engage in autonomous growth and form dense *in vitro* spheroids

Based on the data obtained in the transient siRNA-mediated EGFR silencing assays (Figures 12 and 13) we decided to examine the *in vivo* consequences of silencing EGFR activity. In order to do so, we engineered $VHL^{-/-}$RCC cells with stably inactivated EGFR by expression of shRNA. For these experiments we used the $VHL^{-/-}$ 786-0 RCC cell line, which has been extensively characterized by several other groups (Iliopoulos *et al.,* 1995; Knebelmann *et al.,* 1998; Lieubeau-Teillet *et al.,* 1998; Koochekpour *et al.,* 1999), and the $VHL^{-/-}$ KTCL RCC cell line, which has been less extensively investigated and which we have been characterizing in our lab. Several shRNAs targeted against different regions of the EGFR mRNA were tested for their ability to efficiently suppress EGFR protein production. After initial screening of numerous shRNA-expressing stable clones (Table 2), two different shRNAs, referred to as shRNA EGFR-1 and shRNA EGFR-2 were further characterized. Before proceeding to the *in vivo* nude mice assay, the growth autonomy and spheroid forming abilities of these cells were examined.

$VHL^{-/-}$ RCC cells stably expressing shRNA EGFR-1 or -2 displayed a significant decrease in EGFR protein levels compared to parental cells and cells stably expressing control scrambled shRNA (Figure 14A). Notably, loss of phosphorylated EGFR was also observed in EGFR shRNA-expressing cells, demonstrating a near-complete loss of EGFR

Table 2. Screening clones for shRNA-mediated EGFR silencing

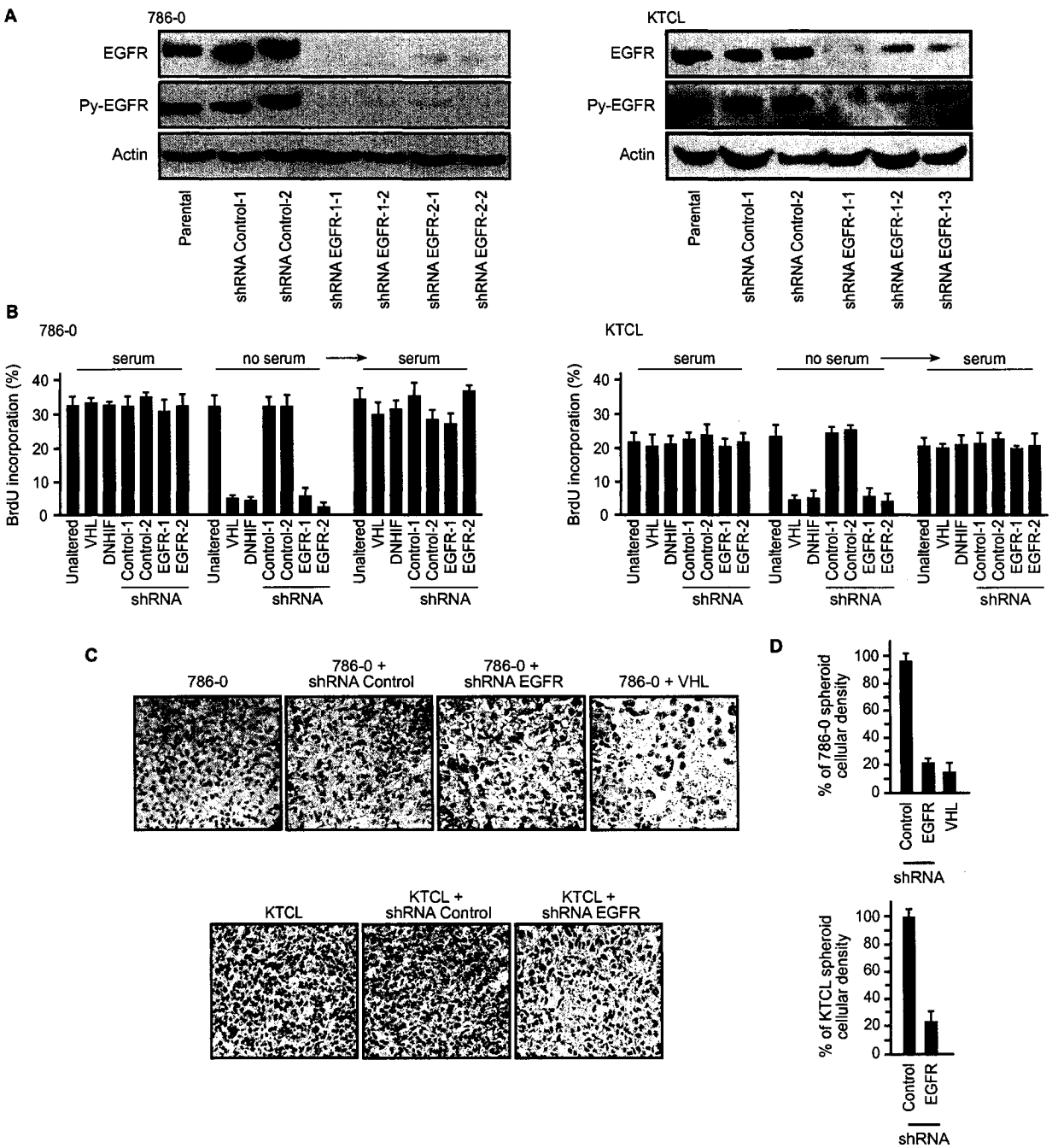
Cell Line	shRNA	DNA sequence derived from mRNA target site (Sense strand 5' to 3')*	Starting position and location of target sequence^	Clones screened	Positive clones
786-0	EGFR-1	CTCTGGAGGAAAAGAAAGT	237 Exons 1&2	29	5
KTCL				26	4
786-0	EGFR-2	CACAGTGGAGCGAATTCCT	448 Exon 3	37	6
KTCL				24	2
786-0	EGFR-3	CGTAAAGGAAATCACAGGG	1357 Exons 10&11	18	0

*Target sequences from Nagy *et al.* (2003). Shown is part of the DNA sequence to be transcribed from the pSilencer expression vector (Ambion) that will form the sense strand of the hairpin structure

^ The starting positions are given for the GI 29725608

function (Figure 14A). We did not notice a significant difference in 2-dimensional growth in the presence of serum between parental 786-0 cells, or 786-0 cells expressing VHL, DNHIF, 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 (Figure 14B). The ability of the shRNA against the EGFR to abolish the growth autonomy of VHL-defective 786-0 RCC cells was similar to that observed upon reintroduction of VHL (Figure 14B), expression of DNHIF (Figure 14B), transient incubation with siRNA against EGFR (Figure 12B), treatment with PD153035 (Figure 12B) or incubation with antisense oligonucleotides against TGF-α mRNA (de Paulsen *et al.,* 2001). EGFR protein levels and phosphorylation were also significantly diminished in the KTCL cell lines stably expressing shRNA EGFR-1-1 (Figure 14A), and the growth effects of suppressing EGFR expression in KTCL cells were very similar to those observed for 786-0 cells (Figure 14B). Furthermore, stable silencing of EGFR by shRNA resulted in low-density spheroids formation by 786-0 and KTCL cells (Figure 14C, D). These experiments confirm that VHL-defective RCC clones stably expressing shRNA directed against EGFR mRNA display a considerable decrease in EGFR protein level, phosphorylation status, and activity, without a significant dominant effect on the cell cycle – as demonstrated by the ability of these cells to proliferate in media supplemented with exogenous growth factors. The data also suggest that observations made in 786-0 cells can be generalized to other $VHL^{-/-}$ RCC cell lines.

Figure 14. Stable shRNA-mediated inhibition of EGFR protein and activity in $VHL^{-/-}$ RCC cells. A) ShRNA directed against EGFR mRNA suppresses EGFR protein expression. $VHL^{-/-}$ 786-0 and KTCL RCC cells were stably transfected with pSilencer expression vectors encoding control shRNA (referred to as shRNA control) or shRNA 1 or 2 directed against EGFR mRNA (referred to as shRNA EGFR-1 or shRNA EGFR-2, respectively). Immunoblotting was performed to detect total EGFR and phosphorylated EGFR. **B)** Stable silencing of EGFR by shRNA abolishes the growth autonomy of $VHL^{-/-}$ RCC cells as efficiently as expression of VHL or DNHIF. $VHL^{-/-}$ RCC cells infected to express VHL or DNHIF, and cells stably expressing control shRNAs or shRNA EGFR-1 or EGFR-2, were incubated in the presence or absence of serum for 72hr prior to labeling with BrdU. A growth rescue experiment (→) was performed after 72hr of serum-starvation by the addition of fresh serum-containing medium for another 48hr. Columns represent the average mean of at least three independent experiments in triplicates; bars represent the SEM. **C)** Stable silencing of EGFR by shRNA prevents dense *in vitro* tumor formation. Parental VHL-deficient RCC cells and clones stably expressing control shRNA or shRNA EGFR-1 were plated for the spheroid assay. VHL-competent 786-0 cells (786-0 + VHL) were also plated. Spheroids were harvested on day six. Histology from spheroids is visualized at a magnification of 400X. **D)** Spheroid density was determined from H&E sections and measured at a magnification of 400X as nuclei/field of view. For each spheroid, density was measured in at least three different areas. For each treatment group, density was compared to the cellular density of spheroids formed by parental cells (786-0 or KTCL), and is presented as a proportion of this cellular density. Columns represent the average mean of at least four independent spheroids; bars represent the SEM.


A
786-0
EGFR
Py-EGFR
Actin
Parental
shRNA Control-1
shRNA Control-2
shRNA EGFR-1-1
shRNA EGFR-1-2
shRNA EGFR-2-1
shRNA EGFR-2-2
KTCL
EGFR
Py-EGFR
Actin
Parental
shRNA Control-1
shRNA Control-2
shRNA EGFR-1-1
shRNA EGFR-1-2
shRNA EGFR-1-3
B
786-0
BrdU incorporation (%)
serum
no serum
serum
Unaltered
VHL
DNHIF
Control-1
Control-2
EGFR-1
EGFR-2
shRNA
KTCL
BrdU incorporation (%)
serum
no serum
serum
shRNA
C
786-0
786-0 + shRNA Control
786-0 + shRNA EGFR
786-0 + VHL
KTCL
KTCL + shRNA Control
KTCL + shRNA EGFR
D
% of 786-0 spheroid cellular density
Control
EGFR
VHL
shRNA
% of KTCL spheroid cellular density
Control
EGFR
shRNA

Results obtained from cells stably expressing EGFR silencing shRNA are consistent with results from cells transiently expressing EGFR silencing siRNA. This argues that data obtained from VHL-deficient cells stably expressing EGFR silencing shRNA are not a consequence or clonal selection. It has been reported that introduction of shRNA vectors into cells can elicit an interferon response, which has a general non-specific inhibitory effect on translation. Twenty-one-nucleotide-long siRNAs are often employed for RNA-mediated gene silencing to circumvent the interferon response (Bridge *et al.,* 2003). Therefore, as similar growth inhibitory results were obtained in both transient and stable EGFR inhibition experiments, the results from the transient siRNA-mediated EGFR silencing experiments also serve to control for any non-specific effects that the shRNA-mediated gene silencing may have had.

3.6 Inhibition of EGFR does not correct other defects associated with VHL loss

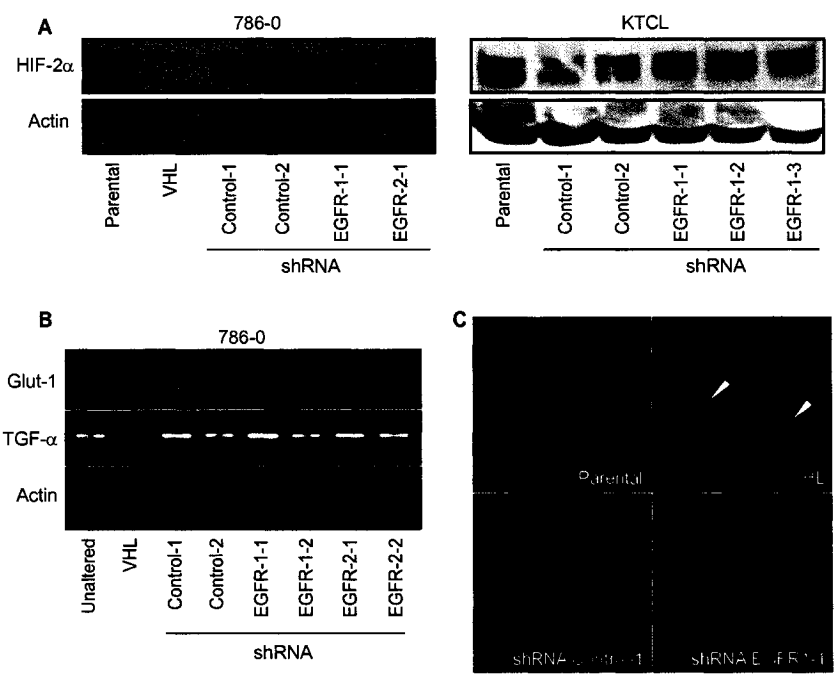
We next considered the effects of EGFR knockdown on defects that are associated with cellular transformation in VHL-loss RCC. In VHL-deficient RCC cells HIF is stabilized resulting in constitutive expression of HIF target genes. Importantly, shRNA-mediated silencing of HIF-2α prevents $VHL^{-/-}$ RCC tumor formation in a nude mice xenograph assay, demonstrating that the constitutive activation of HIF-2 targets is required for VHL-defective RCC tumorigenesis (Kondo *et al.,* 2003). In the present study, shRNA-mediated silencing of EGFR did not affect HIF-2α protein levels in VHL-deficient RCC cells (Figure 15A), nor did it affect the ability of HIF-2 to activate its target genes, such as Glut-1 and TGF-α (Figure 15B).

Another defect associated with VHL-loss RCC is the inability of $VHL^{-/-}$ RCC cells to deposit an extracellular fibronectin matrix. This contrasts with VHL-competent RCC cells, which are able to deposit an extracellular fibronectin matrix. This is associated with the differentiated phenotype of these cells (Ohh *et al.*, 1998) and their ability to form cellular aggregates with signs of organization when plated in the spheroid assay (Lieubeau-Teillet *et al.*, 1998). EGFR knockdown failed to restore the ability of VHL-deficient 786-0 cells to form an extracellular fibronectin matrix (Figure 15C). This finding is consistent with the fact that though less dense, the histology from spheroids formed by VHL-deficient RCC cells expressing EGFR silencing siRNA (Figure 13B) or shRNA (Figure 14C) more closely resembles the histology from spheroids formed by un-manipulated VHL-deficient 786-0 cells than those formed by VHL-competent cells, which are capable of proper fibronectin matrix assembly. These data indicate that shRNA-mediated EGFR silencing in $VHL^{-/-}$ RCC cells specifically corrects the growth autonomy and dense spheroid forming ability of these cells without correcting other malignant characteristics associated with VHL-loss RCC.

3.7 Stable silencing of EGFR abolishes $VHL^{-/-}$ RCC tumor formation *in vivo*

The xenograph assay is an accepted model to examine the tumorigenic potential of RCC cells, as ectopically expressed RCC cells form highly vascularized tumors in nude mice, with similar histology to that observed from RCC tumors excised from human kidneys (Angevin *et al.*, 1999). 786-0 cells form tumors when injected sub-cutaneously in nude mice, and tumor formation can be suppressed by reintroduction of VHL or shRNA-mediated inhibition of HIF-2α (Iliopoulos *et al.*, 1995; Kondo *et al.*, 2003).

Figure 15. Inhibition of EGFR does not correct other defects associated with VHL loss. **A)** Stable inhibition of EGFR does not alter HIF-2α protein levels. Immunoblotting was performed to monitor HIF-2α protein levels in 786-0 and KTCL cells stably expressing shRNA directed against the EGFR. **B)** Stable silencing of EGFR by shRNA does not prevent activation of HIF-2α-regulated genes. RT-PCR was performed from RNA isolated from the indicted clones to detect TGF-α and Glut-1 mRNA. RT PCR for actin transcript was performed for loading comparison. **C)** Inhibition of EGFR does not correct the inability of $VHL^{-/-}$ RCC cells to deposit a fibronectin matrix. VHL-deficient and VHL-competent 786-0 cells, and clones stably expressing control shRNA or shRNA EGFR-1, were grown on glass coverslips for six days in serum-containing media. Fibronectin deposition (arrow heads) was detected by immunofluorescence.


A
786-0
HIF-2α
Actin
Parental
VHL
Control-1
Control-2
EGFR-1-1
EGFR-2-1
shRNA
KTCL
Parental
Control-1
Control-2
EGFR-1-1
EGFR-1-2
EGFR-1-3
shRNA
B
786-0
Glut-1
TGF-α
Actin
Unaltered
VHL
Control-1
Control-2
EGFR-1-1
EGFR-1-2
EGFR-2-1
EGFR-2-2
shRNA
C
Parental

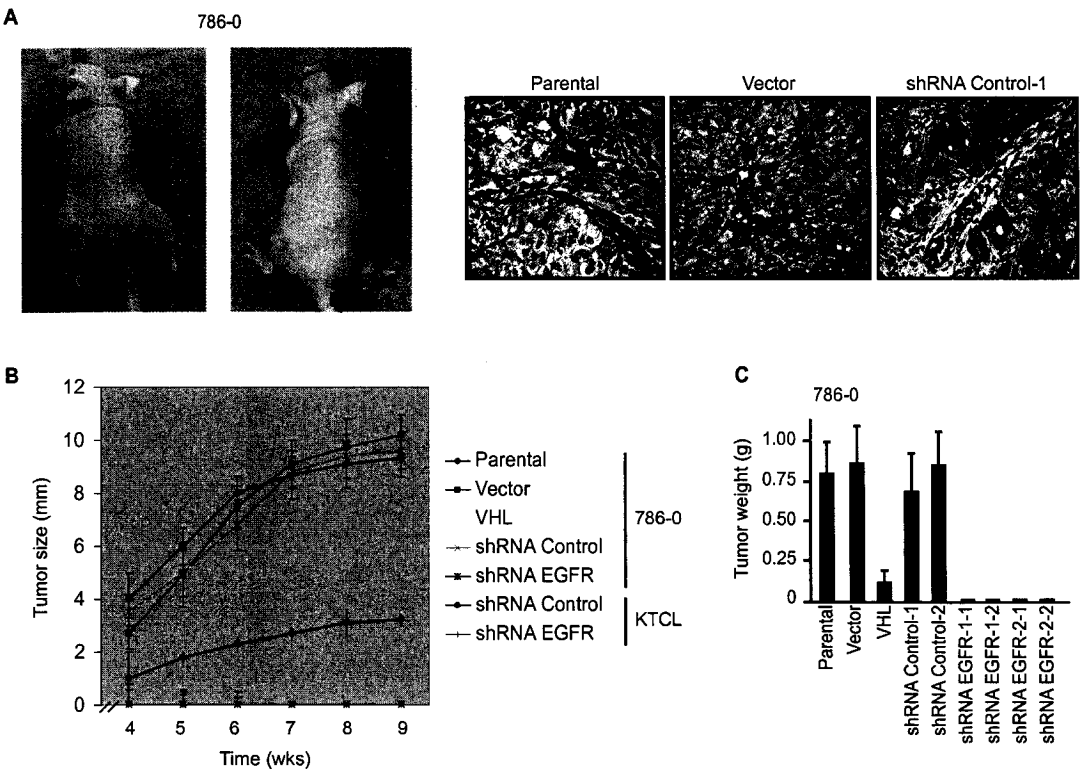
To examine the *in vivo* effects of EGFR knockdown on HIF-2-driven RCC tumorigenesis, we injected cells sub-cutaneously into nude mice and monitored tumor formation (Table 3). Parental 786-0 cells, as well as 786-0 clones expressing pcDNA 3.1 empty vector or control shRNA, produced large tumors (Figure 16A) detectable after about four weeks (Figure 16B). The first few weeks post- injection, the 'tumors' that were measured may still have been the initial cell mass from the injections, as we first noticed a recession in tumor size before tumor growth . The parental, empty vector, and control shRNA tumors reached an average size of about 9.5mm (Figure 16B) and an average weight of about 0.8g (Figure 16C). Furthermore, as is typical of human RCC tumors, these tumors were highly vascularized (Figure 16A, histology). $VHL^{-/-}$ RCC cells expressing reintroduced VHL did not form tumors after nine weeks of incubation (Figure 16A, B). Similarly, VHL-deficient 786-0 cells expressing EGFR silencing shRNA (either shRNA1 or shRNA2) failed to form tumors (Figure 16A-C). It should be noted that prolonged periods of incubation resulted in the formation of small tumors by VHL-competent RCC cells (Figure 16C, column 3). In contrast, we were unable to detect small tumors from shRNA EGFR-expressing cells even after 16 weeks of incubation. This suggests that EGFR knockdown permanently abolishes HIF-2-dependent tumor formation by $VHL^{-/-}$ RCC cells. These findings were extended to the KTCL $VHL^{-/-}$ RCC cell line, though tumors that formed in mice injected with KTCL cells expressing control shRNA were smaller than those tumors observed with the 786-0 cells (Figure 16B). Collectively, these results demonstrate 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-2-dependent tumorigenesis.

Table 3. Cell lines used in xenograph assay

Cell Lines	Tumor	Injection	Tumor*
786-0	Parental	4	4
	Vector	4	4
	VHL	8	0
	shRNA Control -1	8	8
	shRNA Control-2	8	6
	shRNA EGFR-1-1	4	0
	shRNA EGFR-1-2	4	0
	shRNA EGFR-2-1	4	0
	shRNA EGFR-2-2	4	0
KTCL	shRNA Control-1	8	4
	shRNA EGFR-1-1	4	0
	shRNA EGFR-1-2	4	0

* Number of tumors formed 9 weeks post-injection

Figure 16. Silencing of EGFR abolishes $VHL^{-/-}$ RCC tumor formation *in vivo*. A) Representative nude mice injected with $VHL^{-/-}$ 786-0 RCC cells expressing vector alone, reintroduced VHL (VHL), control shRNA or shRNA-1 targeting the EGFR. Also shown is the histology from representative tumors. Cells were injected subcutaneously (10^7 cells) into the hind flanks of female nude mice to compare their relative tumor forming abilities. Nude mice images and histology sections are at nine weeks post-injection. **B)** Graph of average tumor diameters, measured over nine weeks. Shown are the average tumor sizes +/- SEM from weeks four to nine when tumors were detectable. Experiments were blinded. **C)** Data shown are the average tumor masses, nine weeks post-injection, of at least four injection sites/cell line. In all cases when mice were injected with cells expressing EGFR silencing shRNA we failed to detect a tumor even after 16 weeks following the injection. Small tumors were detected after approximately 12 weeks in the cases of 786-0 cells expressing reintroduced VHL (shown is the tumor mass from mice injected with VHL-competent RCC cells and sacrificed 16 weeks post-injection). Columns represent the average mean of tumor weight; bars represent the SEM. Experiments were blinded.


A
786-0
Parental
Vector
shRNA Control-1
B
Tumor size (mm)
Time (wks)
Parental
Vector
VHL
shRNA Control
shRNA EGFR
shRNA Control
shRNA EGFR
786-0
KTCL
C
786-0
Tumor weight (g)
Parental
Vector
VHL
shRNA Control-1
shRNA Control-2
shRNA EGFR-1-1
shRNA EGFR-1-2
shRNA EGFR-2-1
shRNA EGFR-2-2

DISCUSSION

4.1 Mechanism of RCC tumorigenesis

It is of critical importance to ascertain the molecular basis of malignancy in order to develop effective tumor-specific treatment strategies for cancer. In VHL-loss RCC, tumorigenesis appears to be mediated by a pathway involving the HIF-2 transcription factor. Kaelin and collaborators originally proposed that HIF-2 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 (Kondo *et al.,* 2002; Kondo *et al.,* 2003). In the Eker rat model of RCC, tumorigenesis is initiated following loss of function of the Tuberous Sclerosis (Tsc-2) tumor suppressor gene. Similar to loss of VHL function in human RCC, inactivation of the Tsc-2 gene results in stabilization of HIF-2α and upregulation of HIF-2 target genes (Liu *et al.,* 2003). These results suggest that dysregulation of HIF-2α is a common event in the development of RCC.

In previous studies, we elucidated a pathway in which VHL loss of function results in the generation of a HIF-mediated TGF-α/EGFR growth stimulatory loop, responsible for the dysregulated growth of VHL-deficient RCC cells *in vitro*. Here, we uncover a mechanistic link between HIF-2α-dependent tumorigenesis and TGF-α-mediated EGFR signaling. The present study identifies that TGF-α is a HIF-2 specific transcription target, and that HIF-2α stabilization results in the aberrant growth of renal cells by eliciting EGFR signaling by way of TGF-α activation (Figure 17). In contrast, HIF-1α is unable to activate the EGFR, through TGF-α production or through other TGF-α independent mechanisms, and fails to promote autonomous growth of RCC cells. This is consistent with the inability of this HIF-α isoform to promote RCC tumor formation *in vivo* (Kondo *et al.,* 2002; Maranchie *et al.,* 2002). We further show that

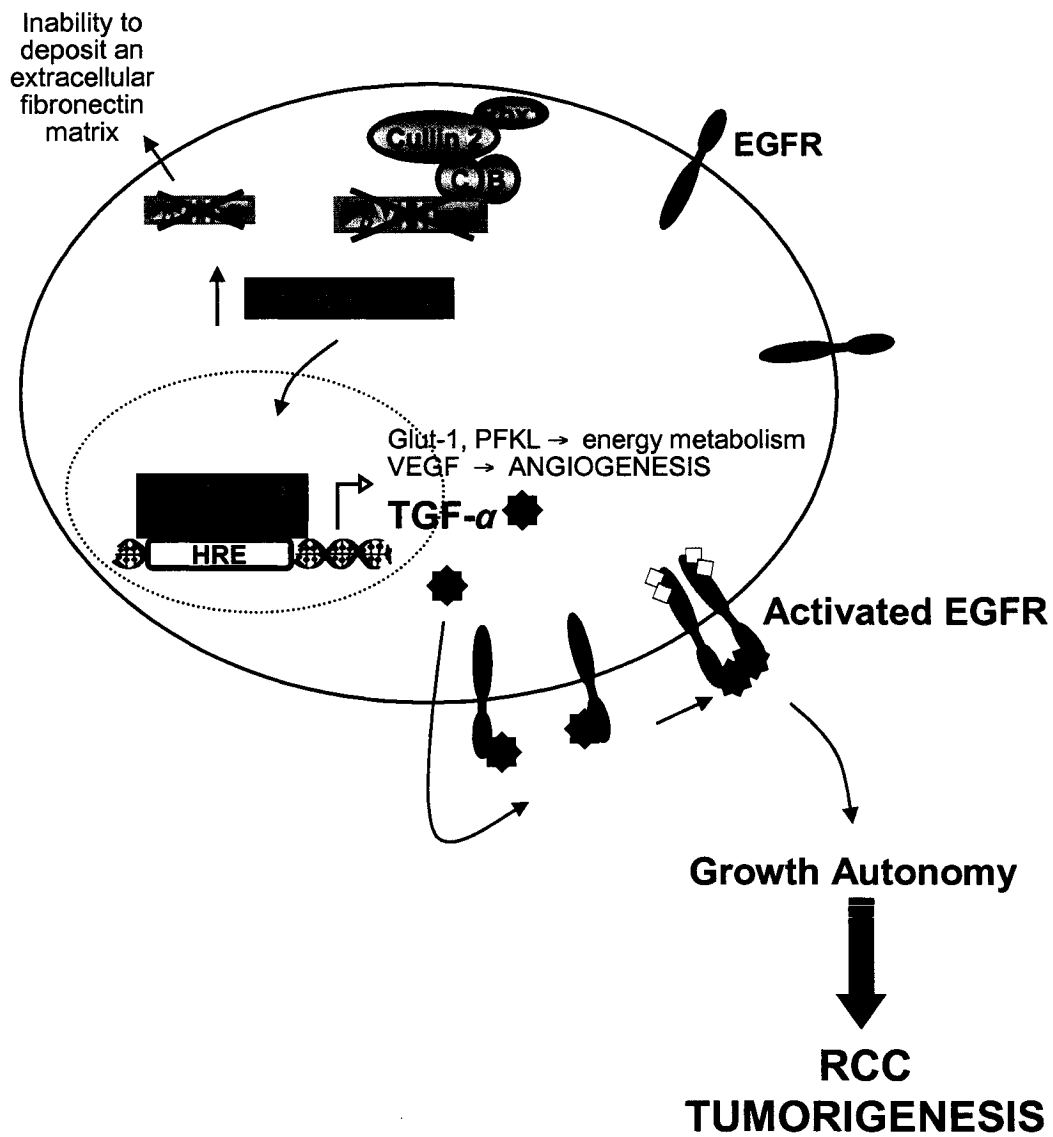
EGFR signaling is required for HIF-2α-dependent growth dysregulation and RCC tumor formation. Disruption of EGFR expression was sufficient to prevent the growth autonomy, *in vitro* tumor formation, and *in vivo* tumorigenesis of VHL-defective RCC cells, demonstrating, at the molecular level, that the EGFR is an ultimate downstream effector of the HIF-2 pathway We thus propose that oncogenic EGFR signaling is the mechanism of HIF-2-dependent VHL-loss RCC tumorigenesis (Figure 17).

4.2 Centrality of the EGFR in $VHL^{-/-}$ RCC tumorigenesis

One of the hallmarks of cancer is the ability of cancer cells to promote tumor vascularization, required for growth and metastasis (Hanahan and Weinberg 2000). The HIF transcription factor is thought to play an important role in tumorigenesis, in part, by inducing the expression of angiogenic genes (Brahimi-Horn and Pouyssegur 2004). RCC tumors are highly vascularized, and though angiogenesis is undoubtedly important for tumor progression, here we suggest that HIF-2-mediated EGFR activation is the growth promoting event driving RCC tumorigenesis.

The spheroid assay allowed us to examine the role of EGFR signaling in RCC tumor formation in a setting that excluded the contribution of angiogenic factors to the initial growth stage of malignancy. The observation that unmanipulated VHL-deficient RCC cells formed avascular spheroids, whereas transient or stable inactivation of the EGFR prevented dense spheroid formation, argues that suppression of tumorigenesis is not an indirect outcome of failed angiogenesis. Though the overexpression of hypoxia inducible genes may explain the highly vascularized nature of $VHL^{-/-}$ RCC tumors, the

Figure 17. Mechanism of HIF-2-dependent tumorigenesis in $VHL^{-/-}$ RCC. In renal epithelial cells biallelic inactivation of the VHL tumor suppressor gene results in the stabilization and accumulation of the alpha subunits of the heterodimeric HIF transcription factor. Accumulation of HIF-2α and activation of the HIF-2 transcription factor results in constitutive TGF-α expression and aberrant EGFR signaling. This provides VHL-loss renal cells with a potent means of autonomous proliferation and malignant transformation. While other defects characteristic of VHL-deficient RCC cells may contribute to the malignant phenotype associated with RCC, constitutive EGFR signaling is central for tumor initiation.


Inability to deposit an extracellular fibronectin matrix
EGFR
Glut-1, PFKL → energy metabolism
VEGF → ANGIOGENESIS
TGF-α
HRE
Activated EGFR
Growth Autonomy
RCC TUMORIGENESIS

data presented here supports the hypothesis that HIF-2α acts as an oncogene in driving tumorigenesis of VHL-loss RCC by promoting aberrant EGFR signaling.

Though we did not examine the transcription of HIF-target genes that are directly involved in angiogenesis, such as VEGF, based on the expression of TGF-α and Glut-1 we assumed that expression of such angiogenic factors was not compromised by silencing EGFR expression. However, signaling through the EGFR has many downstream effects in cells, and has been implicated in angiogenesis through HIF-independent induction of VEGF via a PI3K/Akt/Sp1 pathway (Yen *et al.,* 2002; Pore *et al.,* 2003; Pore *et al.,* 2004). In this regard, one study, examining the antitumor effects of the EGFR tyrosine kinase inhibitor Iressa in RCC, reported that in addition to inhibition of cellular proliferation VEGF production was significantly decreased in RCC cells treated with the drug compared with untreated controls (Asakuma *et al.,* 2004). In a xenograph assay, treatment of mice with Iressa significantly inhibited RCC tumor growth and immunohistochemical analysis of tumor sections revealed that blood vessel formation was inhibited as well (Asakuma *et al.,* 2004). Though we expect that we did not interfere with direct HIF-2-mediated induction of angiogenic factors, we may in effect have reduced the neo-vascularization potential of cells by inhibition of the EGFR pathway itself. Nonetheless, if EGFR signaling does contribute to the induction of angiogenic factors in RCC cells, this would serve as an added incentive to target the receptor in the treatment of RCC.

Other than aberrant EGFR signaling, inactivating mutations of the VHL gene confer a number of molecular defects to RCC cells that contribute to their malignant phenotype, including constitutive HIF activation (upstream of EGFR signaling) and

improper assembly of a fibronectin matrix. Silencing EGFR activity had no effect on the expression of HIF-2α or on the ability of HIF-2 to transactivate its target genes. This suggests that the constitutive expression of HIF-target genes is inadequate to promote $VHL^{-/-}$ RCC tumorigenesis in the absence of EGFR expression. As well, EGFR silencing did not correct the inability of VHL-deficient RCC cells to deposit an extracellular fibronectin matrix. Since silencing of EGFR was sufficient to block RCC tumorigenesis, overriding the effect of VHL loss, HIF-2 activation and failed fibronectin deposition, our results validate a pivotal role for EGFR activation in mediating HIF-2-dependent RCC tumor formation. These results, however, are not meant to downplay the role of other defects associated with $VHL^{-/-}$ RCC cells in tumor development.

4.3 Tissue specificity in the development of RCC

Both VHL and the HIF-α subunits are ubiquitously expressed in cells, yet VHL inactivation is only linked to the development of tumors in a limited number of tissues (Maher and Kaelin 1997). This suggests that the role of VHL in tumorigenesis is dependent on tissue specific function, (Kaelin 2002) and may reflect tissue specific expression and regulation of different proteins. Previously, it has been demonstrated that cyclin D1, a protein involved in the G1 to S phase transition in the cell cycle, is negatively regulated by VHL and is hypoxia inducible in RCC cell lines, but is downregulated by hypoxia in non-renal cell lines (Bindra *et al.,* 2002; Wykoff *et al.,* 2004). In another study, Maina *et al.* (2005) found that the expression of GPR56, a G-protein-coupled receptor, was suppressed by VHL inactivation and hypoxia in RCC cell lines, but not affected by hypoxia in breast or bladder cancer cell lines. These findings

suggest that distinct hypoxia-related signaling pathways may exist in renal cells, and may be involved in tissue-specific tumor development following VHL inactivation.

In the development of RCC, a prerequisite for tumor formation following VHL loss may be the preferential expression of HIF-2α over HIF-1α. In an immunohistochemical study examining HIF-α expression in the evolution of neoplastic kidney lesions in VHL disease, Mandriota *et al.* (2002) found that HIF-1α protein was detectable in the earliest foci, with maintained expression in more advanced lesions. Conversely, HIF-2α was virtually undetectable in the earliest foci, but staining of high intensity was observed in multicellular foci displaying abnormal histology, including renal cysts and overt RCC. Therefore, it may be that tumors only arise from VHL-loss RCC cells when the deregulated expression of HIF-2α predominates over that of HIF-1α.

RCC 786-0 cells express HIF-2α, but not HIF-1α, and preliminary data from our laboratory shows that infecting 786-0 cells to express HIF-1α results in a decrease in TGF-α expression. In this context, the expression of HIF-1α may have some sort of tumor suppressive effect on HIF-2α-mediated oncogenesis. In keeping with this hypothesis, Ratcliffe's group, in collaboration with others, has just published a study showing that HIF-α isoforms display suppressive interactions in RCC cells, with overexpression of HIF-1α suppressing HIF-2α expression and activity, and vice-versa. In reaffirmation of data presented here, and published in Smith *et al.* (2005), they show that HIF-2α positively regulates TGF-α and cyclin D1 (whereas HIF-1α does not), and that overexpression of HIF-1α in 786-0 RCC cells results in down-regulation of HIF-2α and cyclin D1 proteins. In a xenograph assay they demonstrate that overexpression of

HIF-2α in 786-0 cells has a positive effect on tumor growth while overexpression of HIF-1α retards it (Raval *et al.,* 2005).

In certain cell types, the hypoxic expression of HIF-1α has been associated with reduced cellular proliferation and increased apoptosis (Carmeliet *et al.,* 1998), owing, in part, to HIF-1-mediated induction of the cyclin-dependent kinase inhibitors p21 and p27 (Goda *et al.,* 2003; Koshiji *et al.,* 2004). To examine the effects of VHL loss and constitutive HIF activation on cell cycle progression, Mack *et al.* (2005) generated $VHL^{-/-}$ mouse embryonic fibroblasts (MEFs). In these cells HIF-1α proteins were stabilized under normoxic conditions, whereas HIF-2α proteins were undetectable. $VHL^{-/-}$ MEFs exhibited defects in cellular proliferation, which, compared to wild-type MEFs, correlated with increased expression of p21 and p27. Si-RNA-mediated silencing of HIF-1α resulted in a decrease in the expression of these two proteins, indicating that the elevated levels of p21 and p27 result from constitutive HIF-1 activation. These results suggest that the activation of HIF-1α can lead to growth arrest. Consistent with a postulated tumor suppressor role for HIF-1α in RCC, in their study, Raval *et al.* (2005) demonstrated that the proapoptotic gene encoding BNip3 is positively regulated by HIF-1α in RCC cells, and negatively regulated by HIF-2α.

Therefore, subsequent to loss of VHL function, additional genetic or epigenetic events may be required for tumor progression - specifically the evolution of the exclusive or predominant expression of HIF-2α over HIF-1α. In support of this hypothesis, the trend towards expression of HIF-2α alone is seen in various RCC cell lines established from primary human tumors (Maxwell *et al.,* 1999; Turner *et al.,* 2002); and, while RCC cells that exclusively express HIF-2α form tumors in nude mice, to our knowledge cells

that express both HIF-α isoforms are not tumorigenic in xenograph assays. As we report here, HIF-2α mediated TGF-α production and EGFR activation provide RCC cells with a potent growth "engine". Thus, in growth factor-limiting conditions, selection for and clonal expansion of HIF-2α-expressing cells may ultimately drive RCC tumor formation.

4.4 VHL and HIF-2α in cancer

Recently, a Ph.D. student in our laboratory discovered that hypoxia and/or acidosis can trigger the nucleolar sequestration of VHL, inactivating its function (Mekhail *et al.,* 2004). Nucleolar confinement of VHL in the hypoxic acidic core of tumors can promote dysregulated HIF expression and, though still unproven, may also contribute to defects in fibronectin deposition and extracellular matrix assembly. As previously mentioned, inactivating mutations in the VHL tumor suppressor gene have only been associated with the development of tumors in a small number of tissue types (Maher and Kaelin 1997); however, nucleolar sequestration and inactivation of VHL function may prove to be an important epigenetic mechanism mediating the progression of many human cancers.

HIF is commonly activated in the hypoxic core of tumors and induces the expression of a number of genes that contribute to the malignant phenotype of cancer cells. Most well characterized of these are genes encoding proteins involved in anaerobic glycolysis and angiogenesis (Harris 2002; Vaupel 2004). Data presented here suggests that activation of the TGF-α/EGFR oncogenic pathway represents a previously unappreciated mechanism of tumor progression in HIF-2α expressing cancers.

HIF-2 activation provides an environment of continued production of endogenous growth factor (TGF-α) and, in cells expressing the EGFR, of sustained EGFR signaling. Activation of the EGFR enhances processes responsible for tumor growth and progression, by promoting cellular proliferation, angiogenesis, and invasion/metastasis, and by inhibiting apoptosis (Salomon *et al.,* 1995; Arteaga 2002; Marmor *et al.,* 2004). In cancer patients, EGFR overexpression and deregulated EGFR signaling in solid tumors are associated with more aggressive disease and poor responsiveness to therapy (Nicholson *et al.,* 2001).

HIF-2α is induced in the vast majority of human cancer as a consequence of altered microenvironment conditions (e.g. hypoxia, acidosis) found in the core of tumors (Talks *et al.,* 2000; Mekhail *et al.,* 2004). TGF-α overexpression and oncogenic dysregulation of EGFR activity are also common features of human malignancies and are thought to provide permanent self-sufficiency in growth signaling that drives growth autonomy of cancer cells (Salomon *et al.,* 1995). While still unproven, TGF-α/EGFR activation found in many tumors may be explained, in part, by HIF-2α activation, and in keeping with the role of EGFR signaling in tumorigenesis, may shed light on why the hypoxic cores of tumors are associated with increased probability of metastasis and poor prognosis (Subarsky & Hill, 2003). This hypothesis is supported by data gathered in our lab, which suggest that the expression of HIF-2α drives TGF-α production and EGFR activation in many human cancer cell lines from different tissue origins (Smith *et al.,* 2005). Therefore, the tumor microenvironment may present a physiological cue that promotes malignant progression via hypoxic stabilization of HIF-2α and activation of the TGF-α/EGFR tumorigenic pathway.

4.5 The EGFR in RCC – Clinical applications

Surgery, to remove all or parts of the kidney, remains the mainstay treatment for kidney cancer. However, patients undergoing surgery usually present with, or will eventually develop, metastatic disease (Bukowski and Novick 2000). Traditional therapeutics, including chemotherapy and radiation treatment, have exhibited poor results in the treatment of RCC, and cytokine therapy only elicits responses in a small number of patients with long-term survival not significantly affected (Vuky and Motzer 2000). Thus, the development of novel therapeutic strategies to treat people suffering from kidney cancer is of great urgency.

$VHL^{-/-}$ clear cell renal cancer is the most common type of renal tumor, and accounts for about 75% of cases (Linehan *et al.,* 2003). Understanding the VHL pathway and how VHL-loss and HIF activation lead to clear cell kidney cancer provides a great opportunity for the development of disease-specific therapy for patients with RCC. HIF activates an array of genes in RCC, some of which have generated interest as potential therapeutic targets. Drugs targeting VEGF, the VEGF receptor, the PDGF receptor, and the TGF-α/EGFR pathway are in various phases of clinical trials (Sosman 2003). However, as silencing EGFR expression was sufficient to abolish the growth autonomy, avascular tumor formation, and *in vivo* tumorigenesis of $VHL^{-/-}$ RCC cells, our results unambiguously argue that the EGFR should be the prime and *bona fide* therapeutic target for non-surgical treatment of VHL-defective RCC.

A number of EGFR targeted therapies have been developed for cancer treatment, including monoclonal antibodies directed against the receptor that block ligand binding, TK inhibitors that compete with ATP for binding to tyrosine kinase catalytic domains,

ligands conjugated to toxins that can bind to the EGFR and deliver a 'lethal payload', and bi-specific antibodies with one antigen arm that is specific for the EGFR and the other arm that binds to an immunologic effector cell (Mendelsohn and Baselga 2003). Blockade of EGFR signaling with monoclonal antibodies or TKIs has been shown to inhibit the growth autonomy of VHL-deficient RCC cells, and to abolish primary as well as metastatic RCC tumor formation in mice (Kedar *et al.,* 2002; Weber *et al.,* 2003; Asakuma *et al.,* 2004; Foon *et al.,* 2004). Unfortunately, the encouraging effects of anti-EGFR targeted therapies in pre-clinical RCC models have not yet been achieved in human clinical trials (Dancey 2004). In a phase II clinical trial evaluating ABX-EGFR - a fully human monoclonal antibody directed against the EGFR - in 88 patients with metastatic renal cancer, although a single complete response (disappearance of all clinical evidence of tumors) was observed only 5 patients had disease regression (Rowinsky 2004). A phase II trial of the EGFR tyrosine kinase inhibitor Gefitinib (Iressa) in patients with advanced renal cancer did not alter outcome in those patients (Drucker *et al.,* 2003). In a more recent phase II trial of Iressa in 22 patients with advanced metastatic renal cancer, no complete or partial response was observed, though 38% of previously progressing patients had stable disease with an associated prolonged survival.

An important clinical concern is why EGFR-targeted therapies show great promise for the treatment of RCC in pre-clinical experiments with less impressive results in human clinical trials (Drucker *et al.,* 2003; Dancey 2004). The failure of these drugs to elicit complete or partial patient responses may reflect poor delivery to the tumor and an inability to reach the required dose for anti-tumorigenic effects, or may be due to drug-resistance. Mechanisms that mediate resistance to anti-EGFR therapies include: the

presence of transporters that actively extrude drugs from the cells; the presence of alternative receptor tyrosine kinases whose activation results in the activation of overlapping or redundant signal transduction pathways; the selection of cancer cells with altered EGFR-independent survival pathways in tumors, leading to latent tumor growth following anti-EGFR therapy; alterations in mediators of downstream signaling pathways resulting in constitutive activation of these pathways; and EGFR mutations that may influence the sensitivity of the cells to EGFR-targeted therapies (Camp *et al.,* 2005; Hynes and Lane 2005). In support of the latter of these mechanisms, evidence in lung cancer suggests that Iressa may only be active in a minority of patients who harbor specific activating mutations in the EGFR (Lynch *et al.,* 2004; Sordella *et al.,* 2004) - whether or not this is the case with RCC has yet to be determined. Recently, changes in EGFR receptor dynamics have also been shown to influence responses to EGFR-targeted agents; some EGFR-targeted agents may in fact induce upregulation of the EGFR, rendering the receptor insensitive to that treatment (Jimeno *et al.,* 2005).

In the present study, our results confirm at the molecular level that the EGFR is a valid target for the treatment of RCC. Furthermore, given that all of the cell lines used in the present study were derived from patients with clear cell renal cancer, our findings support the continued search for effective therapies that target the EGFR pathway in RCC, and encourage the continued investigation into different parameters affecting the efficacy of anti-EGFR agents in the treatment of this disease.

4.6 Future directions

In the current study we addressed the pivotal role of the EGFR in VHL-loss HIF-2-dependent RCC tumor initiation; however, all of the $VHL^{-/-}$ RCC cell lines that we used were derived from tumors from patients with advanced RCC, and were thus cells that had progressed well beyond the 'initial tumor formation' stage of malignancy. Yet, silencing EGFR expression in these cells was sufficient to inhibit serum-independent growth and tumor formation, suggesting that at the advanced stage of RCC the EGFR is required for some aspects of tumorigenesis.

EGFR activation is implicated in several pathways involved in tumor progression, including those mediating tumor cell proliferation and survival, angiogenesis, motility, and invasion (Salomon *et al.,* 1995; Arteaga 2002; Marmor *et al.,* 2004). As a result, inhibition of EGFR activity may influence multiple aspects of tumor development, above and beyond initial tumor growth. In this regard, it would be of interest to address the fundamentality of EGFR expression and activity in the evolution of RCC. In order to do this, one could employ an inducible system to express EGFR silencing shRNA in VHL-deficient RCC cells once they have already formed tumors in nude mice. This could be accomplished using a tetracycline regulated system of gene expression (Matsukura *et al.,* 2003). For tumor formation, cells could be implanted subcutaneously in nude mice or injected in the tail vein to establish metastases. To induce expression of the shRNA mice would be treated with tetracycline incorporated in their diet, administered by oral gavage, or administered by intraperitoneal injections. By silencing EGFR expression in cells once tumors have already formed we would be able to investigate if abolishing EGFR activity would inhibit further tumor growth or even translate to tumor regression. To

examine the effects of EGFR silencing on cell viability and angiogenesis immunohistochemical staining of tumor specimens to detect apoptotic cells and microvessel density could also be accomplished. This study would either fully validate or totally refute the EGFR as the key target in the treatment of RCC, and would thus be of great clinical relevance.

One investigation that is presently underway in our laboratory is aimed at the observation that in VHL-deficient RCC cells HIF-2 induced TGF-α remains cell-associated with almost no detectable TGF-α protein in the extracellular media (Figures 10 and 11; Derynck *et al.,* 1987). Both membrane-bound and soluble TGF-α have been reported to activate the EGFR and have biological activity (Brachmann *et al.,* 1989). However, Baselga *et al.* (1996) reported that the transmembrane form of TGF-α appears to be preferentially expressed in malignant cells. As well, some reports suggest that membrane-bound pro-TGF-α activates the EGFR more potently than soluble TGF-α (Shi *et al.,* 2000; Yang *et al.,* 2000). On the other hand, it has been reported that inhibitors of ectodomain shedding, or the expression of shedding-resistant pro-TGF-α deletion constructs, prevent juxtacrine activation of the EGFR (Dong *et al.,* 1999; Borrell-Pages *et al.,* 2003). In RCC cells, the cell associated TGF-α may represent un-cleaved proTGF-α that in its membrane bound state somehow facilitates constitutive EGFR signaling. Conversely, the cell associated TGF-α may represent fully processed TGF-α that is secreted into the media but, because of high levels of EGFR expression, is very efficiently captured by the receptor and internalized. The VHL-deficient RCC clones in which shRNA-mediated silencing has inhibited EGFR expression provide us with a direct tool to examine these hypotheses. ELISA results suggest that when EGFR expression is

downregulated in VHL-defective RCC cells, a fair amount of TGF-α protein accumulates in the extracellular media rather than remaining cell associated. The enzyme TACE mediates the processing/ectodomain shedding of TGF-α (Peschon *et al.,* 1998; Sunnarborg *et al.,* 2002), and these results suggest that TACE is functional in $VHL^{-/-}$ RCC cells. A Master's student in our laboratory is currently using drugs targeting TACE activity and RNA interference technology to examine the role of TACE in TGF-α-induced EGFR activation in RCC cells. If TACE is required for the growth autonomy and tumorigenicity of RCC cells, inhibiting its activity may serve as an alternative method of abolishing TGF-α-dependent EGFR signaling. In this regard, TACE may be a valid alternative drug target in the treatment of VHL-loss RCC.

4.7 Conclusions

Here, we provide a mechanistic explanation for HIF-2-dependent RCC tumorigenesis. We show that HIF-2α, but not HIF-1α, is able to promote autonomous growth of RCC cells by specifically activating the EGFR through TGF-α production. Moreover, we identify the EGFR as a vital downstream signaling molecule in HIF-2-dependent RCC tumor formation, since silencing EGFR expression abolished tumor formation of VHL-deficient RCC cells *in vitro* and in nude mice *in vivo*. Our findings demonstrate that the EGFR is essential for HIF-2-dependent tumorigenesis in RCC, and reaffirm that anti-EGFR therapy is the key strategy for the treatment of VHL-loss kidney cancer.

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