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UNDERSTANDING THE ROLE OF HIF-2α IN HUMAN CANCERS

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of
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ABSTRACT

Cancer development is a multi-step process, driven by a series of genetic and environmental alterations, which endows cells with a set of hallmark traits required for tumorigenesis. It is broadly accepted that growth signal autonomy, the first hallmark of malignancies, can be acquired through multiple genetic mutations that activate an array of complex, cancer-specific growth circuits. The superfluous nature of these pathways is thought to limit therapeutic approaches targeting cell proliferation and so this strategy is often abandoned in favor of inhibiting more systemic hallmarks. Here, we report that genetically diverse cancers converge at a rate-limiting oncogenic axis instigated by HIF-2α, a component of the oxygen-sensing machinery. Inhibition of HIF-2α prevents the *in vivo* growth and tumorigenesis of a panel of highly aggressive human cancer cell lines, irrespective of their mutational status and tissue of origin. We further offer mechanistic evidence that HIF-2α exerts its proliferative effects through the translational upregulation and/or activation of select receptor tyrosine kinases, including the EGFR and IGF-IR, and their downstream signaling pathways. Consistently, silencing these receptors or blocking upstream ligand processing events phenocopies the loss of HIF-2α activity, abrogating the serum-independent growth of cancer cells in culture and their tumor formation in athymic mice. Taken together, the data presented in this thesis reveal an important role for HIF-2α in promoting the persistent proliferation of neoplastic cells and suggest that tumors do share common growth elements that can serve as drug targets in the treatment of a wide range of human cancers.

AUTHORIZATIONS

First-Author Manuscripts:

Cancer Research (2006)

Franovic, A., I. Robert, K. Smith, G. Kurban, A. Pause, L. Gunaratnam, and S. Lee. (2006) Multiple acquired renal carcinoma tumor capabilities abolished upon silencing of ADAM17. *Cancer Res.* **66**, 8083-8090.

Proceedings of the National Academy of Sciences of the United States of America (2007)

Franovic, A., L. Gunaratnam, K. Smith, I. Robert, D. Patten, and S. Lee. (2007) Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 13092-13097.

Proceedings of the National Academy of Sciences of the United States of America (2009)

Franovic, A., C. E. Holterman, J. Payette, and S. Lee. (2009) Human cancers converge at the HIF-2α oncogenic axis. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 21306-21311.

Co-Author Manuscripts:

Smith, K., L. Gunaratnam, M. Morley, **A. Franovic**, K. Mekhail and S. Lee. (2005) Silencing of epidermal growth factor receptor suppresses hypoxia-inducible factor-2-driven VHL-/- renal cancer. *Cancer Res.* **65**, 5221-5230.

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LIST OF ABBREVIATIONS

A254	Absorbance reading at a wavelength of 254 nM
ADAM	A disintegrin and metalloproteinase
Ala	Alanine
ANG	Angiopoietin
ARNT	Aryl hydrocarbon receptor nuclear translocator
APC	Adenomatous polyposis coli tumor suppressor
ATP	Adenosine triphosphate
BAF	Bafilomycin
BCA	Bicinchoninic acid
BCR/ABL	Breakpoint cluster region/ v-Abl Abelson murine leukemia viral oncogene homolog
bHLH	Basic-helix-loop-helix
BrdU	5-bromo-2'-deoxy-uridine
BSA	Bovine serum albumin
C225	Cetuximab
CA9	Carbonic anhydrase IX
Cbl	Casitas B-lineage lymphoma
CBP	CREB-binding protein
CCND1	Cyclin D1 gene
CCNG2	Cyclin G2 gene
Cdc53	Cell division cycle 53
CDKN2A	Cyclin-dependent kinase inhibitor 2A; p16 gene

CHO	Chinese hamster ovary
CHX	Cycloheximide
Ci	Curie
CITED2	CBP/p300-interacting transactivator with Glu/Asp carboxy terminal domain, 2
CR1-2	Cysteine-rich regions 1 and 2
CREB	cAMP response element binding
Cre-lox	Cyclization recombinase - Locus of cross-over P1 site
CTAD	C-terminal transactivation domain
CTGF	Connective tissue growth factor
Cul-2	Cullin-2
CXCR4	Chemokine (C-X-C motif) receptor 4
Da	Dalton
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DN-HIF	Dominant-negative HIF
DNA	Deoxyribonucleic acid
E2F1	E2F transcription factor 1
ECAD	E-cadherin
EDTA	Ethylenediaminetetraacetic acid
EGLN	Egl nine homolog (C. elegans); HIF prolyl hydroxylase (PHD)
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor; HER1, ErbB1

ElB	Elongin B
ElC	Elongin C
ELISA	Enzyme-linked immunosorbent assay
EPAS1	Endothelial PAS domain protein 1; HIF-2α, HLF, MOP2
EPO	Erythropoietin
ER	Estrogen receptor
ErbB	Avian erythroblastosis oncogene B; HER
ERK1/2	Extracellular signal-regulated kinase; p44/42 MAPK
ES	Embryonic stem
EtOH	Ethanol
ETS	v-Ets erythroblastosis virus E26 oncogene homolog 1
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FIH1	Factor-inhibiting HIF1
FLK1	Kinase insert domain receptor gene; VEGF receptor 2
FLT1	FMS-related tyrosine kinase 1 gene; VEGF receptor 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GFP	Green fluorescent protein
GLUT	Glucose transporter
GM6001	Ilomastat
H&E	Hematoxylin and eosin

H-ras	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
HB-EGF	Heparin-binding epidermal growth factor
HA	Hemagglutinin
HER	Human epidermal growth factor receptor; ErbB
HIF	Hypoxia-inducible factor
HK	Hexokinase
HLF	HIF-1α-like factor; EPAS1, HIF-2α, MOP2
HRE	Hypoxia response element
HRP	Horseradish peroxidase
IC50	Half maximal inhibitory concentration
IFNα	Interferon-alpha
IGF	Insulin growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-IR	Insulin-like growth factor 1 receptor
IL-2	Interleukin-2
IR	Insulin receptor
ITS	Insulin-transferrin-selenium
KRAS	v-Ki-ras Kirsten rat sarcoma viral oncogene homolog
Ki-67	Antigen identified by monoclonal antibody Ki-67
L1-2	Leucine-rich regions 1 and 2
LAC	Lactacystin
LDHA	Lactate dehydrogenase A
LOX	Lysyl oxidase

MAPK	Mitogen-activated protein kinase
Max	Myc-associated factor X
Mdm-2	Murine double minute oncogene
MEK	Mitogen-activated protein kinase ERK kinase; MAP2K
Met	Mesenchymal-epithelial transition factor; c-Met
miRNA	MicroRNA
Miz1	Myc-associated zinc-finger protein 1
MMP	Matrix metalloprotease
MOI	Multiplicity of infection
MON	Monensin
MOP2	Member of PAS family 2; EPAS1, HIF-2α, HLF
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MVD	Microvessel density
Myc	v-Myc myelocytomatosis viral oncogene homolog
NP-40	Nonidet P-40
NSCLC	Non-small cell lung carcinoma
NTAD	N-terminal transactivation domain
OCT4	Octamer-4
ODD	Oxygen-dependent degradation domain
p	Short-arm of chromosome
p300	E1A binding protein
PAGE	Polyacrylamide gel electrophoresis

PAI	Plasminogen activator inhibitor
PAS	Per/Arnt/Sim
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK	Pyruvate dehydrogenase kinase
Per	Period circadian protein
PET	Polyethylene terephthalate
PGK	Phosphoglycerate kinase
PHD	Prolyl-4-hydroxylase
phEGFR	Phosphorylated epidermal growth factor receptor
ph-S6	Phosphorylated S6 ribosomal protein
PI(3)K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLCγ	Phospholipase C-gamma
PMSF	Phenylmethylsulphonyl fluoride
PR	Progesterone receptor
PSI	Proteasome inhibitor I
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
Raf	v-Raf-1 murine leukemia viral oncogene homolog 1
RB1	Retinoblastoma gene
Rbx-1	Ring-box protein 1

RCC	Clear cell renal carcinoma
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
rTGFα	Recombinant transforming growth factor-alpha
RTK	Receptor tyrosine kinase
[^{35}S]Met	Methionine labeled with radioactive sulfur-35
S	Svedberg sedimentation coefficient
S6	Ribosomal protein S6
SCF	Skp1/Cdc53/F-Box complex
SDF	Stromal cell-derived factor; CXCR ligand
SDS	Sodium dodecyl sulfate
shRNA	Short-hairpin ribonucleic acid
Sim	Single-minded protein
siRNA	Small-interfering ribonucleic acid
Skp1	S-phase kinase-associated protein 1
SRC	v-Src sarcoma (Schmidt-Ruppin A2) viral oncogene homolog
ssDNA	Single-stranded deoxynucleic acid
STAT	Signal transducers and activators of transcription
SV40	Simian vacuolating virus 40
TACE	Tumor necrosis factor-alpha converting enzyme
TAD	Transactivation domain

TGFα	Transforming growth factor-alpha
TGFβ	Transforming growth factor-beta
TIE	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TK	Tyrosine kinase
TM	Transmembrane
TNFα	Tumor necrosis factor-alpha
*TP*53	p53 tumor suppressor gene
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
U	Enzyme activity unit
UPAR	Urokinase plasminogen activator receptor
Val	Valine
VBC/Cul-2	VHL/Elongin B/Elongin C/Cullin-2 complex
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel-Lindau
VHL$^{-/-}$ RCC	RCC harboring bi-allelic inactivating *VHL* mutations
VHL^{+} RCC	*VHL*$^{-/-}$ RCC cells stably re-expressing wild-type *VHL*
WCL	Whole cell lysate
WT7	*VHL*$^{-/-}$ 786-0 RCC cells stably re-expressing wild-type *VHL*
ZD1839	Gefitinib, Iressa

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INTRODUCTION

1. INTRODUCTION

1.1. The Biology of Cancer

1.1.1. The Nature of Cancer.

Cancer is a collective term used to describe over 200 diseases characterized by unrestrained cell proliferation that eventually leads to the invasion and destruction of healthy tissues. The pervasiveness of this disease is reflected in the Canadian Cancer Society's projections that 40-45% of Canadians will develop cancer during their lifetime and 25% will die as a result (Canadian Cancer Society, 2009). Genetic abnormalities account for the vast majority, if not all, human cancers. The accumulation of genetic mutations elicits the emergence of cell populations lacking the intrinsic control mechanisms that ensure their appropriate behavior. In accordance, epidemiological risk factors for cancer development, including age, carcinogen and ionizing radiation exposure, viral/bacterial infection, and heritability, often cause or are associated with the acquisition of genetic aberrations.

1.1.2. The Hallmarks of Cancer.

Cancer development is a multi-step process which endows cells with a set of characteristic traits required for their inappropriate expansion in the face of rigid cell-autonomous homeostatic mechanisms as well as environmental barriers to their growth (Gatenby and Gillies, 2008). These traits, coined the hallmarks of cancer, include the ability to proliferate in a growth signal-independent manner, evade antigrowth and pro-apoptotic signals, replicate limitlessly, induce new blood vessel formation, and invade surrounding tissues (Hanahan and Weinberg, 2000). The latter attributes, essential for

tumor progression, are largely dependent on common physiological parameters and hence involve more widespread mechanisms. In contrast, the proliferative and survival advantages that trigger deregulated cell growth and initiate tumorigenesis are thought to be acquired through the genetic activation of an array of dominant oncogenes or inactivation of tumor suppressor genes (Vogelstein and Kinzler, 2004). As such, the current consensus is that cancers evolve in a parallel manner to attain the same spectrum of malignant phenotypes.

1.1.3. Cancer-Susceptibility Genes.

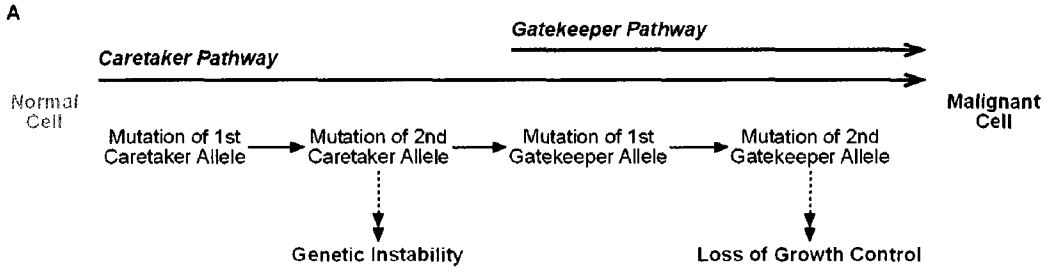
The Darwinian model of tumorigenesis depicts a transformation process whereby genetic mutations induce growth promoting phenotypic changes and ensuing physical obstacles force additional adaptations that support tumor progression (Nowell, 1976; Fearon and Vogelstein, 1990; Gatenby and Gillies, 2008). Whether these genotypic-phenotypic alterations occur at the earliest steps of transformation or instead are accumulated in a chronological fashion is subject to debate. Nonetheless, it is agreed that cancer cells must undergo successive genomic alterations in order to attain the full scope of rate-limiting tumorigenic traits. There are two classes of cancer susceptibility genes which increase the risk of cancer development: gatekeeper and caretaker genes (Kinzler and Vogelstein, 1997). Gatekeeper genes, which encompass most of the classical oncogenes and tumor suppressor genes, are those which are directly involved in the regulation of cell proliferation. Mutation of a gatekeeper gene is thus sufficient to confer cells with a selective growth advantage and initiate tumor formation. Caretaker or stability genes, on the other hand, are those which are involved in maintaining the integrity of the genome. Mutation of a caretaker gene therefore predisposes the afflicted individual to genetic

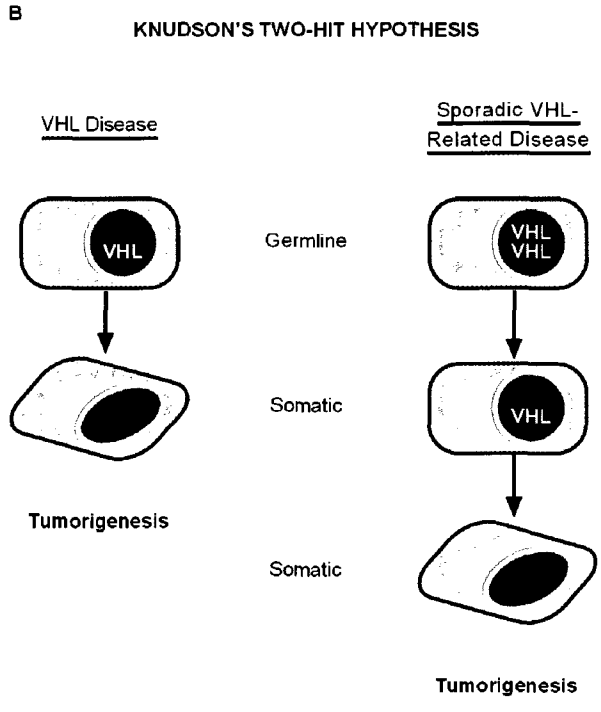
mutations and neoplasia indirectly, ultimately requiring obstruction of a gatekeeper pathway for tumorigenesis to commence (**Fig. 1A**).

1.1.4. Oncogenes and Tumor Suppressor Genes.

Mutations affecting oncogenes and tumor suppressor genes, alike, are associated with defects in the regulatory circuits that govern cell proliferation and differentiation. A dominant activating mutation in a single allele of an oncogene instigates tumorigenesis either by promoting cell growth, inhibiting programmed cell death, or altering the ability of cells to respond to anti-growth signaling (Vogelstein and Kinzler, 2004). Consistently, most of the known oncogenes are elements of mitogenic signal transduction pathways, including receptor tyrosine kinases (RTKs) and their downstream effector and adaptor proteins (Vogelstein and Kinzler, 2004). Conversely, tumor suppressor genes act as safeguards against aberrant cellular division by suppressing cell cycle progression or promoting apoptosis. With few exceptions, both alleles of a tumor suppressor gene must be inactivated for its repressive function to be effectively abrogated, as predicted by Knudson's two-hit hypothesis (Knudson, 1971) (**Fig. 1B**). To date hundreds of mutations thought to affect putative oncogene and tumor suppressor gene pathways have been identified in various hereditary and sporadic cancers. Recent large-scale genomic studies have revealed that approximately 60 genetic mutations are found on average per tumor, which in turn affect a dozen specific signaling pathways per cancer type (C.G.A.R.N., 2008; Jones et al., 2008; Parsons et al., 2008). It is thus commonly believed that cell autonomous proliferative capability, the first hallmark of malignancies, is acquired through complex genetic mutations that activate a number of cancer-specific growth pathways.

Figure 1. Tumor-initiating genetic events. **(A)** Gatekeepers and caretakers represent the two main classes of cancer-susceptibility genes, varying only in the number of genetic events required for complete neoplastic conversion in their pathways. Gatekeepers and caretakers are involved in the regulation of cellular proliferation and the maintenance of genomic integrity, respectively. As such, loss of a gatekeeper can directly initiate tumor formation whereas loss of a caretaker supports the accumulation of genetic mutations but ultimately requires obstruction of a gatekeeper pathway for tumorigenesis to commence. **(B)** Knudson's two-hit hypothesis stipulates that both copies of a tumor suppressor gene must be lost to initiate tumorigenesis. In the case of inherited cancer syndromes, such as VHL disease, the individual is born with one defective *VHL* allele in the germline and develops cancer following the somatic mutation of the second copy. Sporadic VHL-related cancers, however, require that a single cell acquire two independent somatic inactivating mutations affecting each of the *VHL* alleles.


A
Gatekeeper Pathway
Caretaker Pathway
Normal Cell
Malignant Cell
Mutation of 1st Caretaker Allele
Mutation of 2nd Caretaker Allele
Mutation of 1st Gatekeeper Allele
Mutation of 2nd Gatekeeper Allele
Genetic Instability
Loss of Growth Control


B
KNUDSON'S TWO-HIT HYPOTHESIS
VHL Disease
Sporadic VHL-Related Disease
VHL
VHL
VHL
Germline
Somatic
VHL
Somatic
Tumorigenesis
Tumorigenesis

1.1.5. The Oncogene Addiction Theory.

The phenomena referred to as oncogene addiction and tumor suppressor gene hypersensitivity, which have been supported by numerous mechanistic and clinical studies, substantiate that various genetic alterations can confer a selective growth advantage to mutant cells (Weinstein, 2002; Weinstein and Joe, 2006). The oncogene addiction theory contends that, in spite of the myriad of genetic aberrations observed in an individual cancer, the disruption of a central oncogenic pathway should cause growth inhibition, differentiation, apoptosis, and likely tumor regression (Weinstein, 2002). It has, for instance, been demonstrated that continued expression of H-ras^{V12G} and *MYC* is required for maintenance of the tumorigenic state in transgenic mice harboring melanomas and osteogenic sarcomas induced by the corresponding oncogenes (Chin et al., 1999; Jain et al., 2002). Restoration of tumor suppressor function has also been shown to inhibit cancer cell proliferation and tumorigenesis. Reintroduction of a wild-type copy of the Retinoblastoma (*RB1*) or von Hippel-Lindau (*VHL*) tumor suppressor genes in human retinoblastomas and renal carcinomas, respectively, are two extensively studied examples of dominant tumor suppressive effects (Huang et al., 1988; Iliopoulos et al., 1995). Finally, the success of agents targeting BCR/ABL, in the treatment of chronic myeloid leukemia, and HER2, in patients with breast carcinomas, provides important clinical evidence in support of oncogenic addiction (Vogel et al., 2002; Hughes et al., 2003). In fact, mutational status has been the most reliable predictor of drug efficacy thus far (Heinrich et al., 2003; Hughes et al., 2003; Lynch et al., 2004; Paez et al., 2004). Taken together these studies, and others, suggest that human cancers may rely wholly on a single gene, and the pathways it impinges on, to sustain tumor growth.

1.2. The VHL Tumor Suppressor Gene

1.2.1. Von Hippel-Lindau (VHL) Disease.

The genetic basis of human cancers was mainly established through the study of rare familial cancer syndromes and the discovery of their respective susceptibility genes (Fearon, 1997). A well-documented example of this is VHL disease; a heritable condition which was first described over a hundred years ago (Collins, 1894; von Hippel, 1904; Lindau, 1927). Trademark features of VHL disease include the development of highly vascularized retinal angiomas, cerebellar and spinal hemangioblastomas, pheochromocytomas, endolymphatic sac and pancreatic islet tumors, epididymal and broad ligament cystadenomas, and clear cell renal carcinomas (RCC) (Maher and Kaelin, 1997). The formation of multifocal, bilateral renal tumors and cysts occurs in approximately 50-70% of afflicted individuals and is the most common cause of death in VHL disease. While VHL disease presents an autosomal dominant pattern clinically, it is actually associated with a recessive mutation at the molecular level. Linkage analysis and positional cloning led to the mapping and identification of the *VHL* gene on chromosome 3p25 (Seizinger et al., 1988; Latif et al., 1993). In accordance with Knudson's hypothesis, VHL patients are born with a germline mutation on one allele of the *VHL* gene and tumor formation only ensues upon the somatic inactivation of the remaining wild-type allele (Knudson, 1971; Tory et al., 1989; Maher et al., 1990) (**Fig. 1B**). The bi-allelic nature of *VHL* mutations combined with the fact that VHL patients develop hundreds of tumors, in contrast to those carrying caretaker gene mutations who typically develop a single tumor in their lifetime, is suggestive of a gatekeeper function (Kinzler and Vogelstein, 1996; Kinzler and Vogelstein, 1997; Maher and Kaelin, 1997).

1.2.2. The *VHL* Gene and Genotype-Phenotype Correlations.

Over 150 mutations of the *VHL* gene, which consists of 3 exons and is ubiquitously expressed in embryonic and adult tissues (Richards et al., 1996; Corless et al., 1997), have been reported to date and span the bulk of its coding sequence (Zbar et al., 1996). Unlike most hereditary cancers, VHL disease is likewise heterogeneous in its clinical manifestations from family to family. The analysis of such familial variances led to the discovery of prominent genotypic-phenotypic correlations and the establishment of a corresponding disease classification system (Kim and Kaelin, 2004) (**Table I**). Type 1 disease is characterized by a low risk of pheochromocytoma development, whereas patients with type 2 disease are at a higher risk. Type 2 disease is further subdivided to reflect the risk of developing RCC. Type 2A and 2B disease are associated with a low and high risk of RCC, respectively. Individuals with type 2C disease, however, are unique in that they develop pheochromocytoma only. Interestingly, type 1 families often harbor gross *VHL* gene alterations, including deletions and truncating mutations, while those with type 2 disease tend to exhibit subtle missense mutations, suggesting that the *VHL* gene product has multiple, tissue-specific functions (Chen et al., 1995). The biochemical outcomes resulting from and differentiating these genotypic changes are still being unveiled (Clifford et al., 2001; Hoffman et al., 2001; Li et al., 2007; Lee et al., 2009) (**Table I**).

1.2.3. *VHL* Mutations in Hereditary and Sporadic RCC.

VHL gene mutations are not restricted to VHL disease and the vast majority of sporadic or nonhereditary RCC, the most common and deadliest form of kidney cancer, also harbor bi-allelic *VHL* gene defects ($VHL^{-/-}$ RCC). In this case, a single renal epithelial

Table I. Classification of VHL Disease by Genotype-Phenotype Correlations			
Classification	**Clinical Manifestation**	**Genetic Alteration**	**Molecular Outcome**
Type 1	Low Risk of Pheochromocytoma Hemangioblastoma Renal Cell Carcinoma (RCC)	Gross Deletions/ Truncating Mutations	Defect in HIF Regulation
Type 2A	Low Risk of RCC Hemangioblastoma Pheochromocytoma	Missense Mutations	Defect in HIF Regulation Defect in Microtubule Stabilization
Type 2B	High Risk of RCC Hemangioblastoma Pheochromocytoma	Missense Mutations	Defect in HIF Regulation
Type 2C	Pheochromocytoma only	Missense Mutations	Defect in Fibronectin Binding/ Matrix Assembly

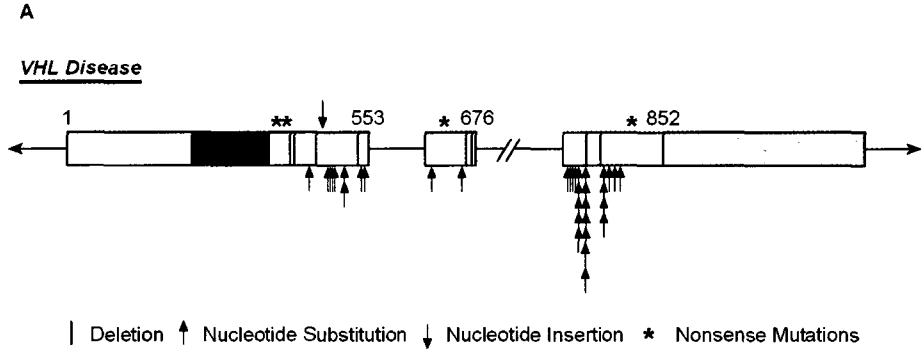
cell must acquire independent somatic inactivating mutations on each of the *VHL* alleles for initiation of RCC tumorigenesis (**Fig. 1B**). Correspondingly, a loss of heterozygosity single *VHL* gene mutation is observed in 98% of sporadic RCC (Gnarra et al., 1994). Genetic and epigenetic inactivation of the second allele presents itself in the form of somatic mutations or promoter hypermethylation in approximately 50% and 10-20% of cases, respectively (Kim and Kaelin, 2004). Notably, a different spectrum of *VHL* gene mutations is detected in hereditary and sporadic forms of RCC. In sporadic RCC, genetic alterations are often concentrated in exon 2 while mutations in this region occur at a lower frequency in VHL disease, and instead tend to afflict the 3' end of exon 1 and 5' end of exon 3 (Gnarra et al., 1994; Chen et al., 1995) (**Fig. 2A**). This disparity may be indicative of non-random somatic mutations resulting from specific carcinogens or the incompatibility of particular genetic alterations in the germline. In line with its putative gatekeeper function, *VHL* loss is an early event in RCC tumorigenesis and is readily observed in the epithelial cells lining benign renal cysts and other preneoplastic kidney lesions (Lubensky et al., 1996). Moreover, the stable reintroduction of a wild-type copy of the *VHL* gene in human sporadic $VHL^{-/-}$ RCC cell lines is sufficient to completely abrogate their ability to form tumors in a nude mouse xenograft assay (Iliopoulos et al., 1995). This result not only suggests that the loss of VHL function is a required event for the formation of $VHL^{-/-}$ RCC tumors *in vivo*, but functionally establishes that VHL acts as a *bona fide* gatekeeper tumor suppressor of kidney epithelial cells.

1.2.4. The VHL Ubiquitin Ligase Complex.

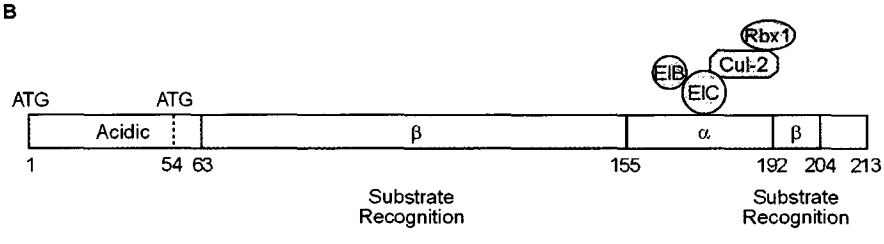
The *VHL* gene encodes a 213 amino acid, 24-30 kDa nuclear-cytoplasmic protein which consists of two functional subdomains (α and β). A second 18-20 kDa form of VHL,

Figure 2. *VHL* mutations obstruct E3 ligase complex formation and substrate binding. **(A)** Distribution of germline and sporadic *VHL* gene mutations across its 3 exons as observed in patients. Inherited *VHL* mutations occur at a high frequency in the 5' end of exon 3, which encodes its α-domain (adapted from Chen et al). Sporadic *VHL* mutations tend to cluster in exon 2, which encodes its β-domain. Dark blue bar denotes eight copies of an acidic tandemly repeated pentamer ($GXEEX_8$) in exon 1 and the light blue bar denotes the 3' untranslated region of exon 3 (adapted from Gnarra et al). **(B)** The *VHL* gene encodes a 213 amino acid protein consisting of two functional subdomains (α and β). A second form of VHL, lacking the N-terminal acidic domain, is also produced from an internal ATG transcription start site. VHL forms an E3 ubiquitin ligase complex with elongin B (ElB), elongin C (ElC), cullin-2 (Cul-2) and Rbx-1 through its α-domain. Its β-domain functions to recruit ubiquitination substrates, such as HIF-α.

A


VHL Disease
1
553
676
852
Deletion
Nucleotide Substitution
Nucleotide Insertion
Nonsense Mutations
Sporadic RCC
1
553
676
852
Deletion/Insertion
Splice Site Mutation
Nucleotide Insertion
Nonsense Mutations

B


Rbx1
Cul-2
EIB
EIC
ATG
ATG
Acidic
β
α
β
1
54
63
155
192
204
213
Substrate Recognition
Substrate Recognition

lacking the N-terminal acidic domain, is also produced but behaves much like the long form in functional assays and so the two are considered interchangeable. Early studies demonstrated that the VHL gene product, whose primary peptide sequence failed to provide any functional indicators, interacts with elongin B, elongin C, cullin-2 (Cul-2), and Rbx-1 to form a stable multimeric complex (VBC/Cul-2 complex) through its C-terminal α-domain (Duan et al., 1995; Kibel et al., 1995; Pause et al., 1997; Kamura et al., 1999) (**Fig. 2B**). Comparison with the structurally similar Skp1/Cdc53/F-Box protein (SCF) complex, found in yeast, led to the confirmation that the VBC/Cul-2 complex analogously boasts E3-ubiquitin ligase activity (Iwai et al., 1999; Lisztwan et al., 1999).

Ubiquitination is a multistep process that marks specific proteins for proteasomal degradation *via* the actions of three enzymes. First the ubiquitin molecule is activated by the E1-ubiquitin activating enzyme, in an ATP-driven reaction. The activated ubiquitin is then transferred to an E2-ubiquitin conjugating enzyme. Finally, the E3-ubiquitin ligase transfers the E2-ubiquitin conjugating enzyme and ubiquitin to a selected substrate targeting it for degradation by the 26S proteasome. The VHL protein acts as the substrate recognition moiety of the VBC/Cul-2 complex recruiting the alpha-subunit of the hypoxia-inducible factor (HIF-α) through its β-domain and thereby targeting it for Cul-2-mediated ubiquitination (Maxwell et al., 1999; Cockman et al., 2000; Ohh et al., 2000).

Mediating the oxygen-dependent degradation of HIF-α is not only the best-described VHL function but is also crucial with respect to its role as a tumor suppressor, as its inappropriate activation is required for VHL tumor formation *in vivo* (Kondo et al., 2002). Tellingly, the most common *VHL* gene mutations interfere with the formation of

the E3 ubiquitin ligase complex (exon 2 mutations) or HIF-α binding (exon 3 mutations) (Gnarra et al., 1994; Chen et al., 1995; Stebbins et al., 1999) (**Fig. 2**).

1.2.5. Tumorigenic Phenotypes Associated with VHL Loss.

Loss of VHL tumor suppressor function is associated with the acquisition of several hallmark cancer traits. *VHL*$^{-/-}$ RCC cells display a loss of cell cycle control and differentiation, defects in extracellular matrix formation and turnover and overproduction of a multitude of pro-tumorigenic molecules including matrix metalloproteases and angiogenic factors (Iliopoulos et al., 1996; Lieubeau-Teillet et al., 1998; Ohh et al., 1998; Pause et al., 1998; Petrella et al., 2005; Kurban et al., 2006). VHL tumors are notoriously well-vascularized with a propensity for the formation of blood vessel tumors and emergence of secondary polycythemia. These cardinal features are largely attributed to the deregulation of hypoxia-inducible genes such as the vascular endothelial growth factor (*VEGF*), platelet-derived growth factor (*PDGF*) and erythropoietin (*EPO*) (Gnarra et al., 1997; Haase et al., 2001; Rankin et al., 2005). Additional HIF-dependent traits including the ability to proliferate in the absence of exogenous growth factors and to transition from an epithelial-to-mesenchymal state are also pivotal in RCC oncogenesis (Gunaratnam et al., 2003; Esteban et al., 2006; Evans et al., 2007). Nonetheless, several rate-limiting phenotypic changes have been linked to HIF-independent VHL functions as well. For example, *VHL*$^{-/-}$ RCC cells display abnormalities in cytoskeletal architecture and fail to properly assemble an extracellular matrix. These processes are modulated through the direct interaction of VHL with various structural components including fibronectin, collagen IV and microtubules, although the precise mechanisms involved are poorly understood (Hergovich et al., 2003; Stickle et al., 2004; Kurban et al., 2008).

Importantly, reintroduction of wild-type *VHL* in *VHL*$^{-/-}$ RCC cells is sufficient to correct all of the above-mentioned defects, further supporting its pivotal tumor suppressive role in kidney epithelial cells.

1.3. Hypoxia and HIF in Human Cancers

1.3.1. The Hypoxic Tumor Microenvironment.

Hypoxia, which is a reduction in cellular or tissue oxygen tension, occurs in both physiological and pathological states. Cancers exhibit intermittent regions of hypoxia which can be accredited to both diffusional limitations as the tumor cells distance themselves from existing capillaries and the structural/functional anomalies characteristic of a tortuous tumor vasculature (Hockel and Vaupel, 2001). The increased energy demand associated with actively proliferating cells similarly contributes to oxygen depletion within solid tumors. Not only does this microenvironment exert strong selective pressures by virtue of the fact that cells undergo necrosis in the absence of oxygen, but hypoxia also causes genomic instability prompting tumor cell evolution through genetic changes (Bristow and Hill, 2008). Cellular adaptations to low oxygen supply, such as increased aerobic glycolysis or the "Warburg effect" which results in the acidification of the microenvironment, further necessitate the establishment of aggressive populations, hence, tumor hypoxia is associated with decreased over-all survival and resistance to radiation and chemotherapies (Gatenby and Gillies, 2004).

A key mediator of the cellular response to hypoxia is the hypoxia-inducible factor (HIF) (Iyer et al., 1998; Semenza, 2001). HIF is a transcriptional activator that drives the expression of a multitude of genes involved in the control of glucose metabolism, neovascularization, pH, and survival (Semenza, 2003). Activation of HIF, thus, ranks

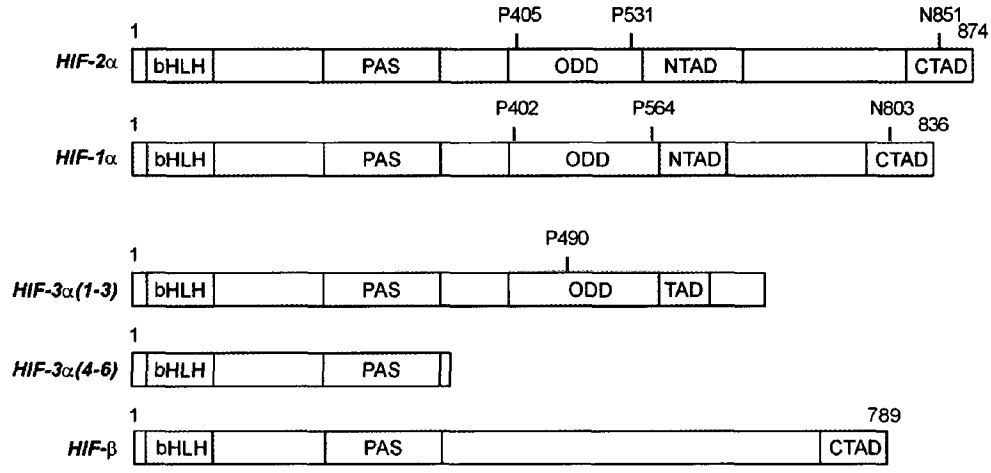
amongst the most important events involved in tumor cell evolution and disease progression under otherwise non-permissive conditions.

1.3.2. The Hypoxia-Inducible Factors.

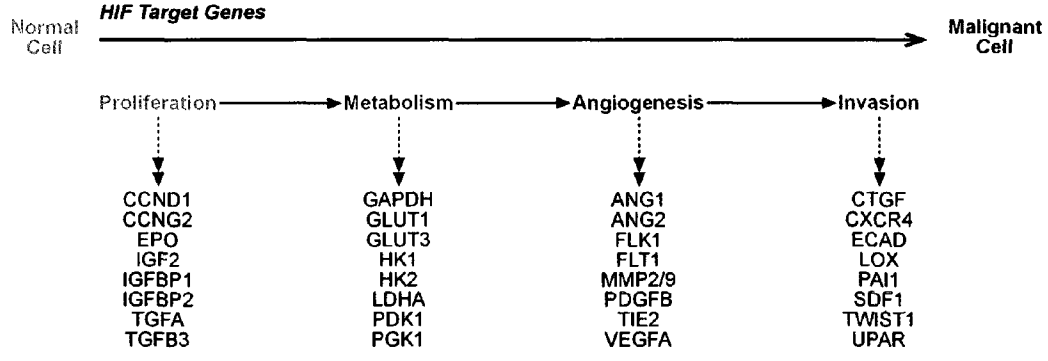
HIF belongs to the Per/ARNT/Sim (PAS) domain family of transcription factors and proteins that sense changes in the cell's immediate environment (Wang et al., 1995). PAS family proteins are classified according to their structural similarities: a basic helix-loop-helix (bHLH) DNA binding domain and a conserved PAS domain which confers dimerization and target gene specificity. HIF is a heterodimeric protein consisting of a 120 kDa alpha-subunit (HIF-α) and a 91-94 kDa beta-subunit (HIF-β/ARNT) (Wang et al., 1995; Wang and Semenza, 1995; Jiang et al., 1996). The HIF-α subunit contains two transcriptional activation domains (TAD), one located in the N-terminal and the other in the C-terminal of the polypeptide (NTAD and CTAD respectively), while HIF-β only has one (**Fig. 3A**). The unique feature of HIF-α is the presence of an oxygen-dependent degradation domain (ODD) that overlaps the NTAD and provides a means of regulating HIF activity (Huang et al., 1998). Three HIF-α isoforms have been identified: HIF-1α, HIF-2α (also referred to as EPAS1, HLF and MOP2) and HIF-3α (Wang and Semenza, 1995; Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Gu et al., 1998; Tian et al., 1998; Maynard et al., 2003). The HIF-3α subunit is often segregated from the remaining isoforms as its splice variants can act as dominant-negative molecules binding to and inhibiting HIF-1α and HIF-2α activity (Makino et al., 2001; Makino et al., 2002). It remains unclear whether the longer HIF-3α variants, which retain a TAD, possess transcriptional activity. The HIF-1α and HIF-2α isoforms, both of which are regulated

Figure 3. The HIF family of transcription factors and their target genes. (A) The HIF proteins display homologous structural elements common to PAS (Per/ARNT/Sim) family members: the basic helix-loop-helix DNA-binding domain (bHLH) and the PAS dimerization domain. With the exception of the dominant-negative HIF-3α splice forms 4-6, the remaining HIF family members also contain one or more transcriptional activation domains (TAD). The main difference between the subunits is the presence of two proline hydroxylation sites in the oxygen-dependent degradation domain (ODD) and asparagine hydroxylation site in the C-terminal TAD (CTAD) of the HIF-α isoforms, which serve an essential function in regulating its stabilization and activation in hypoxia. **(B)** HIF target genes that are commonly deregulated in human cancers and contribute to various malignant processes including aberrant cell growth, increased aerobic glycolysis, the induction of new blood vessel formation, and invasion of surrounding tissues.

A


P405
P531
N851
1
874
HIF-2α
bHLH
PAS
ODD
NTAD
CTAD
P402
P564
N803
1
836
HIF-1α
bHLH
PAS
ODD
NTAD
CTAD
P490
1
HIF-3α(1-3)
bHLH
PAS
ODD
TAD
1
HIF-3α(4-6)
bHLH
PAS
1
789
HIF-β
bHLH
PAS
CTAD

B


Normal Cell
HIF Target Genes
Malignant Cell
Proliferation
Metabolism
Angiogenesis
Invasion
CCND1
CCNG2
EPO
IGF2
IGFBP1
IGFBP2
TGFA
TGFB3
GAPDH
GLUT1
GLUT3
HK1
HK2
LDHA
PDK1
PGK1
ANG1
ANG2
FLK1
FLT1
MMP2/9
PDGFB
TIE2
VEGFA
CTGF
CXCR4
ECAD
LOX
PAI1
SDF1
TWIST1
UPAR

by VHL and are hypoxia-inducible, have a high degree of sequence homology and appear to have largely overlapping transcriptional activities though transgenic studies suggest that they have incongruent biological roles (Iyer et al., 1998; Ryan et al., 1998; Tian et al., 1998; Peng et al., 2000; Compernolle et al., 2002; Scortegagna et al., 2003). Indeed, the HIF-α isoforms have divergent and sometimes opposing transcription-dependent and -independent effects on metabolic and tumorigenic processes (Kondo et al., 2002; Maranchie et al., 2002; Raval et al., 2005; Gordan et al., 2007; Zhang et al., 2007). While the ubiquitously expressed HIF-1α isoform is the best-characterized member, recent reports suggesting critical developmental and oncogenic roles for HIF-2α have increased interest in the latter isoform, which displays a more restricted tissue distribution (Kondo et al., 2002; Holmquist-Mengelbier et al., 2006; Gruber et al., 2007; Rankin et al., 2007; Li et al., 2009; Mastrogiannaki et al., 2009; Qing and Simon, 2009).

1.3.3. HIF Transcriptional Target Genes.

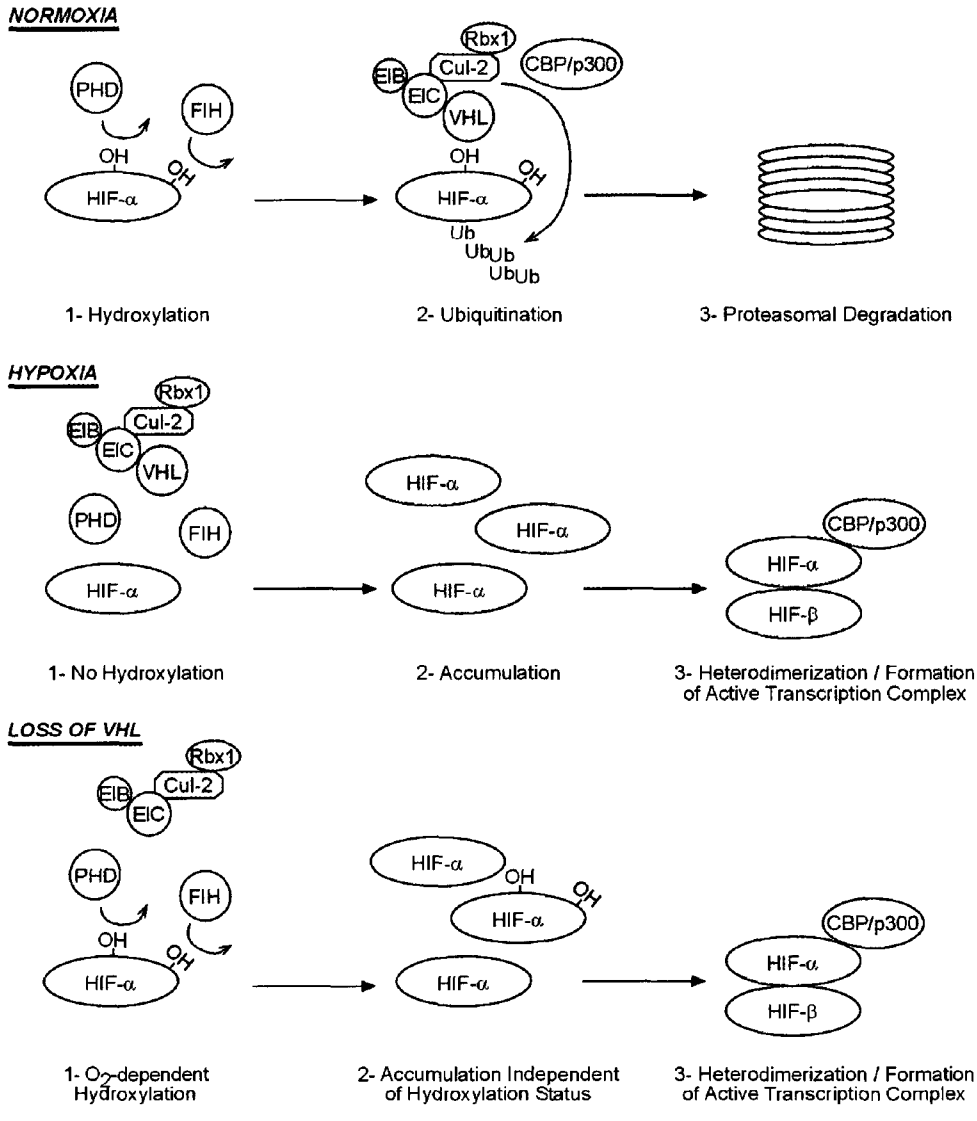
The tight link between oxygen availability and new blood vessel formation was first recognized in studies of vascular and neoplastic diseases (Pugh and Ratcliffe, 2003). This led to the discovery that EPO and a number of glycolytic and angiogenic factors were upregulated under hypoxic conditions by a common transcription factor (Semenza and Wang, 1992; Semenza et al., 1994; Liu et al., 1995). The HIF isoforms bind specifically to *cis*-acting hypoxia response elements (HRE; 5'-RCGTG-3') present in the promoter region of hypoxia-inducible genes, of which over a hundred have been identified, and induce transcription at these sites (Semenza and Wang, 1992; Liu et al., 1995; Forsythe et al., 1996; Jiang et al., 1996). It is now accepted that in addition to the canonical pro-angiogenic and glycolytic HIF target genes, tumor formation and

progression are also profoundly affected by an array of hypoxia-inducible genes involved in cell survival and invasiveness (**Fig. 3B**). While both HIF isoforms are capable of activating the majority of known target genes in reporter assays *in vitro*, there is evidence of preferential gene induction in various cellular systems and animal models (Patel and Simon, 2008). Glycolytic enzymes, for example, are predominantly activated by HIF-1α (Raval et al., 2005; Hu et al., 2006). There is likewise a group of target genes that are specifically responsive to HIF-2α including *CCND1*, *CITED2*, *EPO*, *LOX*, *OCT4*, *TGFA*, *TWIST1*, and *VEGFA* (Raval et al., 2005; Smith et al., 2005; Aprelikova et al., 2006; Covello et al., 2007; Gruber et al., 2007; Gort et al., 2008). Domain swapping experiments suggest that the NTAD confers differential transcriptional activities to the isoforms, yet both transactivation domains are required for full pro-tumorigenic function *in vivo* (Hu et al., 2007; Yan et al., 2007). Synergism with co-activators, such as Ets-1 and Elk-1, which belong to the ETS family of transcription factors, has also been proposed as a mechanism that dictates HIF-2α target gene specificity (Elvert et al., 2003; Aprelikova et al., 2006; Hu et al., 2007; Le Bras et al., 2007). While the intricacies of the HIF gene program haven't been completely resolved, the general consensus is that HIF activity is likely context-specific; contingent on the temporal and spatial distribution of the isoforms and the presence of modulating proteins.

1.3.4. Oxygen-dependent Regulation of HIF Activity.

Given its extensive effects on fundamental metabolic processes HIF expression and activity is tightly regulated, by post-translational modification of its ODD and CTAD, in an oxygen-dependent manner (**Fig. 4**). While the HIF-β subunit is constitutively expressed in the cell, the HIF-α subunit is hydroxylated and degraded when oxygen

Figure 4. Model of oxygen-dependent regulation of HIF-α. In normoxia, the HIF-α subunit undergoes asparaginyl hydroxylation by the factor inhibiting HIF-1α (FIH) and prolyl hydroxylation by prolyl-4-hydroxylases (PHDs). These post-translational modifications have a two-fold effect on HIF activity: 1) hydroxylation of the asparagine prevents HIF-α from associating with its transcriptional co-activators, CBP/p300; and 2) hydroxylation of the proline residues allows its recruitment to the VBC/Cul-2 ubiquitin ligase complex, resulting in its ubiquitination and degradation by the 26S proteasome. In hypoxia, however, unhydroxylated HIF-α accumulates in the cell where it can heterodimerize with the HIF-β subunit and interact with CBP/p300, resulting in the induction of HIF target genes involved in various aspects of glucose metabolism and angiogenesis. Under conditions of *VHL* loss of function, HIF-α is stabilized in the cell irrespective of oxygen tension and its target genes are constitutively overexpressed.


NORMOXIA
PHD
FIH
OH
OH
HIF-α
Rbx1
Cul-2
CBP/p300
EIB
EIC
VHL
OH
OH
HIF-α
Ub
UbUb
UbUb
1- Hydroxylation
2- Ubiquitination
3- Proteasomal Degradation
HYPOXIA
Rbx1
Cul-2
EIB
EIC
VHL
PHD
FIH
HIF-α
HIF-α
HIF-α
HIF-α
CBP/p300
HIF-α
HIF-β
1- No Hydroxylation
2- Accumulation
3- Heterodimerization / Formation of Active Transcription Complex
LOSS OF VHL
Rbx1
Cul-2
EIB
EIC
PHD
FIH
OH
OH
HIF-α
HIF-α
OH
OH
HIF-α
HIF-α
CBP/p300
HIF-α
HIF-β
1- O2-dependent Hydroxylation
2- Accumulation Independent of Hydroxylation Status
3- Heterodimerization / Formation of Active Transcription Complex

tension is normal (Salceda and Caro, 1997; Huang et al., 1998; Kallio et al., 1999; Sutter et al., 2000). The ODD of HIF-α is only recognized by VHL when two key proline residues are hydroxylated (P402/564 in HIF-1α, P405/531 in HIF-2α) (Ivan et al., 2001; Jaakkola et al., 2001; Hon et al., 2002), a process mediated by the EGLN family prolyl-4-hydroxylases (PHD1-3) and requiring molecular oxygen, Fe(II), ascorbate, and 2-oxoglutarate as co-factors (Bruick and McKnight, 2001; Epstein et al., 2001). Upon recruitment by VHL, HIF-α is polyubiquitinated by the VBC/Cul-2 complex and targeted for degradation by the 26S proteasome (Maxwell et al., 1999; Cockman et al., 2000; Ohh et al., 2000). Under hypoxic conditions, however, PHDs are inactivated due to the lack of oxygen availability and so HIF-α is not hydroxylated and cannot be recognized by the VBC/Cul-2 complex. Consequently, HIF-α accumulates within the cell and translocates to the nucleus where it can dimerize with HIF-β, forming the active transcription factor.

A secondary measure in place to curtail inappropriate HIF activity is the normoxic hydroxylation of an asparagine residue in its CTAD domain (N803 in HIF-1α, N851 in HIF-2α) by FIH1 (factor-inhibiting HIF1) (Lando et al., 2002a; Lando et al., 2002b). This modification, which disrupts the physical interaction of HIF with its transcriptional co-activators, CREB-binding protein (CBP)/p300 (Freedman et al., 2002), principally affects HIF-1α activity as the HIF-2α isoform appears to be relatively resistant to the catalytic actions of FIH1 (Koivunen et al., 2004; Bracken et al., 2006; Yan et al., 2007). Interestingly, release of these inhibitory mechanisms appears to occur in a step-wise manner as PHDs are inactivated at higher oxygen tensions than FIH1, allowing for the stabilization of HIF-α protein and expression of NTAD-dependent genes prior to its full transcriptional activation (Koivunen et al., 2004; Dayan et al., 2006).

1.3.5. The Role of HIF in Cancer.

The involvement of HIF in the induction of hypoxia-inducible angiogenic and glycolytic factors raised fundamental questions regarding its general role in tumor development. The demonstration that inactivation of the *HIF1A* gene in embryonic stem (ES) cells leads to reduced VEGF expression, blood vessel density, and/or growth of ES-derived tumors solidified its status as the master regulator of tumor angiogenesis (Carmeliet et al., 1998; Ryan et al., 1998). Ablation of HIF-1α, or its dimerization partner HIF-β, in various transformed cell lines consistently impaired the release of angiogenic factors but its effect on tumor growth was variable, presumably due to differences in the initial vascularization of the sites of injection (Maxwell et al., 1997; Ryan et al., 2000; Blouw et al., 2003). Nonetheless, the expanding HIF gene program has since been implicated in the acquisition of additional hallmark traits in several *in vitro* and *in vivo* cancer models (Semenza, 2003). Its widespread expression in clinical specimens and strong correlation with increased patient mortality further propelled HIF as an esteemed therapeutic target in the treatment of human cancers (Giaccia et al., 2003; Semenza, 2003). Given the abundance of anti-angiogenics it has been suggested that simultaneous HIF inhibition would not only have a complementary effect in blocking tumor vascularization but would also prevent the rise of aggressive cell populations resistant to hypoxic conditions.

1.4. The Role of HIF-2α in RCC Tumorigenesis

1.4.1. Expression of HIF Isoforms in VHL-loss RCC.

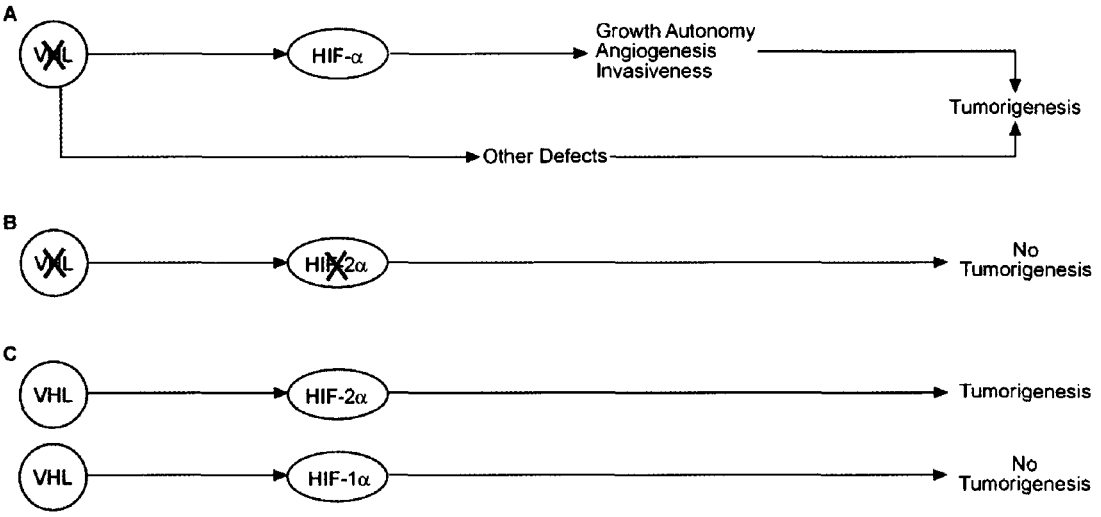
VHL$^{-/-}$ RCC cells, which do not express functional VHL, are characterized by their inability to degrade the HIF-α subunits since they cannot be recruited to the VBC/Cul-2

complex for ubiquitination regardless of the ambient oxygen tension (Krieg et al., 2000). There is, however, an apparent bias towards expression of the HIF-2α isoform in RCC cell lines and patient samples. Whereas HIF-1α protein is readily observed in morphologically normal renal tubular cells, HIF-2α is virtually undetectable in the kidneys of VHL patients (Mandriota et al., 2002; Turner et al., 2002). The appearance of HIF-2α occurs in early multicellular foci in preneoplastic kidney lesions and intensifies with advanced pathologies (Mandriota et al., 2002). This suggests a role specific to the HIF-2α isoform in the conversion of benign renal cysts into frank carcinomas. Tellingly, the preferential induction of HIF-2α mRNA and protein in *VHL*$^{-/-}$ RCC tumor samples compared with adjacent renal tissues has been described and a significant proportion of sporadic RCC cell lines express solely the HIF-2α isoform (Maxwell et al., 1999; Turner et al., 2002). Consistent with the notion that it is the more oncogenic isoform, primary RCC tumors expressing HIF-2α only display higher rates of proliferation and increased volumes when compared to those expressing both HIF-α subunits (Gordan et al., 2008).

1.4.2. Differential Oncogenic Potential of HIF Isoforms.

The constitutive expression of the HIF-α subunits causes *VHL*$^{-/-}$ RCC cells to experience a pseudo-hypoxic response as evidenced by the overproduction of its target genes (Iliopoulos et al., 1996). While the inappropriate expression of hypoxia-inducible angiogenic factors likely accounts for the highly vascular nature of *VHL*$^{-/-}$ tumors, it does not provide much insight into the events initiating RCC tumorigenesis. There are several lines of evidence that suggest a dominant role for HIF-2α, but not HIF-1α, in initiating *VHL*$^{-/-}$ RCC oncogenesis (**Fig. 5**). Firstly, short hairpin RNA (shRNA)-mediated

Figure 5. HIF-2α is the oncogenic HIF variant in *VHL*$^{-/-}$ RCC tumorigenesis. (A) Bi-allelic inactivation of the *VHL* tumor suppressor gene results in the constitutive expression of the HIF-α subunits and their transcriptional target genes, a rate-limiting event in RCC development. **(B)** Inhibition of HIF-2α activity with a dominant-negative form of HIF or shRNA targeted against *HIF2A* mRNA is sufficient to abolish the ability of *VHL*$^{-/-}$ RCC cells to form tumors in nude mice. **(C)** Expression of an oxygen-insensitive HIF-2α, but not HIF-1α, variant overrides VHL tumor suppression and drives the *in vivo* tumorigenesis of *VHL*$^{-/-}$ RCC cells stably re-expressing wild-type VHL.


A
VHL
HIF-α
Growth Autonomy
Angiogenesis
Invasiveness
Tumorigenesis
Other Defects
B
VHL
HIF-2α
No
Tumorigenesis
C
VHL
HIF-2α
Tumorigenesis
VHL
HIF-1α
No
Tumorigenesis

silencing of HIF-2α is sufficient to prevent VHL tumor formation in nude mice, indicating that its activation is a rate-limiting event (Kondo et al., 2003; Zimmer et al., 2004). Secondly, the expression of a HIF-2α variant that evades VHL recognition is sufficient to nullify VHL tumor suppression and promote tumorigenesis of *VHL*$^{-/-}$ RCC cells stably re-expressing a wild-type copy of *VHL* (*VHL*$^{+}$ RCC or WT7) (Kondo et al., 2002). In contrast, *VHL*$^{+}$ RCC cells expressing an oxygen-insensitive variant of HIF-1α fail to form tumors *in vivo* indicating that the activation of common glycolytic and angiogenic HIF target genes is insufficient to drive RCC tumorigenesis (Maranchie et al., 2002). Finally, the HIF-α subunits have opposing effects on *VHL*$^{-/-}$ RCC progression when overexpressed, with HIF-2α enhancing and HIF-1α delaying *in vivo* tumor growth (Raval et al., 2005). Based on these data, it is now generally agreed that HIF-2α activates genes that are required, and perhaps sufficient, to initiate RCC tumorigenesis.

1.4.3. Contrasting Transcriptional Activities of HIF Isoforms.

Early studies conducted with knockout mice uncovered divergent roles for the HIF-α isoforms, which were thought to be functionally redundant for the most part, in normal development (Iyer et al., 1998; Ryan et al., 1998; Tian et al., 1998; Peng et al., 2000; Compernolle et al., 2002; Scortegagna et al., 2003). It is now becoming increasingly evident that the two isoforms have biologically significant differences in their transcriptional activities (Raval et al., 2005; Smith et al., 2005; Covello et al., 2007; Gort et al., 2008). These target gene specificities are particularly well-defined within the context of *VHL*$^{-/-}$ RCC but less so in VHL-competent cells, although the reason for this discrepancy is not clear. Classical hypoxia-inducible genes such as *GLUT1* and *VEGF*

can, for example, be activated by either HIF-α subunit in different settings but are preferentially induced by HIF-2α in VHL-defective cells (Raval et al., 2005). It would thus follow that HIF-2α specifically activates genes involved in cell growth autonomy, accounting for the disparate oncogenic potentials of the HIF isoforms (Maranchie et al., 2002; Kondo et al., 2003; Raval et al., 2005). Indeed, it has been shown that HIF-2α exclusively induces the overproduction of genes implicated in mitogenesis and cell cycle progression, such as transforming growth factor-alpha (TGFα) and cyclin D1, in *VHL*$^{-/-}$ RCC cells (Raval et al., 2005; Smith et al., 2005). These and perhaps other HIF-2α transcriptional targets likely explain the heightened proliferative abilities that underlie RCC tumor formation.

1.4.4. Association of HIF Expression and VHL Disease Types.

The clinical subtypes of VHL disease are based on site-specific risks of tumor development. These distinct pathological manifestations are thought to reflect differential defects in VHL function. The fact that all of the naturally occurring RCC-associated *VHL* mutations result in complete HIF-α deregulation underscores its importance in RCC tumorigenesis (Clifford et al., 2001). Type 2A and 2B VHL disease are distinguished by their low and high proclivity for renal cell carcinoma, respectively. Consistent with the notion that HIF-2α is the oncogenic HIF variant in *VHL*$^{-/-}$ RCC, type 2A VHL mutants retain the capacity to somewhat regulate HIF-2α, but not HIF-1α, whereas type 2B mutants are unable to capture either subunit (Clifford et al., 2001; Li et al., 2007). Stabilization of HIF-2α in the former cells enhances orthotopic kidney tumor growth, which is normally lagging when compared to type 2B VHL mutants (Li et al.,

2007). As in the case of *VHL*-null RCC cells, silencing HIF-2α in type 2B VHL mutant-expressing RCC cells is sufficient to abolish their *in vivo* tumorigenesis (Kondo et al., 2003; Zimmer et al., 2004; Li et al., 2007). Similarly, teratomas derived from murine ES cells expressing the type 2B R167Q VHL ($VHL^{2B/2B}$) mutant, knocked into the wild-type *VHL* locus, display a growth advantage over $VHL^{-/-}$ tumors (Lee et al., 2009). While the $VHL^{2B/2B}$ teratomas exhibit an upregulation of common hypoxia-inducible genes such as *VEGFA* and *EGLN3*, they do not display an induction of HIF-1α-specific targets as do the $VHL^{-/-}$ tumors (Lee et al., 2009). Combined with the finding that $VHL^{-/-}$ RCC cell lines and tumors appear to preferentially express HIF-2α, these data suggest that the relative levels of HIF isoform activation is a major determinant in RCC tumorigenicity.

1.4.5. Genetic Studies in VHL Mouse Models.

Loss of VHL tumor suppressor function is associated with distinct clinical features, including highly vascular clear cell renal carcinomas and hemangiomas of the central nervous system, in patients (Maher and Kaelin, 1997). These manifestations have not, however, been faithfully recapitulated in transgenic mouse models. Homozygous deletion of the *VHL* gene results in embryonic lethality, while *VHL* heterozygotes develop hepatic hemangiomas and angiectasis in multiple organs, a relatively rare occurrence in humans (Gnarra et al., 1997; Haase et al., 2001; Ma et al., 2003). In fact, liver tumors and extensive vascular abnormalities are obtained with a high penetrance in mice of various genetic backgrounds, suggesting that this outcome is strain-independent (Ma et al., 2003). Conditional disruption of the *VHL* gene in hepatocytes phenocopies the aforementioned liver manifestations, and can be reversed by the concurrent inactivation of HIF-2α or HIF-β, but not HIF-1α, once again highlighting a pivotal role

for the former HIF-α isoform in VHL-related pathologies (Haase et al., 2001; Rankin et al., 2005; Rankin et al., 2008). Clear cell renal carcinomas, which are histologically indistinguishable from human samples, do arise upon inactivation of the tuberous sclerosis-2 (*Tsc-2*) tumor suppressor gene in the Eker rat model. Interestingly, the HIF-2α subunit is exclusively expressed in primary rat RCC tumor samples, but is undetectable in control rat renal epithelial cells, and correlates with increased expression of angiogenic HIF targets (Liu et al., 2003). While these data suggest that cell-type specific mechanisms of tumorigenesis are different in both species, it does confirm that *VHL* loss and/or HIF-2α activation results in the formation of highly vascularized neoplasms in animal models.

1.5. Transforming Growth Factor-Alpha and Mitogenic Signaling

1.5.1. Growth Factors and Quiescence.

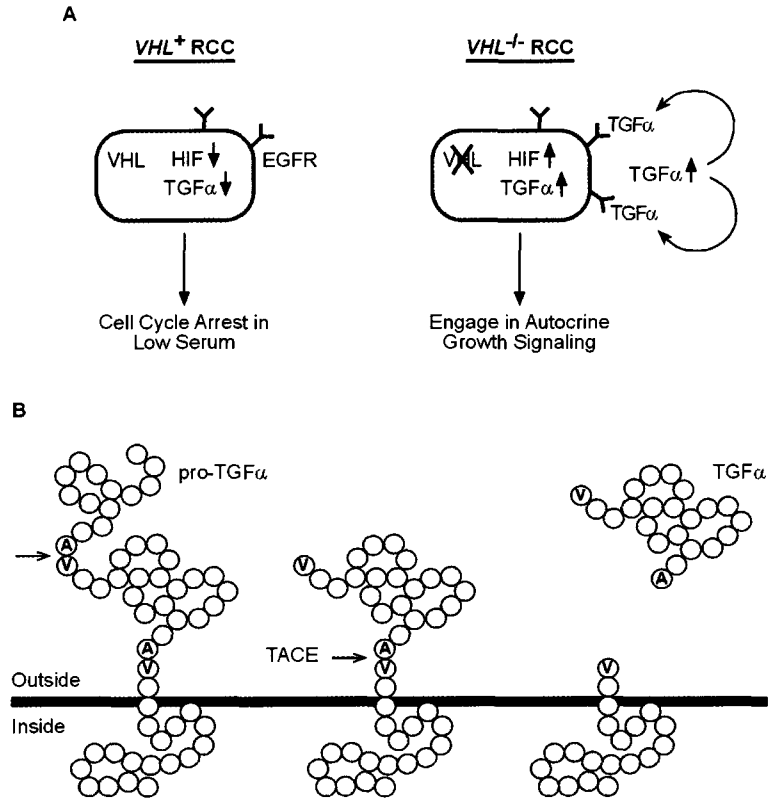
The behavior of normal cells is dictated by several intrinsic mechanisms that ensure their proliferation under favorable conditions and at the appropriate developmental and physiological times. As such, cells are programmed to engage in cellular division only in response to proliferative cues in the form of diffusible growth factors, cytokines, and hormones. This is reflected in their ability to exit the cell cycle and enter quiescence upon serum withdrawal (Pardee, 1974). A loss of dependence on such exogenous growth factors is the first, and debatably most critical, hallmark of cancer cells (Hanahan and Weinberg, 2000). The observation that cells transformed with murine and feline sarcoma viruses as well as primary human cancer cell lines secrete, and are dependent on, their own growth stimulatory peptides led to what is now called the "autocrine hypothesis" (Todaro et al., 1976; De Larco and Todaro, 1978; Ozanne et al., 1980; Todaro et al.,

1980; Kaplan et al., 1982; Sporn and Roberts, 1985). This concept has since been extended to include the actions of oncogenes which are often constitutively activated participants in mitogenic signaling cascades themselves. A suitable example of acquired autocrine loop capacity is observed in *VHL*$^{-/-}$ RCC cells. Renal epithelia require exogenous growth factors to exit quiescence and re-enter the cell cycle (Humes et al., 1991; de Paulsen et al., 2001). In stark contrast, *VHL*$^{-/-}$ RCC cells produce endogenous TGFα permitting their continued proliferation upon serum withdrawal; a condition which can be reversed by the reintroduction of VHL or inhibition of HIF-2α activity (Knebelmann et al., 1998; Pause et al., 1998; de Paulsen et al., 2001; Gunaratnam et al., 2003) (**Fig. 6A**). Notably, inhibition of TGFα/epidermal growth factor receptor (EGFR) signaling is sufficient to prevent the serum-independent growth of RCC cells in culture and their tumor formation *in vivo*, highlighting the pivotal role of this autocrine growth circuit in RCC development (de Paulsen et al., 2001; Gunaratnam et al., 2003; Smith et al., 2005).

1.5.2. Transforming Growth Factor-Alpha.

TGFα is a 50 amino acid, 6 kDa protein belonging to the EGF family of structurally-related ligands (Marquardt et al., 1983; Massague, 1983; Derynck et al., 1984; Lee et al., 1985). The soluble or mature form of TGFα contains the EGF-like consensus sequence ($CX_7CX_{2\text{-}3}GXCX_{10}CXC\ X_3YXGXRC$) which forms a critical three-loop structure, *via* disulfide bonds between its cysteine residues, required for its high affinity binding to the EGFR (Marquardt et al., 1984; Lee et al., 1985; Defeo-Jones et al., 1988). The biochemical distinction between the signals transmitted by TGFα as opposed to the other EGFR ligands has yet to be determined, but, numerous studies suggest that it is the more

Figure 6. Model of TGFα/EGFR autocrine growth signaling in *VHL*$^{-/-}$ RCC cells. **(A)** Normal renal proximal tubule epithelial cells and *VHL*$^{-/-}$ RCC cells stably re-expressing wild-type VHL (*VHL*$^{+}$ RCC) do not produce their own mitogenic signals and thus undergo cell cycle arrest in the absence of serum growth factors. In contrast, *VHL*$^{-/-}$ RCC cells overproduce transforming growth factor-alpha (TGFα) which binds and signals through the epidermal growth factor receptor (EGFR) in an autocrine manner to sustain cell proliferation in the absence of exogenous growth cues. **(B)** TGFα is produced as a transmembrane precursor (pro-TGFα) that undergoes a two-step cleavage process to release the active form of TGFα. The first cleavage step, which is mediated by an unidentified secretase, occurs at the N-terminal, between Ala^{39} and Val^{40}, leaving behind a membrane-bound peptide consisting of mature TGFα attached to its C-terminal tail. The second cleavage step, which is mediated by the tumor necrosis factor-alpha converting enzyme (TACE), occurs between Ala^{89} and Val^{90} at the C-terminal proximal to the transmembrane domain, releasing soluble TGFα into the extracellular space.


A
VHL+ RCC
VHL-/- RCC
VHL
HIF
TGFα
EGFR
TGFα
Cell Cycle Arrest in Low Serum
Engage in Autocrine Growth Signaling
B
pro-TGFα
TGFα
TACE
Outside
Inside

robust inducer of receptor activation and resultant biological responses (Schreiber et al., 1986; Barrandon and Green, 1987; Ebner and Derynck, 1991; Ouyang et al., 1999; Alwan et al., 2003). TGFα is also the most widely expressed EGFR ligand in adult tissues and is upregulated in regenerating epithelial and mesenchymal cell populations during physiological processes such as liver regeneration, wound healing, and mammary gland growth (Gottlieb et al., 1988; Mead and Fausto, 1989; Liscia et al., 1990; Lee et al., 1995). Correspondingly, TGFα expression is sufficient to instigate the proliferation of hepatocytes, keratinocytes and non-pregnant/postlactational cells of mammary glands amongst many other cell types *in vitro* and *in vivo* (Barrandon and Green, 1987; Mead and Fausto, 1989; Snedeker et al., 1991; Lee et al., 1995). Somewhat predictably, an increase in its synthesis and secretion is frequently detected in carcinoma cell lines, primary tumor samples and oncogene-transformed fibroblast and epithelial cell lines alluding to an analogous mitogenic role for TGFα in pathological conditions (Lee et al., 1995).

1.5.3. TACE-mediated Processing of TGFα.

TGFα is synthesized as a 160 amino acid, 20-22 kDa, glycosylated integral membrane precursor (pro-TGFα) that undergoes a two-step proteolytic cleavage process that generates the mature 6 kDa soluble protein (Derynck et al., 1984; Lee et al., 1985; Bringman et al., 1987; Teixido et al., 1990; Pandiella and Massague, 1991) (**Fig. 6B**). The first cleavage step occurs at the N-terminal, between Ala^{39} and Val^{40}, leaving behind a 17 kDa membrane-bound peptide consisting of mature TGFα attached to its C-terminal tail. The final release of soluble TGFα from the C-terminal tail and into the extracellular

space is mediated by TACE (Tumor Necrosis Factor-Alpha Converting Enzyme; also referred to as ADAM-17), a member of the ADAM (A Disintegrin And Metalloproteinase) family of zinc-dependent proteases (Peschon et al., 1998; Sunnarborg et al., 2002). This cleavage step occurs between Ala^{89} and Val^{90} at the C-terminal proximal to the transmembrane domain.

Whether TGFα cleavage is required for its biological activity and receptor activation is a controversial point. Earlier studies involving cells expressing non-cleavable pro-TGFα mutants showed that, when co-cultured with EGFR-expressing cells, these mutants are capable of activating the receptor in a juxtacrine manner (Brachmann et al., 1989; Wong et al., 1989). It has also been reported that the defective cleavage of TGFα results in the expression of partially processed transmembrane forms and increased EGFR activation in colon cancer cells (Yang et al., 2000). In contrast, it has been demonstrated that CHO cells expressing cleavage-incompetent pro-TGFα mutants and shedding-deficient mutant CHO cells transfected with wild-type pro-TGFα fail to form tumors *in vivo*, suggesting that cleavage is required for efficient receptor signaling and tumor formation (Borrell-Pages et al., 2003). In addition to these studies there have been several reports suggesting that the TACE-mediated ectodomain shedding of TGFα, and other EGFR ligands, is absolutely required for EGFR activation and associated downstream biological responses including cell proliferation, migration and survival (Dong et al., 1999; Gschwind et al., 2003; Koon et al., 2004; Schafer et al., 2004). Bearing in mind the caveat that ligand maturation events have been largely studied in artificial cell systems it would be of great interest to examine the importance of TACE-mediated TGFα ectodomain shedding in an endogenous human cancer model.

1.5.4. Overproduction of TGFα in Human Cancers.

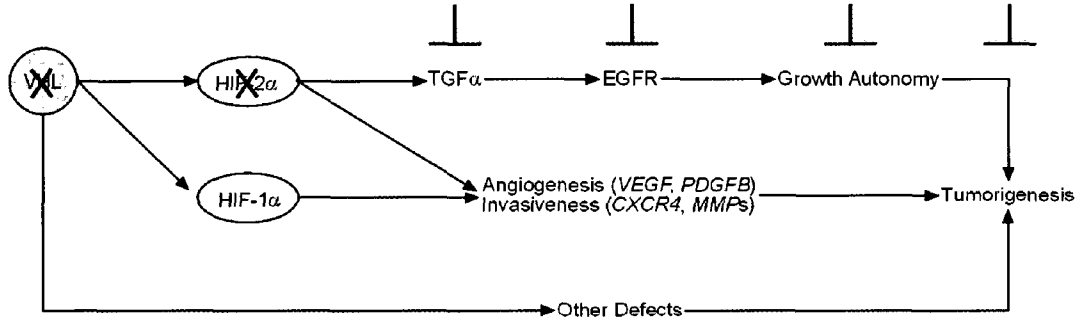
The first lines of evidence supporting the existence of autocrine growth stimulatory loops in cancer cells stemmed from the study of TGFα (Todaro et al., 1976; Todaro et al., 1980; Kaplan et al., 1982; Sporn and Roberts, 1985). Inhibition of this diffusible growth factor or the EGFR, its cognate receptor, with antisense oligonucleotides and monoclonal antibodies, respectively, effectively abrogates its transforming abilities in several malignant cell types (Carpenter et al., 1983; Morishige et al., 1991; Sizeland and Burgess, 1992). With the exception of hematopoietic tumors, the overproduction of TGFα is broadly observed in human cancers including squamous cell head and neck and lung carcinomas, renal and mammary carcinomas, adenocarcinomas of the stomach and endometrium, large cell lung carcinomas, and melanomas (Derynck et al., 1987; Lee et al., 1995). The overexpession of TGFα in transgenic mouse models, however, generally results in epithelial hyperplasia and the establishment of benign growths as opposed to overt carcinomas, which do occur in the mammary gland and liver (Jhappan et al., 1990; Matsui et al., 1990; Sandgren et al., 1990; Vassar and Fuchs, 1991; Lee et al., 1992). Overproduction of ligand thus appears to be insufficient to induce tumorigenesis, an outcome that likely reflects a lack of receptor expression in the target tissues. Nonetheless, TGFα can potentiate the actions of cellular and viral oncogenes, including c-Myc and the SV40 T-antigen, accelerating the rate of tumor formation and resulting in the development of larger masses (Murakami et al., 1993; Sandgren et al., 1993). Collectively, these findings suggest that while it is not directly oncogenic, TGFα can initiate aberrant cellular growth and is likely a major contributor to neoplastic progression.

1.5.5. TGFα Mitogenic Signaling in VHL-loss RCC.

The next conceptual challenge in our understanding of the molecular pathogenesis of RCC was to link HIF-2α to a yet unknown growth stimulatory pathway. To do so, we searched for potential HIF-2α target genes that would be predicted to play a role in growth rather than angiogenesis or glycolysis. The treatment of quiescent renal epithelial and *VHL*$^{+}$ RCC cells with certain growth factors, including the epidermal growth factor (EGF), fibroblast growth factor (FGF) and TGFα, causes them to re-enter the cell cycle (Humes et al., 1991; de Paulsen et al., 2001). The prospective role of TGFα as our candidate mitogen spawned from the findings that it is the only growth factor overproduced by *VHL*$^{-/-}$ RCC cells, is under VHL control and is overproduced in neoplastic renal tissues (Derynck et al., 1987; Gomella et al., 1989; Mydlo et al., 1989; Petrides et al., 1990; Knebelmann et al., 1998; de Paulsen et al., 2001; Gunaratnam et al., 2003) (**Table II**). Our subsequent work led to the demonstration that indeed HIF-2α-mediated activation of the TGFα/EGFR pathway is the major oncogenic axis that drives the autonomous proliferation of *VHL*$^{-/-}$ RCC cells (**Fig. 7**). Briefly, we found that HIF-2α, but not HIF-1α, specifically activates the *TGFA* gene resulting in enhanced EGFR phosphorylation and the autonomous growth of *VHL*$^{-/-}$ RCC cells (Gunaratnam et al., 2003; Smith et al., 2005). Furthermore, inhibition of TGFα production or activity, with an antisense oligonucleotide and EGFR-specific inhibitors respectively, phenocopies reintroduction of VHL or inhibition of HIF, abolishing the ability of *VHL*$^{-/-}$ RCC cells to proliferate in the absence of exogenous growth factors (Pause et al., 1998; de Paulsen et al., 2001; Gunaratnam et al., 2003; Smith et al., 2005). Based on these data, we suggest

Table II. Growth Factor Expression and Activity in *VHL*$^{-/-}$ Renal Cell Carcinoma		
Growth Factor	**Overexpression in *VHL-/-* RCC**	**Mitogenic Activity in *VHL-/-* RCC**
Amphiregulin	No	N/A
Betacellulin	No	N/A
EGF	No	Yes
Epiregulin	No	N/A
FGF	No	Yes
HB-EGF	No	N/A
IGF	No	No
PDGF	Yes	No
TGFα	**Yes**	**Yes**
TGFβ	Yes	No
VEGF	Yes	No

Figure 7. Model of HIF-2α-dependent *VHL*$^{-/-}$ RCC tumorigenesis. Loss of VHL function results in several defects including deregulation of HIF target genes involved in angiogenesis and invasiveness and the inability to deposit an extracellular matrix (other defects). Earlier studies have shown that the HIF-2α, but not HIF-1α, isoform is required and perhaps sufficient to drive *VHL*$^{-/-}$ RCC tumorigenesis. Our group has since then demonstrated that HIF-2α specifically promotes the overproduction of TGFα and activation of the EGFR. Moreover, inhibition of this autocrine signaling loop, using antisense TGFα oligonucleotides, shRNA targeted against *EGFR* mRNA and commercially-available EGFR-based drugs, is sufficient to prevent the autonomous proliferation and/or *in vivo* tumor formation of a panel of human *VHL*$^{-/-}$ RCC cell lines.


VHL
HIF-2α
HIF-1α
TGFα
EGFR
Growth Autonomy
Angiogenesis (*VEGF, PDGFB*)
Invasiveness (*CXCR4, MMPs*)
Tumorigenesis
Other Defects

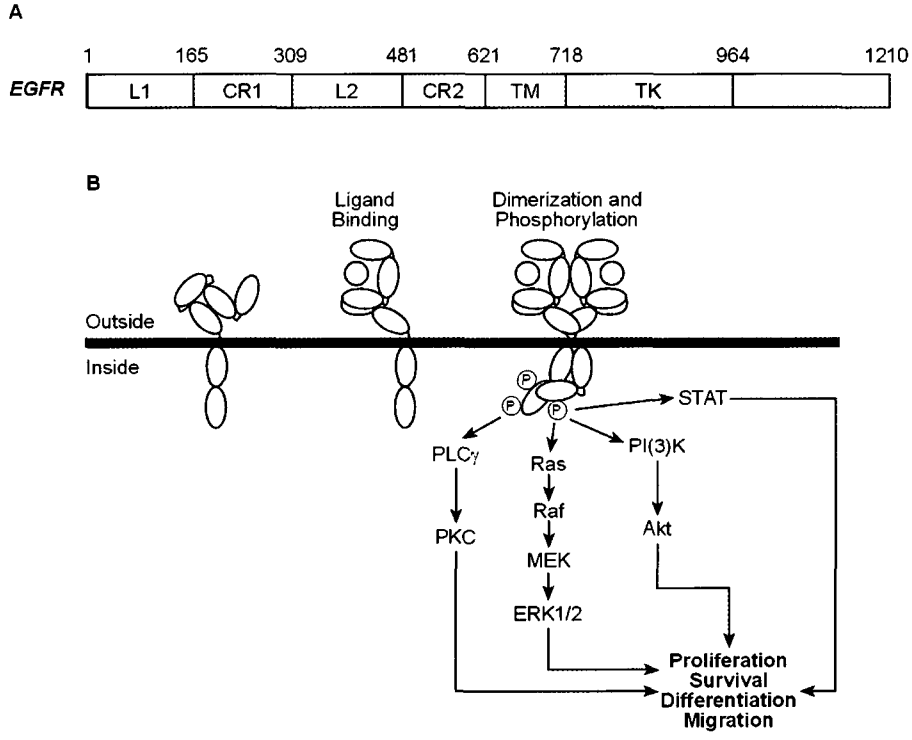
that HIF-2α promotes the growth autonomy and hence tumorigenesis of *VHL*$^{-/-}$ RCC cells by constitutively activating the TGFα/EGFR autocrine signaling loop.

1.6. The Epidermal Growth Factor Receptor

1.6.1. The Epidermal Growth Factor Receptor.

The EGFR, also called ErbB1 or HER1, is a pleiotropic membrane-spanning receptor that directs normal mammalian development and the maintenance of adult tissues through the transduction of extracellular signals (Normanno et al., 2006). A member of the ErbB family of receptor tyrosine kinases, the EGFR is a prototypical single chain transmembrane protein consisting of an extracellular ligand-binding domain, which contains leucine-rich (L1-2) and cysteine-rich (CR1-2) regions, a hydrophobic transmembrane domain, an intracellular tyrosine kinase domain, and a tyrosine-containing C-terminal tail (Downward et al., 1984; Ullrich et al., 1984; Yarden et al., 1985) (**Fig. 8A**). The EGFR is able to bind several ligands including TGFα, EGF, amphiregulin, heparin-binding EGF (HB-EGF), betacellulin, epiregulin, and epigen, which can differentially influence signal selectivity, signal amplification and related negative feedback mechanisms (Cohen, 1964; Anzano et al., 1983; Shoyab et al., 1989; Higashiyama et al., 1991; Shing et al., 1993; Toyoda et al., 1995; Strachan et al., 2001). Ligand binding provokes a conformational change that allows monomeric receptors to homo- or heterodimerize and transphosphorylate each others tyrosine residues (Garrett et al., 2002; Ogiso et al., 2002; Ferguson et al., 2003; Walker et al., 2004; Zhang et al., 2006) (**Fig. 8B**). These phosphorylated tyrosines provide docking sites for diverse adaptor and effector proteins that activate the Ras/MAPK, PLCγ/PKC, PI(3)K/Akt, and

Figure 8. Model of EGFR structure and signaling. **(A)** The EGFR is a membrane-bound protein consisting of an extracellular ligand-binding domain, a hydrophobic transmembrane domain (TM), an intracellular tyrosine kinase domain (TK), and a C-terminal tyrosine-containing tail. The extracellular domain can be further subdivided into two leucine-rich regions (L1-2) and two cysteine-rich regions (CR1-2) whose interactions cause conformational changes that direct receptor dimerization and activation. **(B)** In the absence of ligand, the CR1 and CR2 regions interact to maintain the receptor in a closed, inactive state. Ligand-binding between the L1 and L2 regions causes the peptide to adopt an extended conformation which allows for receptor dimerization through their respective CR regions and the subsequent transphosphorylation of each others C-terminal tyrosine residues (P). The phosphorylated tyrosine residues then serve as binding sites for various adaptor and effector molecules that activate downstream kinase (PKC, ERK, Akt, and STAT) signaling cascades, instigating a variety of biological responses.


A
1
165
309
481
621
718
964
1210
EGFR
L1
CR1
L2
CR2
TM
TK
B
Ligand Binding
Dimerization and Phosphorylation
Outside
Inside
P
P
P
STAT
PLCγ
Ras
PI(3)K
Raf
PKC
Akt
MEK
ERK1/2
Proliferation
Survival
Differentiation
Migration

STAT signaling pathways through direct and indirect mechanisms (Yarden and Sliwkowski, 2001). The biological outputs rendered by the receptor are subject to a number of confounding variables including unique ligand-, dimerization partner- and interacting molecule-dependent effects (Yarden and Sliwkowski, 2001).

1.6.2. Regulation of Receptor Signaling.

Control of receptor signaling is largely mediated by receptor phosphorylation and ubiquitination. Phosphorylated tyrosine residues provide specific docking sites for intracellular signaling molecules, while ubiquitination serves to target the receptor for lysosomal degradation. Following ligand-mediated activation, the phosphorylated receptor is recognized and multiubiquitinated by the E3 ubiquitin ligase, c-Cbl, and endocytosed *via* clathrin-coated pits (Levkowitz et al., 1998; Soubeyran et al., 2002; Mosesson et al., 2003). Once internalized the receptor is either targeted for lysosomal degradation if activated or recycled back to the cell surface for further signaling if unoccupied. A more transient method of attenuating cell signaling is by dephosphorylation. Receptor dephosphorylation by protein tyrosine phosphatases can inhibit receptor kinase activity altogether or temporarily inhibit specific signaling pathways (Haj et al., 2003; Berset et al., 2005; Xu et al., 2005). Unlike receptor degradation, tyrosine dephosphorylation offers a reversible means of negatively regulating receptor signaling. Different ligands also vary in their ability to induce EGFR processing in the midst of endosomal acidification. Upon receptor internalization, the pH progressively decreases during the transition from early endosomes to late endosomes and lysosomes. Association of TGFα with the EGFR is disrupted at a higher endosomal pH and is thus more likely to dissociate from the receptor following internalization

resulting in receptor deubiquitination and recycling (Ebner and Derynck, 1991; French et al., 1995). In contrast, EGF is a more acidic molecule and tends to remain bound to the internalized receptor, in spite of the acidic milieu, ultimately resulting in the degradation of the ligand-receptor pair.

1.6.3. EGFR Activation in Human Cancers.

Amplification of EGFR expression and signaling is a common feature in a variety of human cancers including renal, breast, glioma, non-small cell lung, prostate, pancreatic, and head and neck cancers (Salomon et al., 1995). Ligand-induced activation of the EGFR can instigate a wide range of cellular responses that promote tumorigenesis including growth, de-differentiation, migration, and survival through various signaling pathways (Yarden and Sliwkowski, 2001). Accordingly, it has been shown that persistent activation of the EGFR enables cancer cells to engage in autonomous proliferation, a key tumor initiating event. Moreover, EGFR overexpression, which is observed in over two-thirds of solid tumors, has long since been recognized as a prognostic marker of advanced tumor stage, resistance to standard therapeutic approaches and reduced patient survival (Arteaga, 2002). The dependence of certain cancer cells on the EGFR for growth and survival combined with the above-mentioned factors has directed much attention to the EGFR, which is currently a central target for cancer therapy (Mendelsohn and Baselga, 2000). Aberrant EGFR expression in tumors can arise as a result of amplification of the *EGFR* gene, receptor-activating mutations or evasion from negative regulatory mechanisms (Bache et al., 2004; Normanno et al., 2006). With the exception of glioblastoma multiforme (GBM) and non-small cell lung carcinomas (NSCLC), which display the highest incidence (20-40%) of *EGFR* gene amplifications

and mutations, these oncogenic phenomena are scarcely observed in other tumor types (Salomon et al., 1995; Paez et al., 2004; Kwak et al., 2005; Blehm et al., 2006; Halatsch et al., 2006; Spindler et al., 2006; Sharma et al., 2007). It would, therefore, be reasonable to speculate that the widespread overexpression of the EGFR in human cancer occurs as a consequence of common physiological or oncogenic events in tumors in lieu of *EGFR* gene mutations.

1.6.4. The EGFR in RCC Tumorigenesis.

Overexpression of the EGFR is observed in 70-90% of RCC clinical samples and is predictive of reduced survival and the presence of local and distal metastatic spread (Freeman et al., 1989; Mydlo et al., 1989; Petrides et al., 1990; Moch et al., 1997). At the molecular level, loss of VHL tumor suppressor function and activation of HIF endorses the overproduction and establishment of the TGFα/EGFR autocrine loop which is mitogenic for both normal and malignant epithelial renal tubule cells (Humes et al., 1991; Atlas et al., 1992; Ramp et al., 2000; de Paulsen et al., 2001; Gunaratnam et al., 2003). In view of its appreciated role in human cancer progression and the impending generation of several anti-EGFR agents, the EGFR quickly emerged as a promising antitumor target in the treatment of RCC. Indeed, blockade of EGFR signaling with the monoclonal antibody C225 (Cetuximab) or the receptor tyrosine kinase inhibitors ZD1839 (Gefitinib/Iressa) and PKI-166 suppresses the growth of RCC xenografts in preclinical studies (Prewett et al., 1998; Keder et al., 2002; Weber et al., 2003; Asakuma et al., 2004). More recently, we formally demonstrated that shRNA-mediated silencing of the EGFR in sporadic $VHL^{-/-}$ RCC cell lines is sufficient to abolish their ability to proliferate autonomously, form three-dimensional spheroids *in vitro* and form tumors in nude mice

(Smith et al., 2005). Silencing the EGFR does not, however, correct defects in fibronectin deposition or dampen the constitutive HIF target gene production also associated with loss of VHL function. These results thus authenticate the EGFR as a critical determinant in HIF-2α-dependent RCC growth and tumorigenesis and its credibility as a therapeutic target (**Fig. 7**).

1.6.5. Therapeutic Targeting of the EGFR in RCC.

Kidney cancer carries the burden of a particularly poor prognosis and significant risk of mortality. It is estimated that 12,000 Canadians are afflicted with kidney cancer and 1,600 of these patients die of disease-related complications annually (Canadian Cancer Society, 2009). RCC, which account for approximately 70-80% of all kidney cancers, are notoriously resistant to standard chemotherapeutic and radiation therapies such that the mean survival time of patients is only 6-12 months due to a lack of treatment options (Drucker, 2005). Historically, nephrectomy has served as the principal curative measure in the management of both localized and metastatic RCC (Abel and Wood, 2009). Apart from surgical intervention, high dose cytokine-based (IL-2 and IFNα) therapies have proven to be the most effective providing a meager 10-20% response rate and modestly extending the median survival time (Biswas and Eisen, 2009). The advent of small molecule inhibitors of specific pathways has offered much promise in the treatment of RCC. The identification of rational drug targets such as the EGFR, which is overexpressed in renal tumors and is required for *in vivo* RCC tumorigenesis, has provided the basis for a slew of clinical trials (Freeman et al., 1989; Mydlo et al., 1989; Petrides et al., 1990; Prewett et al., 1998; Keder et al., 2002; Weber et al., 2003; Asakuma et al., 2004; Smith et al., 2005). While EGFR tyrosine kinase inhibitors, such

as gefitinib, failed to produce an objective response, disease stabilization was attained in 38-54% of patients in various trials (Drucker et al., 2003; Motzer et al., 2003; Jermann et al., 2006). Similarly, it was reported that Lapatinib, an EGFR/HER2 inhibitor, provided a survival benefit in a subset of patients that displayed high levels of EGFR expression (Ravaud et al., 2008). It is now suggested that EGFR inhibitors with different pharmacokinetic properties and combination therapies with approved first-line agents, such as multikinase (VEGFR/PDGFR/Raf) and mTOR (Mammalian Target Of Rapamycin) inhibitors, be considered in the treatment RCC patients (Gemmill et al., 2005; Rathmell et al., 2005; Ravaud et al., 2008).

1.7. The Role of the HIF-2α-TGFα-EGFR Axis in Non-RCC Cancers

1.7.1. Expression of HIF-2α in Non-RCC Tumors.

The tumor microenvironment is often hypoxic and this near-universal property of malignancies is sufficient to induce HIF activation in solid tumors (Harris, 2002; Semenza, 2003). The specific ability of the HIF-2α isoform to drive *VHL*$^{-/-}$ RCC oncogenesis is considered to be somewhat of an anomaly, given that both HIF-α subunits are expressed and have seemingly redundant functions in most human cancer cell lines. It is, however, becoming increasingly apparent that HIF-2α may play the more dominant role in non-RCC tumorigenesis as well. Not only is HIF-2α protein detected in the vast majority of solid tumors, but it is also expressed in tumor-associated macrophages whose presence is indicative of advanced disease stage (Talks et al., 2000). Accordingly, HIF-2α expression correlates with disease dissemination and poor patient outcome in gliomas, NSCLCs, neuroblastomas, childhood astrocytomas, hepatocellular carcinomas, and

melanomas (Giatromanolaki et al., 2001; Giatromanolaki et al., 2003; Khatua et al., 2003; Yoshimura et al., 2004; Holmquist-Mengelbier et al., 2006; Li et al., 2009). It has likewise been demonstrated that HIF-2α is preferentially induced at near-physiological oxygen tensions and in chronic hypoxia in normal epithelial and cancerous cell lines (Wiesener et al., 1998; Uchida et al., 2004; Holmquist-Mengelbier et al., 2006; Li et al., 2009). This suggests that HIF-2α activation confers relatively well-oxygenated tumors with an angiogenic phenotype and heterogeneous cell differentiation profile typically provoked by hypoxic conditions.

1.7.2. Stabilization of HIF-2α by Oncogene/Tumor Suppressor Gene Pathways.

In addition to being the primary cellular response to hypoxia, oxygen-independent HIF activation is also mediated by growth signaling pathways that increase its synthesis and/or stability (Harris, 2002; Semenza, 2003). The fact that tumors exploit multiple mechanisms of launching the HIF transcriptional program reaffirms its importance in conferring cancer cells with proliferative and metastatic capabilities. The translational upregulation of HIF-α by mTOR has generated a great deal of interest given its frequent deregulation in human cancers (Hudson et al., 2002; Thomas et al., 2006; Toschi et al., 2008). Upstream *PTEN* tumor suppressor gene inactivation or oncogenic activation of PI(3)K signaling cascade participants therefore provides a means of promoting mTOR-dependent HIF-α accumulation irrespective of its ubiquitination status (Zhong et al., 2000; Zundel et al., 2000). HIF activity has also been shown to be positively regulated by growth factor signaling through the p44/42 MAPK pathway which leads to phosphorylation of HIF-α and its transcriptional cofactors (Richard et al., 1999; Sang et al., 2003). While it has been reported that the p53 tumor suppressor mediates Mdm-2-

dependent HIF-α ubiquitination and degradation, this finding has yet to be independently corroborated, as such loss of VHL function remains the principle means by which HIF-α stabilization is achieved in tumors (Maxwell et al., 1999; Ravi et al., 2000).

1.7.3. HIF-2α Promotes Aggressive Tumor Phenotypes.

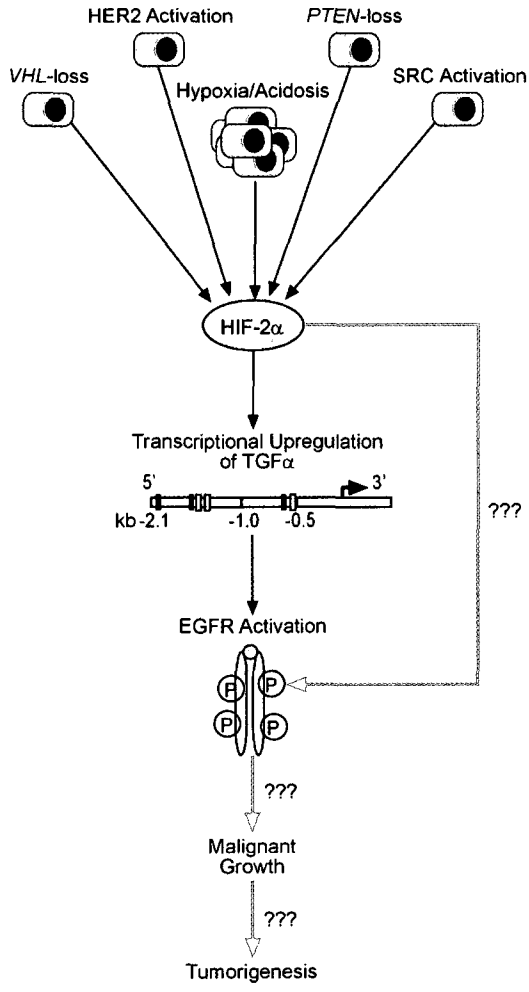
While its canonical role as the master regulator of the hypoxic cellular response and inducer of the angiogenic phenotype is well-appreciated, recent studies have revealed functions specific to the HIF-2α isoform that likely explain its oncogenic nature. Its ability to transcriptionally activate TGFα and promote the autonomous growth of *VHL*$^{-/-}$ RCC is, of course, well-documented (Gunaratnam et al., 2003; Smith et al., 2005). There is now evidence that HIF-2α can promote malignant phenotypes through transcription-independent mechanisms as well. In particular, it has been demonstrated that HIF-2α can promote hypoxic cell cycle progression and de-differentiation *via* direct cross-talk with the c-Myc and Notch pathways, respectively (Gustafsson et al., 2005; Gordan et al., 2007). The physical interaction of HIF-2α with c-Myc facilitates the recruitment of its transcriptional co-factors Sp1, Miz1 and Max and the subsequent activation of targets such as cyclin D2 and E2F1 (Gordan et al., 2007). This is in sharp contrast to HIF-1α, which antagonizes c-Myc activity and ultimately promotes its proteasomal degradation (Koshiji et al., 2004; Zhang et al., 2007). HIF can similarly interact with and stabilize the intracellular domain of Notch, which drives the expression of various genes that control cell fate decisions (Gustafsson et al., 2005). The finding that HIF-2α-positive neuroblastoma cells express neural crest and early sympathetic progenitor marker Notch-responsive genes as well as pro-angiogenic HIF target genes suggests that this isoform

may be the main contributor to the aggressive neuroblastoma phenotype (Holmquist-Mengelbier et al., 2006; Pietras et al., 2008) . In support of this notion, transient silencing of HIF-2α, but not HIF-1α, is sufficient to abrogate the *in vivo* growth of neuroblastoma xenografts (Holmquist-Mengelbier et al., 2006).

1.7.4. HIF-2α Activates the TGFα/EGFR Autocrine Loop in Non-RCC Cancers.

The specificity of HIF-2α transcriptional targets outside of prescribed cellular contexts, including *VHL*$^{-/-}$ RCC cell lines that solely express this isoform, remains unresolved (Qing and Simon, 2009). Instead it is thought that the relative abundance of the HIF-α isoforms in the cell type or tissue of interest dictates the responsiveness of these genes (Lofstedt et al., 2007). Given its preponderance in human malignancies it is reasonable to speculate that, reminiscent of the situation in *VHL*$^{-/-}$ RCC, HIF-2α is competent to activate the TGFα/EGFR signaling pathway and contribute to the growth of other cancer types (Talks et al., 2000; Gunaratnam et al., 2003; Smith et al., 2005) (**Fig. 9**). In line with this prospect, TGFα and EGFR are overexpressed in most human cancers compared with adjacent normal tissues and are known to play a role in the growth of several tumor types, including breast, colorectal and lung carcinomas (Salomon et al., 1995). Consistently, we showed that HIF-2α specifically promotes the transcriptional upregulation and production of TGFα, enhancing EGFR phosphorylation, in a variety of non-RCC human cancer cell lines (Smith et al., 2005). This finding implies that common physiological and oncogenic factors can promote the autonomous proliferation of non-RCC cancers by eliciting the HIF-2α-mediated establishment of the TGFα/EGFR autocrine loop.

Figure 9. Proposed model of HIF-2α mediated human cancer progression. Activation of HIF-2α in solid tumors is endorsed by both genetic and physiological mechanisms in solid tumors. Various oncogene and tumor suppressor pathways promote upregulation of HIF-2α mRNA translation as well as its increased activation through enhanced recruitment of transcriptional co-activators and binding to target gene promoter regions. This effect is likely amplified by environmental stressors, such as hypoxia, which stabilize HIF-2α levels to induce the expression of its target genes involved in oxygen consumption, glucose metabolism and angiogenesis. Given its established role in the transcriptional upregulation of TGFα and the establishment of the TGFα/EGFR autocrine growth circuit which drives *VHL*$^{-/-}$ RCC tumorigenesis, I propose that HIF-2α activation, as a consequence of genetic or environmental cues, would similarly contribute to the autonomous growth and tumorigenesis of other human cancer types (Obj. 2). Based on reports that the EGFR is likewise overexpressed in *VHL*$^{-/-}$ RCC cells, and many other human cancers in the absence of *EGFR* gene mutations, I suggest that HIF-2α may play a hand in this observation simultaneously promoting TGFα/EGFR signaling at the level of the ligand and the receptor (Obj. 1). If so, a better understanding of the molecular mechanisms involved in these processes may provide innovative and effective means of inhibiting the HIF-2α/TGFα/ EGFR growth axis for the treatment of *VHL*$^{-/-}$ RCC and non-RCC human cancers alike (Obj. 3).


HER2 Activation
PTEN-loss
VHL-loss
Hypoxia/Acidosis
SRC Activation
HIF-2α
Transcriptional Upregulation of TGFα
5'
3'
kb -2.1
-1.0
-0.5
???
EGFR Activation
P
???
Malignant Growth
???
Tumorigenesis

1.7.5. Potential Role for HIF-2α in EGFR Regulation.

EGFR overexpression has been implicated in the pathogenesis of several human cancers yet *EGFR* gene alterations are relatively rare and tend to be confined to specific cancer types (Salomon et al., 1995; Paez et al., 2004; Kwak et al., 2005; Blehm et al., 2006; Halatsch et al., 2006; Spindler et al., 2006; Sharma et al., 2007). In spite of the plethora of studies aimed at divulging mechanisms by which this deregulation occurs, the cause of EGFR overexpression in human cancers remains unresolved. We, and others, have observed that $VHL^{-/-}$ RCC cells display higher EGFR protein levels compared to their wild-type VHL^{+} counterparts (Gunaratnam et al., 2003; Gemmill et al., 2005). This finding is somewhat counterintuitive as one would expect that TGFα-expressing $VHL^{-/-}$ RCC cells would experience a higher degree of ligand-mediated receptor turnover resulting in reduced total EGFR levels. This suggests the existence of a mechanism that sustains autocrine growth signaling in these cells by counteracting or interfering with receptor downregulation. Notably, a correlation between tumor hypoxia and EGFR expression has also been reported (Nishi et al., 2002; Swinson and O'Byrne, 2006; Wang et al., 2007). It would thus be interesting to speculate that HIF-2α activation might serve a dual purpose in sustaining TGFα/EGFR signaling through enhanced ligand production and receptor stabilization (**Fig. 9**).

SUMMARY AND GENERAL RATIONALE FOR THE PROPOSED STUDY

Work from our laboratory has uncovered the central role of HIF-2α-mediated TGFα/EGFR autocrine signaling in the autonomous growth and tumorigenesis of *VHL*$^{-/-}$ RCC (de Paulsen et al., 2001; Gunaratnam et al., 2003; Smith et al., 2005). Notably, HIF-2α is expressed in the core of various human tumors where its activation occurs as a consequence of tumor hypoxia and/or genetic alterations (Talks et al., 2000; Harris, 2002; Semenza, 2003). Given that virtually all cancers exploit HIF to attain the angiogenic phenotype, these findings raise the intriguing possibility that they might funnel through the HIF-2α pathway as a systemic means of acquiring growth autonomy in an analogous manner (**Fig. 9**). In line with this prospect, we found that HIF-2α specifically induces TGFα production and EGFR activation in a panel of non-RCC human cancer cell lines (Gunaratnam et al., 2003; Smith et al., 2005). Additionally, evidence that loss of VHL function results in increased EGFR expression infers that HIF-2α may play a direct role in regulating not only ligand but receptor levels as well (Gunaratnam et al., 2003; Gemmill et al., 2005). This could provide a long sought-after explanation for the EGFR overexpression frequently observed in solid tumors in the absence of genetic alterations (Salomon et al., 1995). If the model is correct, this would provide a tumor microenvironment-induced oncogenic molecule common to many human cancers. I propose to continue investigating the mechanisms by which HIF-2α drives *VHL*$^{-/-}$ RCC tumorigenesis and explore the relevance of the HIF-2α/TGFα/EGFR axis in the development of non-RCC cancers. I suggest that targeting HIF-2α may be of broad clinical interest and intend to address its therapeutic potential as outlined here.

HYPOTHESIS

HIF-2α is a common determinant in the autonomous growth of human cancers. Activation of the HIF2α/TGFα/EGFR circuit, as a consequence of genetic mutations or environmental cues, confers growth autonomy to RCC and non-RCC cancers.

OBJECTIVES

1. To further delineate mechanisms involved in HIF-2α-mediated tumorigenesis. HIF-2α-mediated TGFα overproduction allows *VHL*$^{-/-}$ RCC to proliferate autonomously. Notably, the EGFR is also upregulated in *VHL*$^{-/-}$ RCC. I will determine if this occurence is also HIF-dependent, and at what level of regulation, using dominant-negative and siRNA-based approaches, RT-PCR analysis and metabolic labeling techniques.

2. To determine if HIF-2α activation promotes the growth of non-RCC cancers. HIF-2α plays a pivotal role in *VHL*$^{-/-}$ RCC oncogenesis. Since it is often expressed in non-RCC tumors, I will explore the effect of silencing HIF-2α on the *in vivo* tumorigenesis of genetically diverse cancers. The effect of its inhibition on various pro-tumorigenic traits will be assessed by histological and immunohistochemical analyses of xenograft tumor specimens.

3. To identify approaches of therapeutically targeting the HIF-2α oncogenic axis. Given the lack of HIF-2α-based therapies, I will investigate the validity of targeting ligand processing events upstream of EGFR activation as an alternative method of antagonizing the HIF-2α/TGFα/EGFR growth axis. To this end, the effect of silencing TACE, the major TGFα sheddase, on EGFR signaling and *VHL*$^{-/-}$ RCC tumorigenesis will be examined.

IMPORTANCE

Previous reports from our laboratory, and others, have revealed an oncogenic role for HIF-2α in the development of *VHL*$^{-/-}$ RCC and have provided novel therapeutic targets for the treatment of kidney cancer (de Paulsen et al., 2001; Gunaratnam et al., 2003; Kondo et al., 2003; Zimmer et al., 2004; Smith et al., 2005). Given its frequent overexpression in solid tumors, an investigation of the contribution of HIF-2α to non-RCC tumorigenesis could better our understanding of how common environmental and oncogenic stressors affect tumor cell proliferation in otherwise dissimilar contexts. With this in mind, a more comprehensive examination of the intricacies involved in the HIF-2α-mediated activation of the TGFα/EGFR autocrine circuit could provide new mechanistical insights and avenues of antagonizing this growth pathway in the treatment of RCC and other human cancers alike.

MATERIAL AND METHODS

2. MATERIALS AND METHODS

2.1. Cell Lines and Culture.

The 786-0 (*VHL*$^{-/-}$) clear cell renal carcinoma cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD). 786-0 cells stably transfected to express hemagglutinin (HA)-tagged VHL (*VHL*$^{+}$ or WT7) were a kind gift from Dr. W.G. Kaelin (Harvard University, Boston, MA). The KTCL renal carcinoma cell line was a kind gift from Dr. Peter Ratcliffe (University of Oxford, Oxford, United Kingdom). The U87MG glioma and A549 lung carcinoma cell lines were a kind gift from Dr. Ian Lorimer (Ottawa Regional Cancer Center, Ottawa, ON). The MDA-MB-231 breast and PC-3 prostate adenocarcinoma cell lines were a kind gift from Dr. John Bell (Ottawa Regional Cancer Center). The remaining cell lines, including the HCT116 colorectal carcinoma cell line, were also obtained from the ATCC and propagated as suggested. Typically, normoxic cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) or McCoy's 5A Modified Medium (HCT116) supplemented with 5-10% (v/v) fetal bovine serum (FBS) at 37°C in a 5% CO_2 environment. Hypoxic cells were incubated in a hypoxia chamber at 37°C in a 1% O_2, 5% CO_2 and N_2-balanced atmosphere for the indicated time periods. Serum-free medium consisted of the appropriate base medium supplemented with 1% (v/v) insulin-transferrin-selenium (ITS; Invitrogen, Burlington, ON).

2.2. *In Vitro* Tumor Spheroids.

Multicellular spheroids were prepared by the liquid overlay technique as previously described (Sutherland, 1988; Lieubeau-Teillet et al., 1998; Smith et al., 2005). Briefly, cells (10^5) were plated in 24-well plates coated with preheated 1% Seaplaque agarose

(Cambrex, Rockland, ME) in a total volume of 1 mL per well. To promote cell-cell adhesion, the plates were gently swirled in a circular motion 30 min after plating. Spheroids were grown in normoxic conditions for the indicated times (1-7 days) in serum-containing medium. Prior to immunoblotting, spheroids were harvested and lysed in 4% SDS in PBS. Alternatively, spheroids were fixed in 10% formaldehyde for 30 min at room temperature, embedded in paraffin, sectioned, and mounted on slides for histological and immunohistochemical analysis.

2.3. Western Blot Analysis.

Cells were harvested and lysed in 4% SDS in PBS. Protein concentrations were quantified using a BCA protein assay kit (Pierce, Rockford, IL) and samples (25-50 μg protein) were separated by SDS-PAGE and transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane (NEN, Boston, MA). Prior to immunodetection, membranes were blocked with 5% (w/v) skim milk powder in a 0.2% Tween-PBS solution for 1 hr at room temperature. Monoclonal antibodies were used to detect total EGFR (Ab-12; LabVision, Fremont, CA), HIF-1α (BD Transduction Laboratories, Lexington, KY) and Met (Cell Signaling Technology Inc., Danvers, MA). Polyclonal antibodies were used to detect Actin (Sigma, St. Louis, MO), Akt (Cell Signaling), ERK1/2 (Promega, Madison, WI), HIF-2α (Novus, Littleton, CO), IGF-IR-β (Cell Signaling), PDGFRα (Cell Signaling), phosphorylated Akt (Thr308; Cell Signaling), phosphorylated EGFR (Tyr1173; Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated p44/42 MAPK (T202/Y204; Cell Signaling), phosphorylated S6 ribosomal protein (Ser235/236; Cell Signaling), total S6 ribosomal protein (Cell Signaling), and pro-TACE (sc-13973; Santa Cruz Biotechnology). Membranes were

subsequently blotted with horseradish peroxidase (HRP)-conjugated anti-mouse (Amersham Biosciences, Piscatway, NJ) or anti-rabbit (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) secondary antibodies for 1 hr at room temperature on a rocking platform. Bands were detected by enhanced chemiluminescence (Pierce).

2.4. RT-PCR Analysis.

Total RNA was extracted using TriPure Isolation Reagent (Roche Molecular Biochemicals, Indianopolis, IN) according to the manufacturer's protocol. RT-PCR was performed on 1 μg of RNA using the AccessQuick RT-PCR System (Promega) and 0.6 μM of each primer. Cycling conditions were 50°C (30 min), 94°C (2 min), 23-25 cycles of 94°C (20 sec), 55°C (20 sec), 72°C (30 sec), with a final extension at 72°C (10 min). Primer sets were designed as follows (5'→3'): *CA9* (GTTGCTGTCTCGCTTGGAAGA and GCGGTAGCTCACACCCCCTTT), *CITED2* (GCAGAGTGAGCTGTTGACTC and CTGGAGAAAGTGAGGGAAAG, *EGFR* (ACCTGCGTGAAGAAGTGTCC and CAC ATCTCCATCACTTATCTCC), and *HIF2A* (CGGAGAGGAGGAAGGAGAAG and GCCATTCATGAAGAAGTCCC). All primers and cycle details for RT-PCR analysis of *VEGFA*, *GLUT1*, *TGFA* and *ACTB* expression were described elsewhere (Gunaratnam et al., 2003). Products were resolved by agarose gel electrophoresis and ethidium bromide staining was visualized with a Kodak Digital Science IC440 system.

2.5. RNA Interference.

For transient silencing, cells (2×10^5) were transfected with commercially-available double-stranded 21-nucleotide-long small interfering RNA (siRNA) targeting *EGFR* (ID# 4833), *HIF1A* (ID# 42840), *EPAS1/HIF2A* (ID #106447), *TACE/ADAM17* (ID# 12917),

or negative control siRNA (Ambion, Austin, TX) for 48-72 hrs using Effectene reagent (Qiagen, Valencia, CA). ON-TARGETplus SMARTpool siRNA targeting *IGF1R* mRNA was obtained from Dharmacon Inc. (Lafayette, CO). The cell lines were also stably transfected to express shRNA targeted against the following mRNA sequences (5'→3'): *HIF1A* (AAUGGAACAUGAUGGUUCACU), *HIF2A* (GACAAGGUCUGCA AAGGGUUU and GGGGGCUGUGUCUGAGAAGAG) and *TACE* (AAGCUUGAUUC UUUGCUCUCA and CAGGAUGUAAUUGAACGAUUU) (Shao et al., 2003; Mizukami et al., 2004; Zimmer et al., 2004). For each sequence, two complimentary ssDNA oligonucleotides designed with overhangs encoding *Bam*HI/*Hind*III restriction enzyme sites were synthesized and annealed by incubating in 1x DNA Annealing Solution for 3 min at 90°C followed by 1 hr at 50°C (Ambion). The annealed inserts were subsequently ligated into a p*Silencer* 3.1-H1 neo vector (Ambion). All constructs were verified by standard DNA sequencing. A p*Silencer* 3.1-H1 neo vector encoding scrambled shRNA was purchased and served as a negative control. Positive clones were selected and maintained in neomycin-containing medium. The 786-0 cell line stably expressing shRNA targeting *EGFR* mRNA was previously described (Smith et al., 2005).

2.6. Adenoviruses.

Adenoviruses encoding FLAG-tagged GFP, wild-type VHL, dominant-negative HIF-2α (DN-HIF), and the oxygen-insensitive HIF-α variants were generated using a Cre-lox recombination system as previously described (Groulx and Lee, 2002; Gunaratnam et al., 2003; Smith et al., 2005). Vectors encoding the mutated HIF-1α (P402A and P564A) and HIF-2α (P405A and P531A) subunits were a kind gift from Dr. W.G. Kaelin. Cells were infected with equal multiplicity of infection (MOI) of each virus in all experiments.

2.7. Radioisotope Labeling.

Cells were grown in 10 cm plates for 72 hrs in serum-free medium, incubated for 30 min in glutamine-, methionine- and cysteine-free DMEM and then labeled with [^{35}S]methionine ([^{35}S]Met) (50 μCi/mL) for indicated times. For pulse-chase experiments cells were labeled with [^{35}S]Met for 2 hrs, washed with 1x PBS and cold chased in DMEM supplemented with 1% ITS for indicated times. Following immunoprecipitation and separation by SDS-PAGE, semiquantitative analysis of radiolabel incorporation in the protein of interest was performed by measurement of band intensities, less the background readings for equivalent areas, as determined using Adobe Photoshop 7.0 software.

2.8. Immunoprecipitation.

Cells were washed with 1x PBS and lysed with modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM Na_3VO_4, 1 mM NaF) by rotating for 30 min at 4°C. Cell lysates were clarified by centrifugation at 14000 x g for 15 min and supernatants were collected and incubated overnight at 4°C with an agarose-conjugated EGFR antibody (Santa Cruz Biotechnology) or with an anti-EGFR antibody (LabVision) coupled with Protein A/G PLUS Agarose (Santa Cruz Biotechnology) while mixing on a rotating platform. Immunoprecipitates on beads were washed three times with modified RIPA by centrifugation at 1000 x g, dissolved in electrophoresis sample buffer, and boiled for 5 min. Samples were separated on a 6% SDS-polyacrylamide gel. Following electrophoresis, gels were dried at 80°C and exposed to autoradiography film (Kodak BioMax MS Film) overnight.

2.9. Polysomal Analysis.

Cells (2.5 x 10^6) were plated in 15 cm culture dishes and infected with equal MOI of adenovirus encoding GFP or DN-HIF and grown in serum-free media for 48 hrs. For isolation of intact polysomes, 100 μg/mL of cycloheximide was added to cells for 5 min at 37°C prior to harvesting. Polysome lysates were prepared in RNA lysis buffer (15 mM Tris-HCl [pH 7.4], 15 mM $MgCl_2$, 0.3 M NaCl, 1% Triton X-100, 0.1 mg/mL cycloheximide, and 100 U/mL RNasein), and subjected to sucrose gradient (10-50%) centrifugation at 39000 rpm for 90 min at 4°C. Gradients were then collected into 10 equal fractions while the absorbance at 254 nm was continuously monitored. Each fraction was digested with proteinase K for 1 hr at 55°C, and total RNA was isolated by phenol-chloroform extraction and ethanol precipitation. Equal volumes of total RNA were then used for RT-PCR analysis of *EGFR* mRNA levels. Semi-quantitative analysis of the polysome gradients was performed by measurement of band intensities, less the background readings for equivalent areas, using Adobe Photoshop 7.0 software.

2.10. Proliferation Assays.

Cell growth autonomy was measured using a 5-bromo-2'-deoxy-uridine (BrdU) Labeling and Detection Kit I (Roche Molecular Biochemicals) as previously described (Gunaratnam et al., 2003). Briefly, cells were plated at low density (~10^5) on glass coverslips and incubated overnight in DMEM supplemented with FBS. At the start of experiments cells were washed and supplemented with fresh serum-containing or serum-free medium (1% ITS). Following indicated time periods/ treatments, cells were labeled with BrdU for 2 hrs, fixed and immunostained according to the manufacturer's protocol. The coverslips were then counterstained with Hoechst reagent (Hoechst 33258; Sigma)

and the percentage of proliferating cells was reported as the percentage of BrdU-labeled cells per Hoechst-stained nuclei as assessed by fluorescence microscopy (Zeiss Axiovert S100TV microscope, Thornwood, NY). To determine cell doubling times in the presence of serum, cells were plated in 6 cm culture plates and incubated for indicated times in DMEM supplemented with FBS. For each time point, plates were trypsinized and Trypan Blue-excluding cells were counted in a hemocytometer.

2.11. EGFR Tyrosine Kinase Inhibition.

Cells were treated with increasing concentrations (1-100 nM) of EGFR tyrosine kinase inhibitors, PD153035, AG1478 or ZD1839 (Gefitinib/Iressa; AstraZeneca Inc.) for 2 hrs at 37°C followed by stimulation with 20 ng/mL of human recombinant TGFα (Chemicon, Temecula, CA) for 10 min. Cells were harvested and lysed in 4% SDS in PBS prior to immunoblotting. IC50 values were obtained graphically by plotting the percent inhibition of receptor phosphorylation versus the log values of the drug concentrations tested using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Total receptor levels at the IC50 values were determined by measurement of band intensities, less the background readings, using Adobe Photoshop 7.0 software.

2.12. Nude Mouse Xenograft Assays.

Nude mouse xenograft assays were performed as previously described (Iliopoulos et al., 1995; Smith et al., 2005). Briefly, female CD-1 nude mice (Charles River, Wilmington, MA) were injected subcutaneously in their flanks with 10^7 cells diluted in 200 μL sterile 1x PBS. Control (parental or scrambled shRNA) and test (*HIF1A*, *HIF2A* or *TACE* shRNA) cells were injected in the left and right flanks, respectively. Mice were

sacrificed 6-9 weeks post-injection according to ACVS protocol (University of Ottawa) or earlier in cases of significant morbidity. Tumor dimensions were recorded weekly and their final volumes measured upon excision at the time of sacrifice. All experiments were performed double-blinded.

2.13. Histology and Immunohistochemistry.

All histochemical and immunostaining of formalin-fixed paraffin-embedded spheroid and tumor sections was performed by technical assistants from the Department of Pathology and Laboratory Medicine (University of Ottawa). Cell proliferation and microvessel density were evaluated by inspection of Ki-67 and H&E-stained sections respectively. To quantify these parameters at least 5 representative fields were counted at a 400x magnification per triplicate section. Cell death was likewise evaluated by inspection of triplicate TUNEL- and H&E-stained sections. Here, the sections were divided into identical fields at a magnification of 100x and the total area and the area of necrosis were measured using Northern Eclipse image analysis software (Empix Imaging, Inc., Mississauga, ON).

2.14. Phosphorylated Receptor Tyrosine Kinase Array.

Cells were harvested and lysed in NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 10 μg/mL aprotinin, 10 μg/mL leupeptin) by rocking for 30 min at 4°C. Relative levels of receptor tyrosine kinase (RTK) phosphorylation were determined using a Human Phospho-RTK Array Kit according to the manufacturer's protocol (R&D Systems Inc., Minneapolis, MN). Briefly, blocked arrays were incubated with cell lysates (1 mg) overnight at 4°C, washed

three times with the provided buffer, and then incubated with an anti-Phospho-Tyrosine-HRP detection antibody for 2 hrs at room temperature. Positive signals were detected by enhanced chemiluminescence (Pierce) and quantified by measurement of spot intensities, less the background readings for equivalent areas, using Adobe Photoshop CS software.

2.15. Measurement of TGFα Protein Levels.

An equal number of cells were plated and incubated for indicated times in DMEM supplemented with FBS. In experiments involving matrix metalloprotease inhibitors, cells were treated with 10-100 μM GM6001 (Calbiochem, La Jolla, CA) or corresponding volumes of DMSO as a vehicle control. The cell lysates and conditioned media were collected and TGFα levels analyzed according to the ELISA kit instructions (Calbiochem). Total protein concentrations were determined using a BCA protein assay kit (Pierce) to ensure equivalence between samples.

2.16. Fibronectin Deposition.

Cells were plated at a low density on glass coverslips and incubated for 6 days in DMEM supplemented with 10% FBS. Cells were then fixed and permeabilized in prechilled 95% ethanol for 30 min at -20°C. The ethanol was aspirated and coverslips were allowed to air dry at 4°C. Cells were then immunostained with anti-fibronectin antibody (Abcam Inc., Cambridge, MA) for 1 hr at room temperature and fibronectin deposition was assessed as previously described (Bonicalzi et al., 2001).

2.17. Migration and Invasion Assays.

Colorimetric cell migration assays (Chemicon) were performed according to the manufacturer's protocol. Briefly, 5.0 x 10^4 cells were plated in Boyden chambers

consisting of an 8 μM pore size polycarbonate membrane. Cells were allowed to migrate for 16 hrs towards serum-containing (10% FBS) or serum-free (5% BSA) media. The chambers were then removed and placed in staining reagent provided in the kit. The dye was solubilized and the absorbance was measured on a 96-well microplate reader at 574 nm. Colorimetric cell invasion assays were performed on BD BioCoat Matrigel invasion chambers consisting of an 8 μM pore size polyethylene terepthalate (PET) membrane uniformly coated with basement membrane matrix (BD Biosciences, San Jose, CA). Cells (1.5×10^5) were plated in the chambers in the presence (10% FBS) or absence (1% ITS) of serum and incubated for 48 hrs. The chambers were subsequently removed and stained with crystal violet. The dye was then solubilized with 10% acetic acid and its absorbance was read on a 96-well microplate reader at 574 nm.

RESULTS

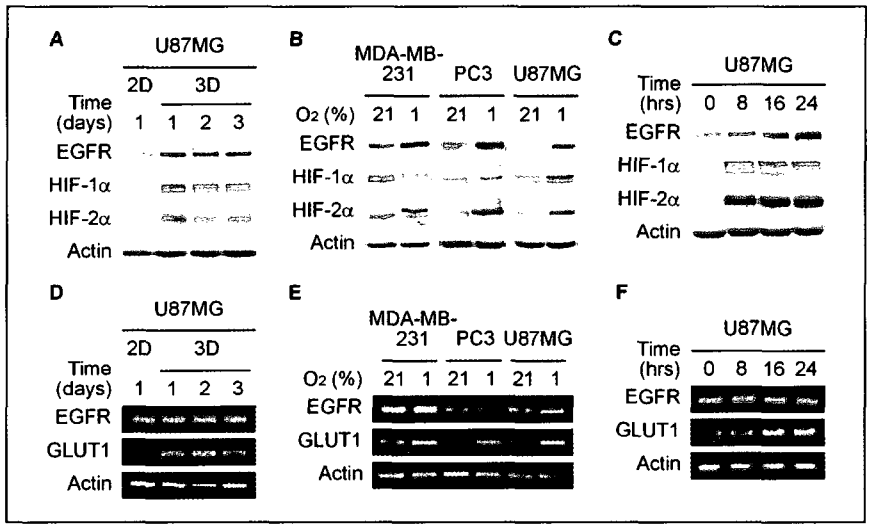
3. RESULTS

3.1. HIF-2α activation elicits the translational upregulation of the EGFR

3.1.1. The hypoxic tumor microenvironment triggers EGFR expression.

The EGFR has been implicated in the pathogenesis of many human cancers. The widespread overexpression of the receptor in such cancers remains unexplained as mutations of the *EGFR* gene are seldom observed (Salomon et al., 1995). We, and others, have noted that *VHL*$^{-/-}$ RCC cells, which constitutively overexpress HIF target genes, exhibit higher levels of EGFR protein than do their wild-type VHL expressing counterparts (Gunaratnam et al., 2003; Gemmill et al., 2005). We hypothesized that overexpression of the EGFR may be the result of common microenvironmental stressors, such as tumor hypoxia and HIF activation, rather than genetic alterations. We first examined if factors in the tumor microenvironment can in fact lead to the accumulation of EGFR using a three-dimensional multicellular tumor spheroid model to mimic such conditions *in vitro* (Sutherland, 1988; Lieubeau-Teillet et al., 1998). We focused on the human U87MG glioma cell line since it expresses low levels of wild-type EGFR and yet amplification of the EGFR is believed to be a fundamental event in the development of GBM, an advanced form of brain cancer (Furnari et al., 2007). Western blot analysis of cell lysates from U87MG spheroids revealed a marked induction of EGFR protein levels compared to the control cells grown in two-dimensional culture (**Fig. 10A**). This result suggests that EGFR protein levels can be upregulated due to inherent features of the tumor microenvironment. It has been well established that the core of tumors is hypoxic, and that this hypoxic microenvironment is sufficient to induce activation of HIF and expression of hypoxia-inducible genes that promote tumor growth and invasiveness

Figure 10. The hypoxic tumor microenvironment triggers EGFR expression. (A) Western blot analysis of total EGFR and HIF-α subunit protein levels in U87MG glioma cells grown as three-dimensional (3D), multicellular spheroids for 1-3 days. Control cells were grown in two-dimensional (2D) culture. **(B)** Western blot analysis of EGFR and HIF-α levels in U87MG, MDA-MB-231 breast and PC3 prostate carcinoma cells exposed to normoxia (21% O_2) or physiological hypoxia (1% O_2) for 24 hrs. **(C)** Western blot analysis of EGFR and HIF-α levels in U87MG cells exposed to hypoxia for the indicated times. **(D-F)** RT-PCR analysis of *EGFR* and *GLUT1* mRNA levels in cells described in A-C. Actin served as a loading control in A-F.


A
U87MG
2D
3D
Time (days)
1
1
2
3
EGFR
HIF-1α
HIF-2α
Actin
B
MDA-MB-231
PC3
U87MG
O2 (%)
21
1
21
1
21
1
EGFR
HIF-1α
HIF-2α
Actin
C
U87MG
Time (hrs)
0
8
16
24
EGFR
HIF-1α
HIF-2α
Actin
D
U87MG
2D
3D
Time (days)
1
1
2
3
EGFR
GLUT1
Actin
E
MDA-MB-231
PC3
U87MG
O2 (%)
21
1
21
1
21
1
EGFR
GLUT1
Actin
F
U87MG
Time (hrs)
0
8
16
24
EGFR
GLUT1
Actin

(Harris, 2002; Semenza, 2003). Fittingly, we observed stabilization of the two isoforms of the α-subunit of HIF, HIF-1α and HIF-2α, in the U87MG spheroids (**Fig. 10A**). This was in contrast to cells cultured in standard growth conditions that failed to express detectable levels of either HIF-α isoform. We thus reasoned that the elevated EGFR levels observed in human cancers might be the result of tumor hypoxia.

To assess if the EGFR is in fact hypoxia-inducible, we measured EGFR levels in three independent human cancer cell lines after exposure to physiological hypoxia (1% O_2). The U87MG glioma, MDA-MB-231 breast and PC-3 prostate carcinoma cell lines all expressed increased EGFR protein levels after exposure to hypoxia for 24 hours (**Fig. 10B**). Similar results were obtained with the A549, H1650 and H1975 lung carcinoma cell lines, suggesting that hypoxic induction of EGFR protein is a widespread occurrence (**Fig. 20A**, **Fig. 21A**). The HIF-2α isoform was strongly upregulated in all three cell lines, while there was variability in HIF-1α stabilization (**Fig. 10B**). This is in accordance with numerous studies that have shown that HIF-2α expression is induced at higher oxygen tensions and is more persistent over time in certain cell types (Wiesener et al., 1998; Uchida et al., 2004; Holmquist-Mengelbier et al., 2006).

To better examine the kinetics involved in the hypoxic upregulation of EGFR protein levels, we conducted a time course experiment using the U87MG cell line. There was a measurable increase in EGFR protein levels within 8 hours of hypoxic exposure (**Fig. 10C**), suggesting that an efficient mechanism is involved in this response. To determine if this increase in EGFR protein was a consequence of transcriptional upregulation we examined *EGFR* mRNA levels in tumor spheroids (**Fig. 10D**) and in hypoxic cancer cells (**Fig. 10E-F**). As expected, hypoxic induction of glucose transporter-1 (*GLUT1*) mRNA,

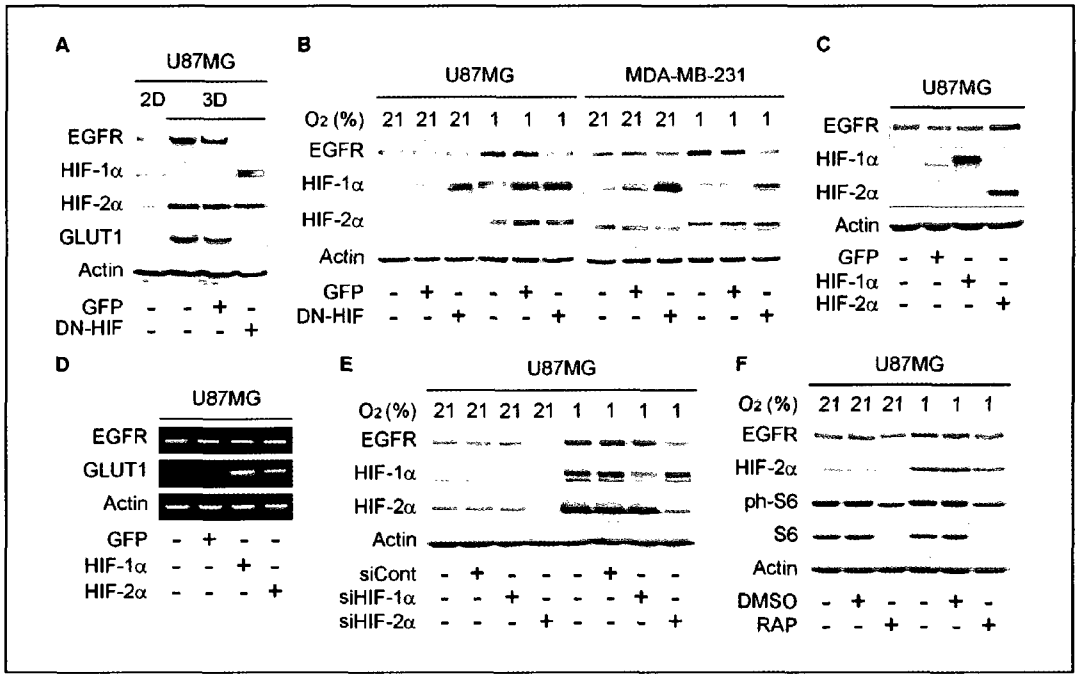
a benchmark HIF target gene, was observed in both settings as assessed by RT-PCR analysis. An analogous increase in *EGFR* mRNA levels was not observed, indicating that the EGFR is not induced at the transcriptional level under hypoxic conditions (**Fig. 10D-F**). These data suggest the existence of a general mechanism by which the hypoxic tumor microenvironment elicits upregulation of EGFR protein, but not mRNA, levels in human cancer cells.

3.1.2. HIF-2α activation results in the induction of EGFR protein levels.

We next examined whether the observed induction of EGFR protein in tumor spheroids and in hypoxic cells was a function of HIF activity. U87MG cells were infected with adenovirus expressing a dominant-negative form of HIF (DN-HIF) or FLAG-tagged GFP (GFP) as a viral control, and cultured as three-dimensional spheroids. Expression of DN-HIF diminished *GLUT1* levels, as expected, and EGFR upregulation in U87MG spheroids, demonstrating that the increased EGFR levels observed in this model system of the tumor microenvironment is indeed a consequence of HIF activation (Gunaratnam et al., 2003) (**Fig. 11A**). Similarly, inhibition of HIF activity by DN-HIF prevented EGFR upregulation in U87MG and MDA-MB-231 cells exposed to hypoxia, indicating that HIF activity is also required for the hypoxic induction of EGFR protein levels (**Fig. 11B**). Interestingly, expression of DN-HIF appeared to stabilize HIF-1α protein levels in certain instances; an observation which is likely attributable to the repression of HIF-dependent negative feedback mechanisms (**Fig. 11A-B**).

Next, we evaluated the individual contributions of the two HIF-α isoforms in the upregulation of EGFR protein expression. To this end, we attempted to recapitulate EGFR upregulation under normoxic conditions by infecting U87MG cells with HIF-1α

Figure 11. HIF-2α activation results in the induction of EGFR protein levels. (A) Western blot analysis of EGFR, HIF-α and GLUT1 protein levels in U87MG cells infected with adenovirus expressing FLAG-tagged GFP (GFP) or a dominant-negative form of HIF (DN-HIF). Cells were grown as three-dimensional (3D) spheroids for 3 days. Control cells were grown in two-dimensional (2D) culture for the same period of time. **(B)** Western blot analysis of EGFR and HIF-α levels in U87MG and MDA-MB-231 cells infected with GFP or DN-HIF for 24 hrs prior to exposure to normoxia or hypoxia for an additional 24 hrs. **(C)** Western blot and **(D)** RT-PCR analysis of EGFR protein and mRNA levels in U87MG cells infected to express GFP or oxygen-insensitive HIF-α variants (HIF-1α and HIF-2α) for 72 hrs in normoxia. **(E)** Western blot analysis of EGFR and HIF-α levels in U87MG transiently transfected with siRNA (50 nM) targeting HIF-1α (siHIF-1α), HIF-2α (siHIF-2α) or a scrambled control sequence (siCont) for 48 hrs prior to exposure to hypoxia for an additional 24 hrs. **(F)** Western blot analysis of EGFR and HIF-2α levels in U87MG pre-treated with rapamycin (RAP; 10 nM), or DMSO as a vehicle control, for 1 hr and then exposed to normoxia or hypoxia for an additional 16 hrs. The phosphorylation status of the S6 ribosomal protein (ph-S6) served as a control for drug activity. Actin served as a loading control in A-F.


A
U87MG
2D
3D
EGFR
HIF-1α
HIF-2α
GLUT1
Actin
GFP
DN-HIF
B
U87MG
MDA-MB-231
O2 (%)
21 21 21 1 1 1 21 21 21 1 1 1
EGFR
HIF-1α
HIF-2α
Actin
GFP
DN-HIF
C
U87MG
EGFR
HIF-1α
HIF-2α
Actin
GFP
HIF-1α
HIF-2α
D
U87MG
EGFR
GLUT1
Actin
GFP
HIF-1α
HIF-2α
E
U87MG
O2 (%)
21 21 21 21 1 1 1 1
EGFR
HIF-1α
HIF-2α
Actin
siCont
siHIF-1α
siHIF-2α
F
U87MG
O2 (%)
21 21 21 1 1 1
EGFR
HIF-2α
ph-S6
S6
Actin
DMSO
RAP

or HIF-2α variants, whose ODDs have been modified (P→A) and are consequently stable in the presence of oxygen (Kondo et al., 2002; Maranchie et al., 2002; Smith et al., 2005). Expression of HIF-2α, but not HIF-1α, was sufficient to generate an increase in EGFR protein under normoxic conditions (**Fig. 11C**). While expression of either HIF-1α or HIF-2α resulted in an induction of *GLUT1* mRNA, verifying that both variants are transcriptionally active, neither isoform promoted the accumulation of *EGFR* mRNA (**Fig. 11D**). Consistent with these observations, siRNA-mediated silencing of HIF-2α was sufficient to abolish EGFR induction under hypoxic conditions and even reduced basal receptor levels in normoxia, suggesting its general involvement in EGFR expression (**Fig. 11E**). No such effect was observed when cells were transfected with siRNA targeted against HIF-1α or scrambled control siRNA. Finally, we examined the role of the mTOR pathway, which is involved in *HIF1A* and *HIF2A* mRNA translation, in the upregulation of EGFR protein levels (Hudson et al., 2002; Thomas et al., 2006; Toschi et al., 2008). Predictably, pre-treatment of U87MG cells with rapamycin prevented accumulation of HIF-2α and hence the EGFR in hypoxia (**Fig. 11F**). Thus activation of HIF, and more specifically of HIF-2α, is required and sufficient for upregulation of EGFR protein in hypoxic cancer cells.

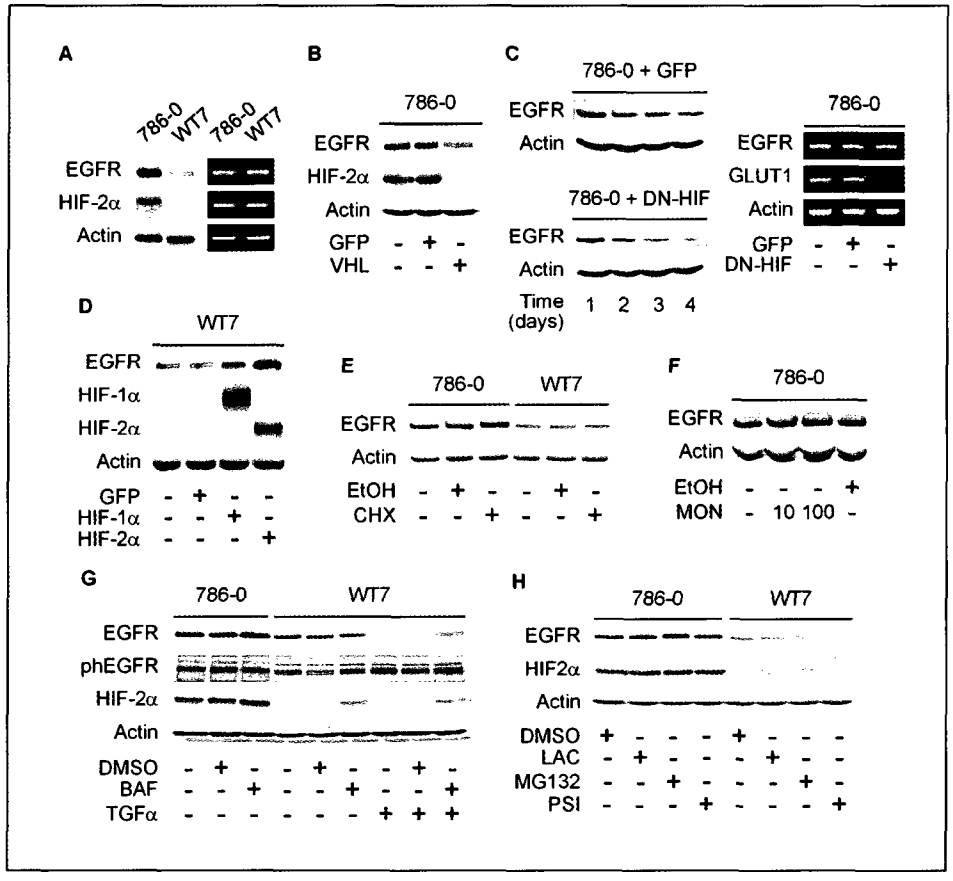
3.1.3. HIF-2α activation does not prevent ligand-mediated EGFR downregulation.

We next wanted to determine at what level HIF-2α activation affects EGFR protein expression. To address this question, we exploited *VHL*$^{-/-}$ RCC cells, that express only HIF-2α and irrespectively of oxygen tension, as a model system for studying EGFR metabolism in the presence of endogenous HIF-2α activity (Maxwell et al., 1999). The

VHL$^{-/-}$ RCC 786-0 cell line expresses significantly higher levels of EGFR protein, but not mRNA, compared with 786-0 cells stably re-expressing wild-type VHL (*VHL*$^{+}$ RCC or WT7), which prevents the normoxic accumulation of HIF-2α, consistent with the data presented in Figures 10-11 and reports by another group (Gemmill et al., 2005) (**Fig. 12A**). Moreover, similar results were obtained with 786-0 cells infected to express GFP-tagged VHL, indicating that the previously observed reduction in EGFR protein upon reintroduction of VHL is not due to clonal variation (**Fig. 12B**). To confirm that the elevated EGFR protein levels in 786-0 cells were a result of HIF-2α activation rather than loss of VHL, we infected 786-0 cells with adenovirus expressing GFP or DN-HIF for up to 4 days. Inhibition of endogenous HIF-2α activity with DN-HIF resulted in a time-dependent decrease in total EGFR protein levels indicating that HIF-2α activity is required for the high levels of EGFR protein in *VHL*$^{-/-}$ RCC as well (**Fig. 12C**). Expression of DN-HIF prevented induction of *GLUT1* mRNA, controling for inhibition of HIF activity, but once again failed to affect *EGFR* mRNA levels (**Fig. 12C**). Finally, we examined the roles of the two HIF-α isoforms in the upregulation of EGFR protein expression in *VHL*$^{-/-}$ RCC. Once again, expression of the HIF-2α, but not HIF-1α, variant resulted in an increase in EGFR protein in *VHL*$^{+}$ RCC cells under normoxic conditions, substantiating the pivotal role of this HIF isoform in the regulation of EGFR protein levels (**Fig. 12D**).

Given that there was no difference in *EGFR* mRNA levels in the presence and absence of HIF, we hypothesized that the elevated levels of EGFR in HIF-2α-expressing cells may be caused by increased *EGFR* mRNA translation or receptor stabilization relative to other cells. We began our investigation by assessing the effect of blocking these processes,

Figure 12. HIF-2α activation does not prevent ligand-mediated EGFR degradation. **(A)** Western blot (left panels) and RT-PCR (right panels) analysis of EGFR protein and mRNA levels in *VHL*$^{-/-}$ 786-0 RCC cells and 786-0 cells stably re-expressing wild-type VHL (WT7). **(B)** Western blot analysis of EGFR protein and mRNA levels in 786-0 cells infected to express GFP or GFP-tagged VHL for 72 hrs. **(C)** Western blot (left panels) and RT-PCR (right panels) analysis of EGFR protein and mRNA levels in 786-0 cells infected to express GFP or DN-HIF for 24-96 hrs. **(D)** Western blot analysis of EGFR protein levels WT7 cells infected with GFP, HIF-1α or HIF-2α for 72 hrs. **(E)** Western blot analysis of EGFR levels in cells treated with cycloheximide (CHX; 100 μg/mL), an inhibitor of protein synthesis, or ethanol (EtOH), as a vehicle control, for 4 hrs. **(F)** Western blot analysis of EGFR levels in 786-0 cells treated with monensin (MON; 10-100 μM), an inhibitor of receptor recycling, for 6 hrs. **(G)** Western blot analysis of EGFR, phosphorylated EGFR (phEGFR) and HIF-2α levels in cells treated with bafilomycin (BAF; 0.25 μM), an inhibitor that blocks lysosomal degradation of the receptor, or DMSO, as a vehicle control, for 16 hrs. Cells were stimulated with recombinant TGFα (20 ng/mL) for an additional 6 hrs where indicated, as a control for drug activity against ligand-mediated EGFR degradation. **(H)** Western blot analysis of EGFR and HIF-2α levels in cells following treatment with 20 μM of the proteasomal inhibitors lactacystin (LAC), MG132 or proteasome inhibitor I (PSI) for 2 hrs. Actin served as a loading control in A-H.


A
786-0
WT7
786-0
WT7
EGFR
HIF-2α
Actin
B
786-0
EGFR
HIF-2α
Actin
GFP - + -
VHL - - +
C
786-0 + GFP
EGFR
Actin
786-0 + DN-HIF
EGFR
Actin
Time (days) 1 2 3 4
786-0
EGFR
GLUT1
Actin
GFP - + -
DN-HIF - - +
D
WT7
EGFR
HIF-1α
HIF-2α
Actin
GFP - + - -
HIF-1α - - + -
HIF-2α - - - +
E
786-0
WT7
EGFR
Actin
EtOH - + - - + -
CHX - - + - - +
F
786-0
EGFR
Actin
EtOH - - - +
MON - 10 100 -
G
786-0
WT7
EGFR
phEGFR
HIF-2α
Actin
DMSO - + - - + - - + -
BAF - - + - - + - - +
TGFα - - - - - - + + +
H
786-0
WT7
EGFR
HIF2α
Actin
DMSO + - - - + - - -
LAC - + - - - + - -
MG132 - - + - - - + -
PSI - - - + - - - +

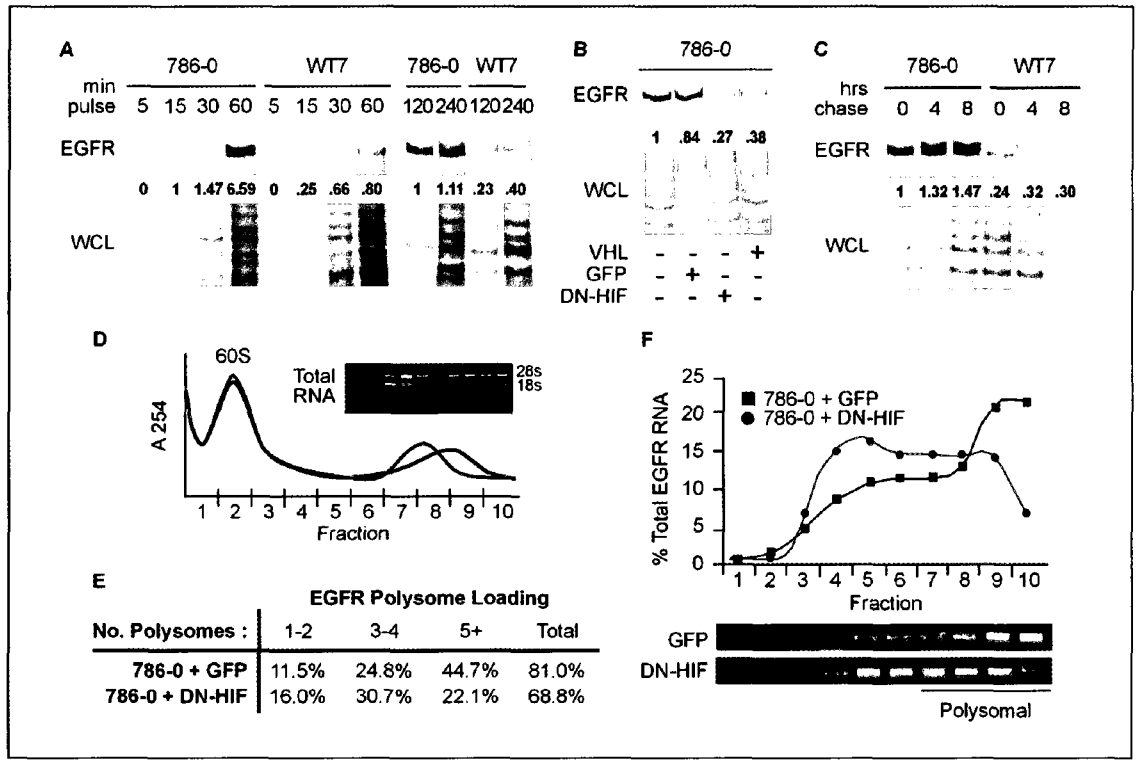
with commercially-available inhibitors, on EGFR protein expression in the 786-0 and WT7 cell lines. We first blocked mRNA translation in both cell lines by treatment with cycloheximide (CHX) for 4 hours. Within this time frame, inhibition of *de novo* protein synthesis had no demonstrable effect on steady-state EGFR protein levels, regardless of VHL or HIF status (**Fig. 12E**). Since this outcome did not prove to be overly informative, we proceeded to consider the possibility that $VHL^{-/-}$ RCC cells display increased receptor recycling or defective ligand-mediated receptor degradation. Antagonizing receptor recycling with the carboxylic ionophore, monensin (MON), did not alter total EGFR levels in the 786-0 cell line, indicating that enhanced recycling does not contribute to the elevated EGFR levels in this setting (**Fig. 12F**). Next, we compared EGFR turnover in VHL^{+} and $VHL^{-/-}$ RCC cells by inhibiting receptor degradation with bafilomycin (BAF), a potent inhibitor of vacuolar H^{+}-ATPases. Treatment of the cells with BAF for 16 hours did not instigate an upsurge in WT7 EGFR levels as might be expected if the receptor was being degraded at a faster pace (**Fig. 12G**). These data suggest that differential HIF-dependent EGFR expression profiles are probably a function of increased receptor synthesis in the presence of HIF rather than increased receptor turnover in its absence; an effect that is most likely missed with short-term CHX treatment due to long receptor half-lives (**Fig. 12E**). Importantly, treatment with BAF prevented ligand-dependent EGFR downregulation in WT7 cells stimulated with recombinant TGFα, confirming its functional efficacy (**Fig. 12G**). As in the case of BAF treatment, addition of proteasome (lactacystin (LAC), proteasome inhibitor I (PSI) and MG132) and lysosome (MG132) inhibitors, whose activity was verified by the accumulation of normoxic HIF-2α, did not affect total EGFR protein levels in either cell

line within the tested time frames (**Fig. 12H**). These results further corroborate that increased receptor turnover does not account for the decreased EGFR levels observed in *VHL*$^{+}$ RCC cells.

3.1.4. HIF-2α activation results in increased EGFR mRNA translation.

To better ascertain the intricacies of EGFR metabolism in *VHL*$^{-/-}$ RCC cells compared with their *VHL*$^{+}$ counterparts, the rate of EGFR protein synthesis was assessed using metabolic labeling techniques. The *VHL*$^{-/-}$ 786-0 and *VHL*$^{+}$ WT7 RCC cell lines were labeled with [^{35}S]methionine ([^{35}S]Met) for different lengths of time, lysed and immunoprecipitated with an antibody specific to the EGFR (**Fig. 13A**). There was a marked and consistent increase in [^{35}S]Met incorporation in the 786-0 cells compared to the WT7 cells even within very short labeling periods (**Fig. 13A**). Importantly, radiolabel incorporation during short pulses generally reflects rates of protein synthesis rather than protein turnover. This increase was abolished upon adenoviral infection of 786-0 cells with DN-HIF, demonstrating that the increased rate of EGFR protein synthesis is HIF-2α-dependent (**Fig. 13B**). To ensure that the difference in [^{35}S]Met incorporation was not due to very rapid receptor turnover in WT7 cells, a pulse chase experiment was conducted with both cell lines. No receptor degradation was observed within 8 hours of chase in either cell line, in line with studies that have reported receptor half-lives of 15-30 hours in the absence of exogenous ligand, such that the observed difference in [^{35}S]Met incorporation could not be attributed to enhanced receptor degradation in *VHL*$^{+}$ RCC (Decker, 1984; Gamou and Shimizu, 1987) (**Fig. 13C**). The increase in radiolabel incorporation observed after 4 hours of chase is similarly consistent with reports of post-translational modification of the receptor (Decker, 1984; Gamou and Shimizu, 1987).

Figure 13. HIF-2α activation results in increased *EGFR* mRNA translation. (A) EGFR radiolabel incorporation in *VHL*$^{-/-}$ 786-0 and *VHL*$^{+}$ WT7 cells pulsed for the indicated times with [^{35}S]methionine ([^{35}S]Met) and immunoprecipitated with an anti-EGFR antibody. **(B)** EGFR [^{35}S]Met incorporation in 786-0 cells infected to express GFP or DN-HIF for 72 hrs and labeled for 1 hr. **(C)** EGFR [^{35}S]Met incorporation in cells labeled for 2 hrs and cold chased for the indicated times. Whole cell lysates (WCL) served as radiolabel incorporation and loading controls in A-C. **(D)** Polysomal profiles of 786-0 cells infected with GFP (blue) or DN-HIF (red) for 48 hrs and subjected to sucrose gradient fractionation. From left to right, fractions lacking ribosomes (fractions 1-2), containing ribosomal subunits (fractions 3-5), or containing polysomes of increasing molecular weights (fraction 6-10). Inset is a representative image of the relative abundance of 28s and 18s rRNAs as visualized by agarose gel separation and ethidium bromide staining. **(E)** The percent of total *EGFR* mRNA associated with polysomes in 786-0 cells described in D. **(F)** RT-PCR analysis of *EGFR* mRNA levels in polysomal fractions of 786-0 cells described in D. The percent of total *EGFR* mRNA in each fraction is plotted.

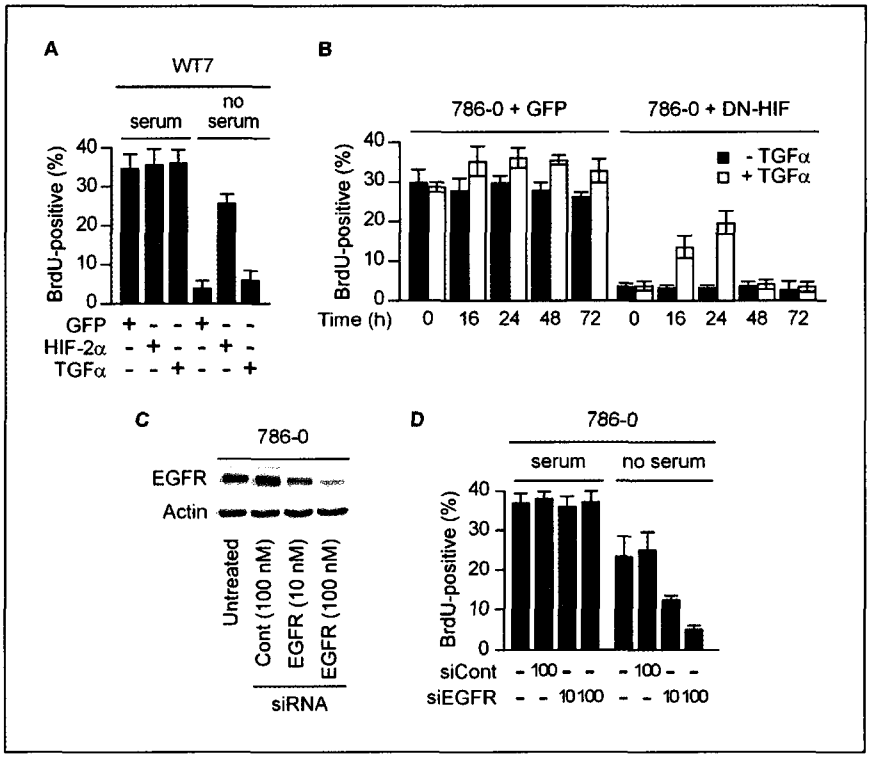

A
786-0
WT7
786-0
WT7
min
pulse
5 15 30 60 5 15 30 60 120 240 120 240
EGFR
0 1 1.47 6.59 0 .25 .66 .80 1 1.11 .23 .40
WCL
B
786-0
EGFR
1 .84 .27 .38
WCL
VHL - - - +
GFP - + - -
DN-HIF - - + -
C
786-0
WT7
hrs
chase
0 4 8 0 4 8
EGFR
1 1.32 1.47 .24 .32 .30
WCL
D
60S
Total
RNA
28s
18s
A 254
1 2 3 4 5 6 7 8 9 10
Fraction
E
EGFR Polysome Loading
No. Polysomes :
1-2
3-4
5+
Total
786-0 + GFP
11.5%
24.8%
44.7%
81.0%
786-0 + DN-HIF
16.0%
30.7%
22.1%
68.8%
F
786-0 + GFP
786-0 + DN-HIF
% Total EGFR RNA
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Fraction
GFP
DN-HIF
Polysomal

Next we were interested in determining the relative amount of ribosome-bound *EGFR* mRNA, which is associated with but not definitively indicative of active translation, in the presence and absence of HIF-2α activity. To this end, ribosome/mRNA complexes from 786-0 cells expressing DN-HIF, or GFP as a control, were fractionated by sucrose gradient centrifugation. Interestingly, expression of DN-HIF resulted in the redistribution of the polysomal profile of 786-0 cells, which may be reflective of a global suppressive effect on protein synthesis (**Fig. 13D**). More specifically and in accordance with the radiolabeling data, inhibition of HIF-2α activity in 786-0 cells with DN-HIF resulted in a shift of *EGFR* mRNA to the monosomes from the actively translating polysomal fraction, as assessed by RT-PCR analysis (**Fig. 13E-F**). Taken together, these data provide evidence that EGFR protein synthesis is upregulated under hypoxic conditions and uncover a novel role for HIF-2α in the translational control of the EGFR.

3.1.5. Overexpression of the EGFR supports cancer cell growth autonomy.

Overproduction of TGFα is thought to be required for the growth autonomy of a variety of human cancers (Lee et al., 1995). Both TGFα and EGFR expression are necessary for the autocrine signaling that drives the serum-independent growth of $VHL^{-/-}$ RCC cells (de Paulsen et al., 2001; Smith et al., 2005). It is thus of interest to distinguish whether HIF-2α-mediated production of TGFα is sufficient or if the above-described boost in EGFR protein synthesis is also required for RCC growth autonomy. VHL^{+} WT7 cells stimulated with exogenous TGFα failed to grow in the absence of serum, as measured by BrdU incorporation, underscoring that overproduction of ligand alone is not sufficient to sustain growth autonomy (**Fig. 14A**). Conversely, adenoviral expression of HIF-2α was sufficient to promote the serum-independent growth of WT7 cells in normoxia, indicating

Figure 14. Overexpression of the EGFR supports cancer cell growth autonomy. (A) *VHL*$^{+}$ WT7 cells were infected with adenovirus to express HIF-2α, GFP as a viral control or stimulated with recombinant TGFα (TGFα; 20 ng/mL) and then cultured in the presence or absence of serum for 72 hrs prior to labeling with 5-bromo-2'-deoxy-uridine (BrdU). **(B)** BrdU incorporation in *VHL*$^{-/-}$ 786-O cells infected with GFP or DN-HIF in serum-free media supplemented with TGFα for the indicated times. **(C)** Western blot analysis of EGFR levels in 786-0 cells transiently transfected with increasing concentrations of siRNA (10-100 nM) targeted against *EGFR* mRNA or a scrambled control sequence for 72 hrs. Actin served as a loading control. **(D)** BrdU incorporation in cells described in C. Data are presented as mean ± s.d. of at least three independent experiments in A, B, and D.


A
WT7
serum
no serum
BrdU-positive (%)
40
30
20
10
0
GFP + - - + - -
HIF-2α - + - - + -
TGFα - - + - - +
B
786-0 + GFP
786-0 + DN-HIF
- TGFα
+ TGFα
BrdU-positive (%)
Time (h) 0 16 24 48 72 0 16 24 48 72
C
786-0
EGFR
Actin
Untreated
Cont (100 nM)
EGFR (10 nM)
EGFR (100 nM)
siRNA
D
786-0
serum
no serum
BrdU-positive (%)
siCont - 100 - - - 100 - -
siEGFR - - 10 100 - - 10 100

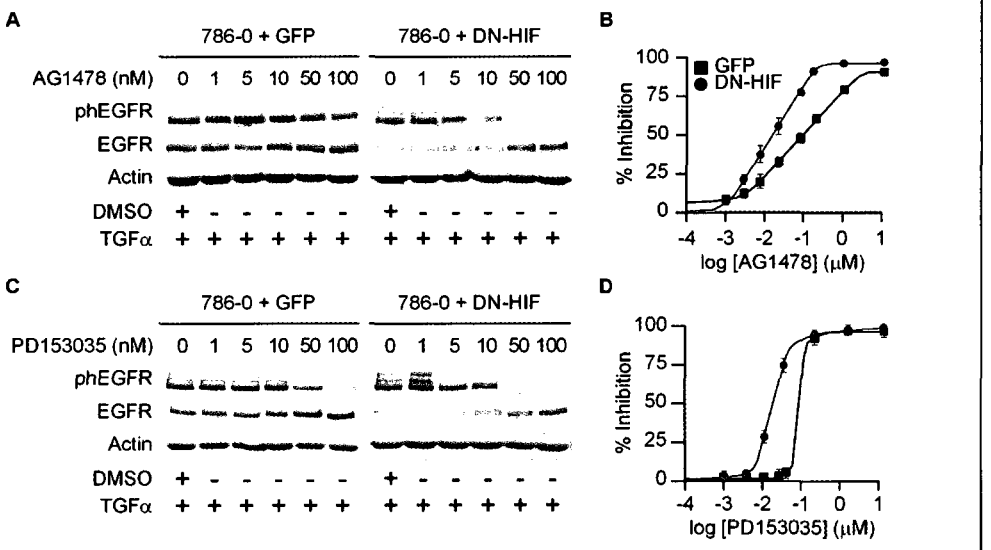
that HIF-2α has a function other than the induction of TGFα that is required for autonomous growth (**Fig. 14A**). As expected, inhibition of HIF-2α activity by adenoviral DN-HIF expression in *VHL*$^{-/-}$ 786-0 cells abolished the ability of the cells to incorporate BrdU in the absence of serum (Gunaratnam et al., 2003) (**Fig. 14B**). While stimulation with excess ligand instigated a slight rise in the number of proliferating GFP-infected control cells, it failed to rescue autonomous growth in the absence of HIF-2α activity (**Fig. 14B**). We did note a transient growth spurt in the latter cells, demonstrating that there is an immediate proliferative response to exogenous TGFα that is not sustained over time in cells expressing low levels of EGFR. This is likely attributable to negative regulatory mechanisms that restrict ligand-dependent signaling (Sweeney and Carraway, 2004). Finally, we wanted to directly examine the effect of varying EGFR levels on the ability of 786-0 cells to engage in autonomous proliferation. EGFR protein levels were diminished in a dose-dependent manner by transient silencing of the receptor with increasing concentrations of siRNA (**Fig. 14C**). There was a corresponding decrease in the serum-independent growth of cells expressing siRNA targeting the EGFR, confirming that the ability of cells to proliferate in the absence of exogenous cues is dependent on the amount of receptor expressed (**Fig. 14D**). As such, these results highlight the importance of HIF-2α in maintaining adequate EGFR expression and availability for the autocrine ligand action that enables the autonomous proliferation of cancer cells.

3.1.6. HIF-2α activity does not affect EGFR sensitivity to RTK inhibitors.

The pivotal role of EGFR signaling in the development and progression of *VHL*$^{-/-}$ RCC, and other human cancers, has been well established (Salomon et al., 1995; Prewett et al.,

1998; Keder et al., 2002; Asakuma et al., 2004; Smith et al., 2005). The existing RTK inhibitors, including gefitinib and erlotinib, are highly specific small molecules that bind the ATP-binding site of the EGFR and competitively inhibit its phosphorylation. These anti-EGFR agents have, however, provided somewhat disappointing results in clinical trials (Drucker et al., 2003; Dancey, 2004; Jermann et al., 2006). Given its effect on EGFR protein levels, we hypothesized that activation of HIF-2α may also cause resistance to EGFR-based therapies by upregulating total receptor levels and thereby making it more difficult for drugs to compete. We thus examined the dose-responsiveness of 786-0 cells infected to express DN-HIF, or GFP control virus, to the EGFR-specific tyrosine kinase inhibitors, AG1478 (**Fig. 15A-B**) and PD153035 (**Fig. 15C-D**). HIF-2α-mediated EGFR overproduction in the control cells did not appear to affect its inherent susceptibility to the actions of the RTK inhibitors as reflected by decreased ligand-dependent receptor phosphorylation at the higher drug concentrations (**Fig. 15A-D**). Notably, inhibition of HIF-2α activity with DN-HIF did result in a 4-5 fold decrease in the IC50 of drug required to block EGFR tyrosine kinase activity on a per cell basis, however when normalized to total receptor levels no significant differences in sensitivity were observed (**Fig. 15E-F**). This result is of particular clinical importance as it emphasizes that, irrespective of HIF status, the IC50 is a function of the total amount of receptor and therefore the concomitant inhibition of HIF-2α and the EGFR itself should render improved patient responses. Taken together, our results reaffirm the central role of the EGFR in conferring cancers with growth autonomy and endorse its therapeutic targeting not only in *VHL*$^{-/-}$ RCC but in all other HIF-2α-expressing cancers as well.

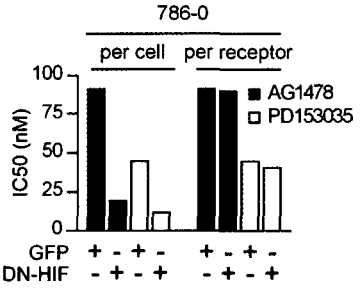
Figure 15. HIF-2α activity does not affect EGFR sensitivity to RTK inhibitors. Western blot analysis of total EGFR and phEGFR levels in serum-starved 786-O cells infected with adenovirus to express GFP or DN-HIF for 48 hrs and then treated with increasing concentrations of the EGFR-specific receptor tyrosine kinase (RTK) inhibitors, **(A)** AG1478 or **(C)** PD153035, or DMSO as a vehicle control, for 2 hrs prior to stimulation with TGFα (20 ng/mL). **(B, D)** Graphs depicting the percent inhibition of EGFR phosphorylation plotted against the log of the tested RTK inhibitor concentrations for experiments described in A and C, respectively. Data are presented as mean ± s.d. of at least three experiments. **(E)** The IC50 concentrations for drug inhibition of kinase activity on a per cell basis as determined by interpolation on graphs obtained in B and D and then normalized to the relative levels of total EGFR expressed by the cells. **(F)** Bar graph depicting the IC50 concentrations of the receptor tyrosine kinase inhibitors, AG1478 and PD153035, for inhibition of EGFR kinase activity as quantified in E.



E

	AG1478		
	IC50 (nM) per cell	Relative EGFR Levels	IC50 (nM) per receptor
786-0 + GFP	89.9	1.0	89.9
786-0 + DN-HIF	16.0	0.18	88.9

	PD153035		
	IC50 (nM) per cell	Relative EGFR Levels	IC50 (nM) per receptor
786-0 + GFP	45.0	1.0	45.0
786-0 + DN-HIF	11.0	0.27	40.7

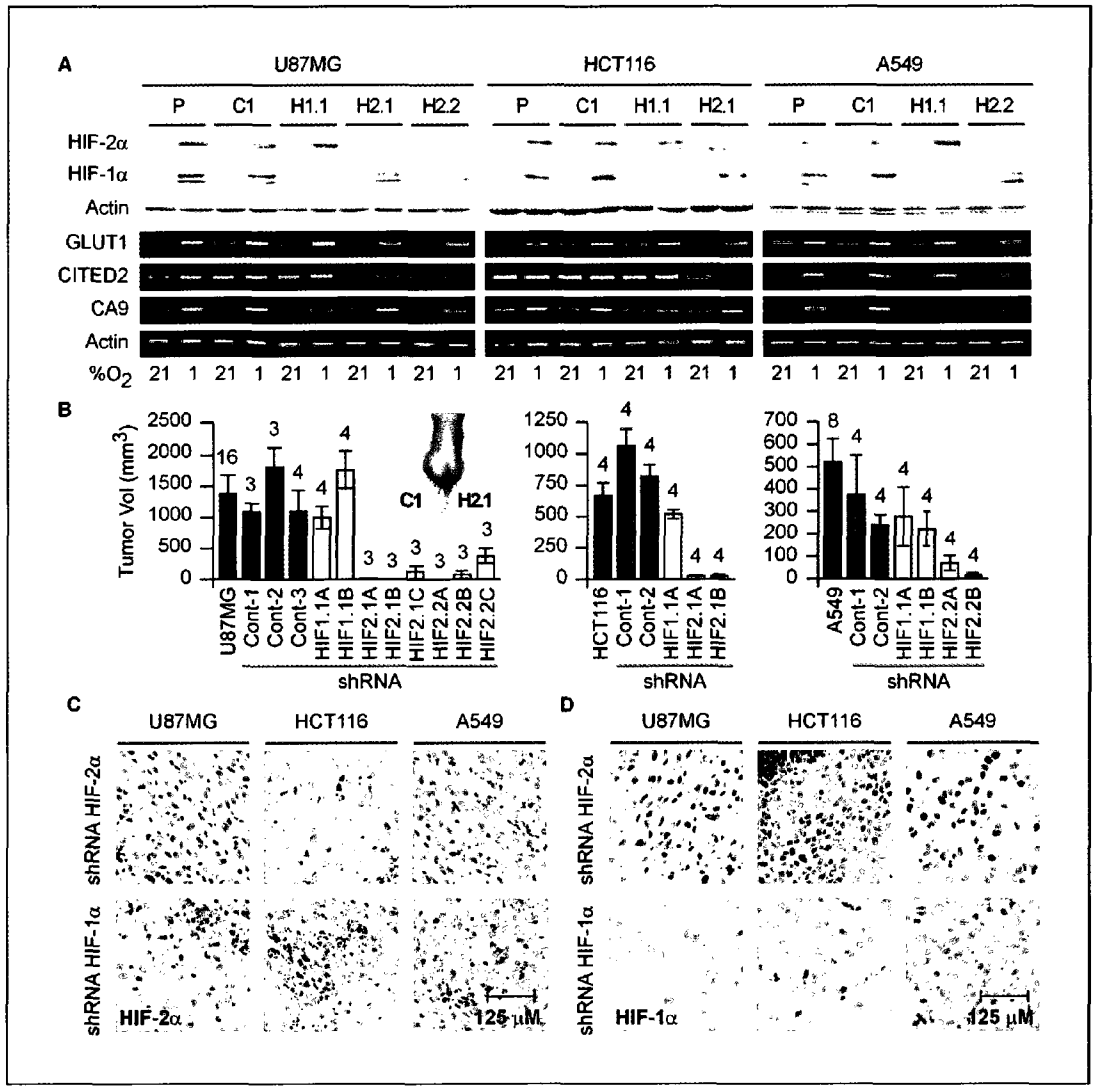


3.2. Genetically diverse human cancers converge at the HIF-2α oncogenic axis

3.2.1. Silencing HIF-2α prevents the tumorigenesis of human cancers *in vivo*.

The unique ability of HIF-2α to drive *VHL*$^{-/-}$ RCC growth autonomy and tumorigenesis is well-documented (Kondo et al., 2002; Maranchie et al., 2002; Kondo et al., 2003; Zimmer et al., 2004). Since HIF-2α is frequently expressed in the core of human tumors and can upregulate TGFα transcriptionally and the EGFR translationally (**Fig. 10-11**) in non-RCC cancer cells, we reasoned that activation of this growth pathway may contribute to the development of other cancer types (Talks et al., 2000; Smith et al., 2005). To address this prospect we initially selected the U87MG glioma, HCT116 colorectal and A549 lung carcinoma cell lines which differ considerably both genetically and histopathologically; the former being *PTEN*-null and the latter two harboring activating *KRAS* mutations (Valenzuela and Groffen, 1986; Jiang et al., 1989; Li et al., 1997). HIF-2α was stably silenced using one of two previously published shRNA sequences (shHIF2.1 and shHIF2.2), achieving an 85-90% reduction in protein levels in all three cell lines (Zimmer et al., 2004) (**Fig. 16A**). The cells were also stably transfected with shRNA targeting HIF-1α (shHIF1.1) or a non-targeting scrambled shRNA (Cont) as controls (Mizukami et al., 2004). Fittingly, HIF-2α knockdown restricted the hypoxic expression of *CITED2*, a HIF-2α-specific target gene, but not common HIF (*GLUT1*) and HIF-1α-specific (carbonic anhydrase-9; *CA9*) target genes and the reverse was true where HIF-1α was silenced (Raval et al., 2005; Aprelikova et al., 2006) (**Fig. 16A**). Notably, *CITED2* was often maximally expressed in normoxia, suggesting that basal HIF-2α levels are sufficient for target gene induction, consistent with reports that it is active at

Figure 16. Silencing HIF-2α prevents the tumorigenesis of human cancers *in vivo*. **(A)** Western blot (upper panels) and RT-PCR (lower panels) analysis of HIF-α subunit and HIF target gene expression in serum-starved, parental (P) U87MG glioma, HCT116 colorectal and A549 lung carcinoma cells compared to those stably expressing scrambled (C) or HIF-1α shRNA (H1.1), as controls, or one of two shRNA sequences targeting HIF-2α (H2.1 and H2.2) following incubation in normoxia (21% O_2) or hypoxia (1% O_2) for 24 hrs. **(B)** Endpoint tumor volumes observed in xenograft assays performed with cell lines described in A. The number of injections per cell line is specified above the corresponding bar. Data are presented as mean ± s.e.m. Inset is a representative image of an athymic mouse subcutaneously injected in the flanks with U87MG cells expressing control (left) or HIF-2α shRNA (right). Representative images of **(C)** HIF-2α and **(D)** HIF-1α stained initial mass tumor sections, excised 1 week post-injection, derived from cell lines described in A. Immunostaining was visualized at a magnification of 400x.


A
U87MG
HCT116
A549
P
C1
H1.1
H2.1
H2.2
HIF-2α
HIF-1α
Actin
GLUT1
CITED2
CA9
Actin
%O2
21 1 21 1 21 1 21 1 21 1
B
Tumor Vol (mm3)
C1
H21
U87MG
Cont-1
Cont-2
Cont-3
HIF1.1A
HIF1.1B
HIF2.1A
HIF2.1B
HIF2.1C
HIF2.2A
HIF2.2B
HIF2.2C
shRNA
HCT116
Cont-1
Cont-2
HIF1.1A
HIF2.1A
HIF2.1B
shRNA
A549
Cont-1
Cont-2
HIF1.1A
HIF1.1B
HIF2.2A
HIF2.2B
shRNA
C
U87MG
HCT116
A549
shRNA HIF-2α
shRNA HIF-1α
HIF-2α
125 μM
D
U87MG
HCT116
A549
shRNA HIF-2α
shRNA HIF-1α
HIF-1α
125 μM

higher oxygen tensions (Wiesener et al., 1998; Uchida et al., 2004; Holmquist-Mengelbier et al., 2006).

Next, we confronted our main question and examined the effect of silencing HIF-2α on the ability of the cell lines to form tumors in a nude mouse xenograft assay. Parental and control cells formed large xenograft tumors within 4-6 weeks of injection, reflecting the particularly aggressive nature of these cancer types (**Fig. 16B**). Remarkably, silencing HIF-2α either completely abolished or significantly impeded (≥5-fold reduction in tumor volume) the ability of these highly malignant cell lines to form tumors in nude mice (n=34) (**Fig. 16B**). Importantly, silencing HIF-2α did not affect HIF-1α expression *in vivo*, and hence this strong phenotype is due to the specific loss of the former isoform (**Fig. 16C-D**). Silencing HIF-1α did not, however, dramatically affect tumorigenesis (n=20) (**Fig. 16B**), consistent with previous studies conducted with human cancer cell lines (Li et al., 2005; Holmquist-Mengelbier et al., 2006; Favaro et al., 2008). We verified that HIF-1α protein levels remained knocked-down *in vivo*, such that the ability of these cells to form tumors cannot be attributed to a loss of shRNA expression (**Fig. 16D**). This result further underscores a distinction between the HIF isoforms in tumor biology and reveals HIF-2α as a rate-limiting molecule in the development of genetically diverse human cancers.

3.2.2. Silencing HIF-2α prevents tumor cell proliferation *in vivo*.

Given that cells expressing HIF-2α shRNA failed to form palpable tumors, we extracted the initial cell masses, one week post-injection, to ascertain if this outcome stemmed from their inability to proliferate *in vivo* or if other hallmark traits were affected.

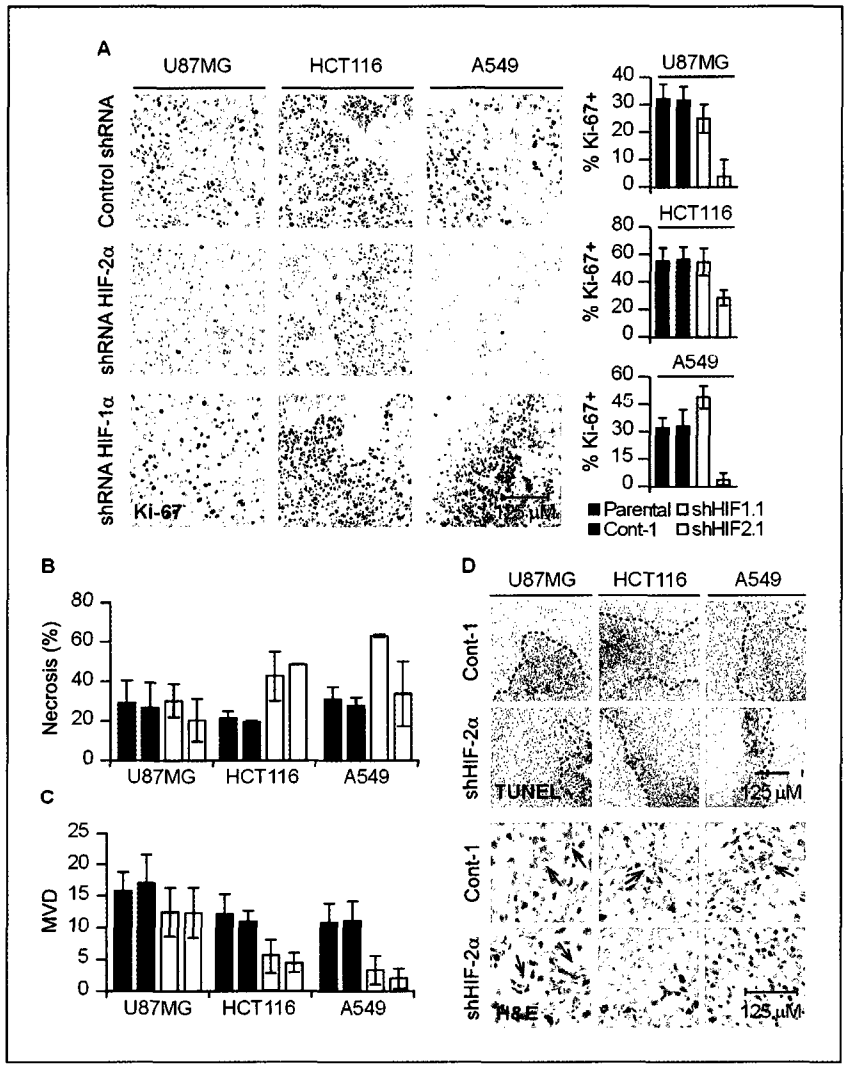
Silencing HIF-2α inhibited tumor cell growth considerably, as denoted by a marked reduction in Ki-67 staining compared to sections derived from parental and scrambled control shRNA-expressing cells (**Fig. 17A**). Silencing HIF-1α did not significantly affect tumor cell proliferation and even resulted in a slight increase in Ki-67 staining in A549 cells (**Fig. 17A**), an effect that was recapitulated *in vitro* (**Fig. 18B**). This result highlights the unique ability of HIF-2α to drive cellular proliferation, providing a mechanistic explanation for the divergent tumorigenic potentials of the HIF isoforms.

We next visually inspected TUNEL- and H&E-stained initial mass tumor sections to determine if silencing HIF-2α had an effect on tumor cell death and vascularization. The initial mass tumors derived from cells expressing HIF-2α shRNA did in some cases display increased tumor cell death (**Fig. 17B, D**) and decreased blood vessel density (**Fig. 17C-D**), however, no gross differences were observed when compared to those lacking HIF-1α. While it is interesting that the isoforms were unable to rescue one another in these regards, it is nonetheless unlikely that silencing HIF-2α inhibits *in vivo* tumor formation by negatively affecting pro-angiogenic or anti-apoptotic pathways alone. Taken together, these data suggest that HIF-2α plays a central role in promoting the persistent proliferation and tumorigenesis of these cancers, and likely others, of variable genetic backgrounds and tissue distributions.

3.2.3. Silencing HIF-2α prevents the autonomous growth of human cancers.

In light of the fact that silencing HIF-2α led to a defect in tumor vascularization, it was desirable to uncouple its role in tumor cell growth from its pro-angiogenic function. The ability of the stable cell lines to form avascular, three-dimensional spheroids *in vitro* was

Figure 17. Silencing HIF-2α prevents tumor cell growth *in vivo*. **(A)** Representative images of Ki-67-stained initial mass tumor sections, excised 1 week post-injection, derived from cells stably expressing control shRNA or shRNA targeting the HIF-α subunits, visualized at a magnification of 200x. Bar graph on the right indicates quantitative analysis of the percentage of Ki-67 positive cells per tumor section. **(B)** Percentage of necrotic areas in initial mass tumor sections as assessed by TUNEL-staining and histological analysis. **(C)** Initial mass tumor vascularization expressed as a function of microvessel density (MVD) per field. **(D)** Representative images of TUNEL- and H&E-stained initial mass tumor sections visualized at magnifications of 100x and 400x, respectively. Dashed lines segregate necrotic and viable regions and arrows indicate the presence of blood vessels. Data in A-C are expressed as mean ± s.d. as measured in at least three independent tumor sections.

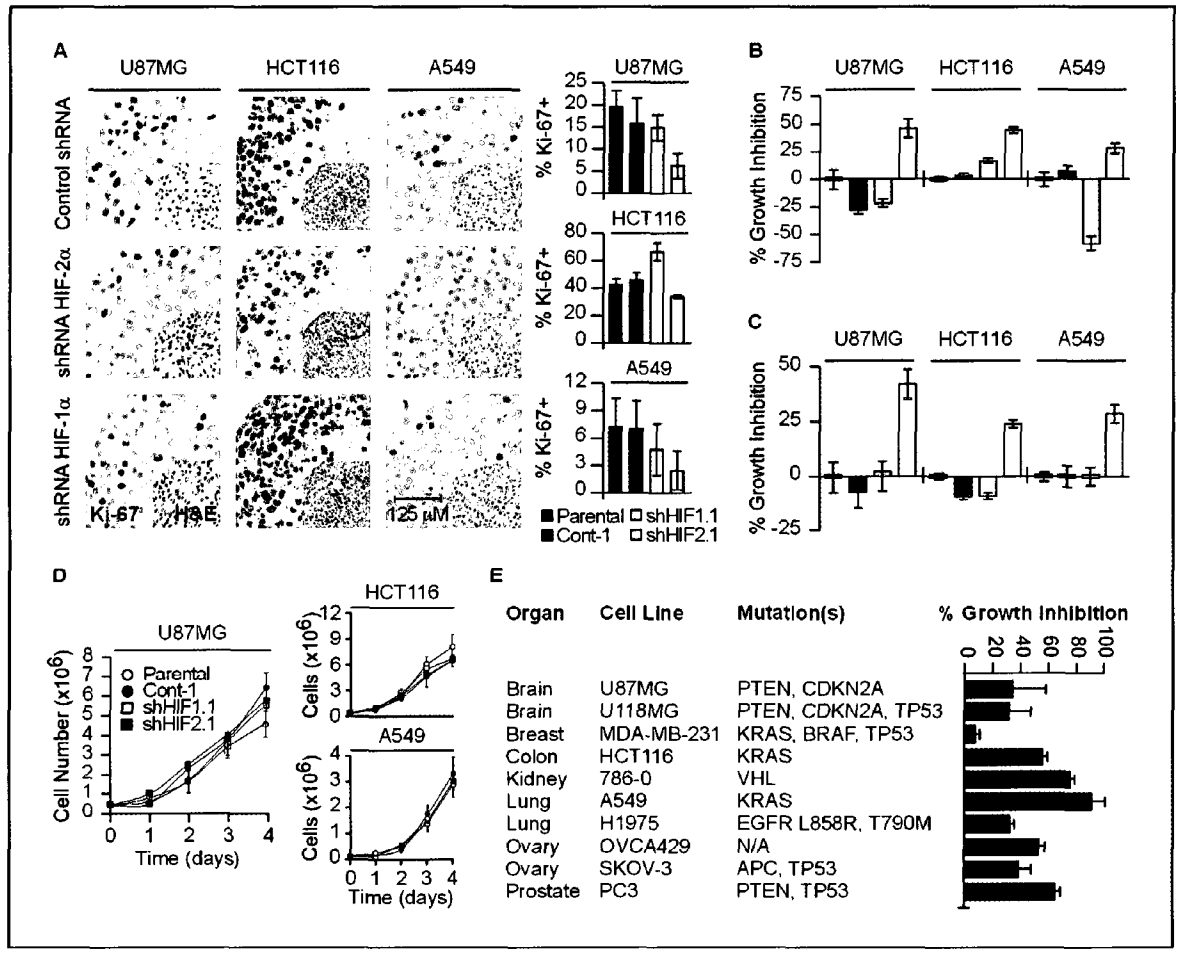

A
U87MG
HCT116
A549
Control shRNA
shRNA HIF-2α
shRNA HIF-1α
Ki-67
125 μM
% Ki-67+
Parental
shHIF1.1
Cont-1
shHIF2.1
B
Necrosis (%)
C
MVD
D
Cont-1
shHIF-2α
TUNEL
H&E

thus considered. Several layers of actively proliferating cells, expressing Ki-67, were detected at the periphery of control and HIF-1α shRNA-expressing spheroids (**Fig. 18A**). Fewer cells in the HIF-2α knockdown spheroids, however, stained positive for Ki-67, substantiating its direct involvement in cell proliferation (**Fig. 18A**).

To better distinguish if silencing HIF-2α had a deleterious effect on cell growth autonomy *per se*, we assessed the ability of the cancer cells to proliferate in the absence of serum growth factors *in vitro*, the standard approach to measuring this capability (Pause et al., 1998; Hanahan and Weinberg, 2000). In contrast to the controls, cells expressing HIF-2α shRNA did not exhibit sustained growth upon serum withdrawal in hypoxia (**Fig. 18B**) or normoxia (**Fig. 18C**), as measured by BrdU incorporation. These results corroborate that HIF-2α is functional in normoxia (**Fig. 16A**) and confers cells with their autonomic growth capabilities. Notably, silencing HIF-1α did not impede, and even stimulated in some instances, the serum-independent growth of the cells (**Fig. 18B-C**). Importantly, cell doubling times in the presence of serum were unaffected indicating that no massive defects in cell cycle progression were incurred upon shRNA introduction and that cell growth could be rescued by exogenous growth factors (**Fig. 18D**).

We then extended our studies and examined the effect of abrogating HIF function on the serum-independent growth of a panel of human cancer cell lines harboring various genetic mutations. Expression of DN-HIF effectively caused most of the cell lines tested, including cell lines harboring *EGFR* activating mutations, to undergo quiescence in the absence of exogenous growth factors, further stressing its key role in conferring cancers with growth signal autonomy (**Fig. 18E**). Collectively, these data suggest that human

Figure 18. Silencing HIF-2α prevents the autonomous growth of human cancers. **(A)** The ability of human cancer cell lines stably expressing control shRNA or shRNA targeting the HIF-α subunits to form multicellular spheroids and proliferate in an avascular setting was assessed by Ki-67-staining. Representative images were visualized at a magnification of 400x. Bar graph on the right indicates quantitative analysis of the percentage of Ki-67 positive cells per spheroid section. The ability of cells described in A to proliferate in the absence of serum growth factors was measured by BrdU labeling following incubation in **(B)** hypoxia or **(C)** normoxia for 48 hrs. The percentage of BrdU-positive cells was determined at a magnification of 100x in at least three independent fields per experiment and is expressed as the percentage growth repression relative to parental cell lines. **(D)** Cell proliferation under growth (10% FBS) conditions in normoxia was measured by serial cell counts over time. **(E)** BrdU incorporation in a panel of serum-starved human cancer cell lines infected with adenovirus to express DN-HIF for 48 hrs in normoxia. Data is expressed as the percentage growth repression relative to GFP-expressing controls. Data in A-E are presented as mean ± s.d. of triplicate experiments.

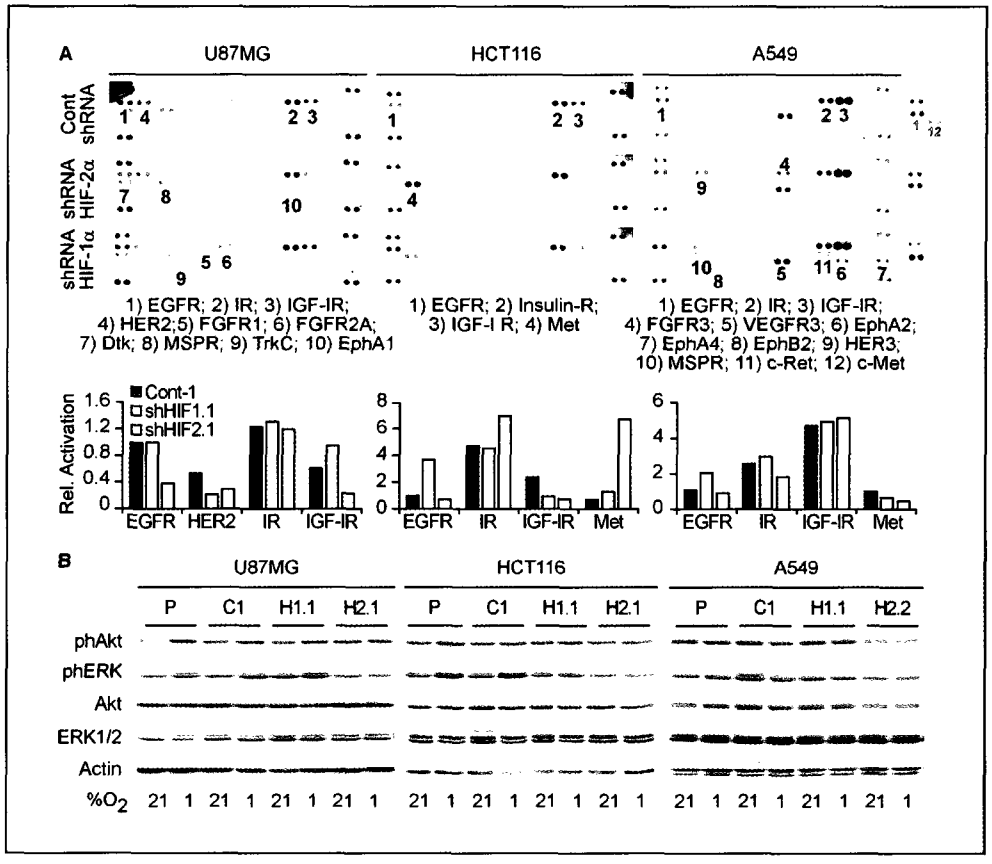

A
U87MG
HCT116
A549
Control shRNA
shRNA HIF-2α
shRNA HIF-1α
Ki-67
H&E
125 μM
% Ki-67+
Parental
Cont-1
shHIF1.1
shHIF2.1
B
% Growth Inhibition
C
D
Cell Number (x10^6)
Time (days)
Cells (x10^6)
E
Organ
Cell Line
Mutation(s)
% Growth Inhibition
Brain U87MG PTEN, CDKN2A
Brain U118MG PTEN, CDKN2A, TP53
Breast MDA-MB-231 KRAS, BRAF, TP53
Colon HCT116 KRAS
Kidney 786-0 VHL
Lung A549 KRAS
Lung H1975 EGFR L858R, T790M
Ovary OVCA429 N/A
Ovary SKOV-3 APC, TP53
Prostate PC3 PTEN, TP53

cancers ultimately converge at a growth axis driven by HIF-2α, challenging the notion that cancers utilize independent pathways to gain a selective proliferative advantage.

3.2.4. Silencing HIF-2α prevents the activation of RTK signaling pathways.

The ability of serum to rescue the growth of cells lacking HIF-2α suggests that their inability to proliferate autonomously is a consequence of defective RTK signaling as opposed to cell cycle progression. Whereas EGFR signaling is pivotal in the pathogenesis of *VHL*$^{-/-}$ RCC, there is evidence that multiple, redundant RTKs are co-activated in other cancers to support tumorigenic phenotypes (de Paulsen et al., 2001; Smith et al., 2005; Stommel et al., 2007). We thus assessed the global effect of silencing HIF-2α on endogenous RTK activation in serum-starved cells using an antibody array. Surprisingly few RTKs were detected with the EGF, insulin-like growth factor-1 (IGF-IR) and insulin receptors (IR) being the most highly and consistently activated across the cell lines (**Fig. 19A**). Significantly, phosphorylation of the EGFR and IGF-IR was HIF-2α-dependent in the cell lines, with the exception of the IGF-IR in A549 cells (**Fig. 19A**). While decreased Met and increased IGF-IR activation was observed in the latter cell line in the absence of HIF-2α the reverse was true in HCT116 cells, indicating cell-type specific RTK regulation. Interestingly, silencing HIF-1α resulted in increased EGFR activation and/or expression in all of the cell lines (**Fig. 19A, 20A**) and enhanced A549 cell growth *in vitro* and *in vivo* (**Fig. 17A, 18B**), suggesting that a negative pressure had been lifted. Given that the HIF-α isoforms display reciprocally suppressive interactions in *VHL*$^{-/-}$ RCC, it is within reason to speculate that these positive effects on RTK signaling and cell growth might be due to the de-repression of HIF-2α function

Figure 19. Silencing HIF-2α prevents the activation of RTK signaling pathways. **(A)** Relative levels of RTK phosphorylation in serum-starved human cancer cell lines stably expressing control shRNA or shRNA targeting the HIF-α subunits, after incubation in hypoxia for 24 hrs, as assessed using an antibody array. Higher exposure (far right) allows for visualization of Met activation in A549 stable cell lines. Numbers identify the specific RTK represented by duplicate spots. Spots in upper and lower corners are phosphorylation (positive) controls. Bar graphs depict the relative differences in activation of the most highly expressed RTKs in each cell line. Data are presented as the mean of duplicate samples. **(B)** Western blot analysis of the phosphorylation status of ERK1/2 and Akt (phERK and phAkt) in cells described in A compared with those incubated in normoxia for 24 hrs. Actin served as a loading control.


A
U87MG
HCT116
A549
Cont shRNA
shRNA HIF-2α
shRNA HIF-1α
1) EGFR; 2) IR; 3) IGF-IR; 4) HER2;5) FGFR1; 6) FGFR2A; 7) Dtk; 8) MSPR; 9) TrkC; 10) EphA1
1) EGFR; 2) Insulin-R; 3) IGF-I R; 4) Met
1) EGFR; 2) IR; 3) IGF-IR; 4) FGFR3; 5) VEGFR3; 6) EphA2; 7) EphA4; 8) EphB2; 9) HER3; 10) MSPR; 11) c-Ret; 12) c-Met
Rel. Activation
Cont-1
shHIF1.1
shHIF2.1
EGFR
HER2
IR
IGF-IR
Met
B
U87MG
HCT116
A549
P
C1
H1.1
H2.1
H2.2
phAkt
phERK
Akt
ERK1/2
Actin
%O2
21 1 21 1 21 1 21 1

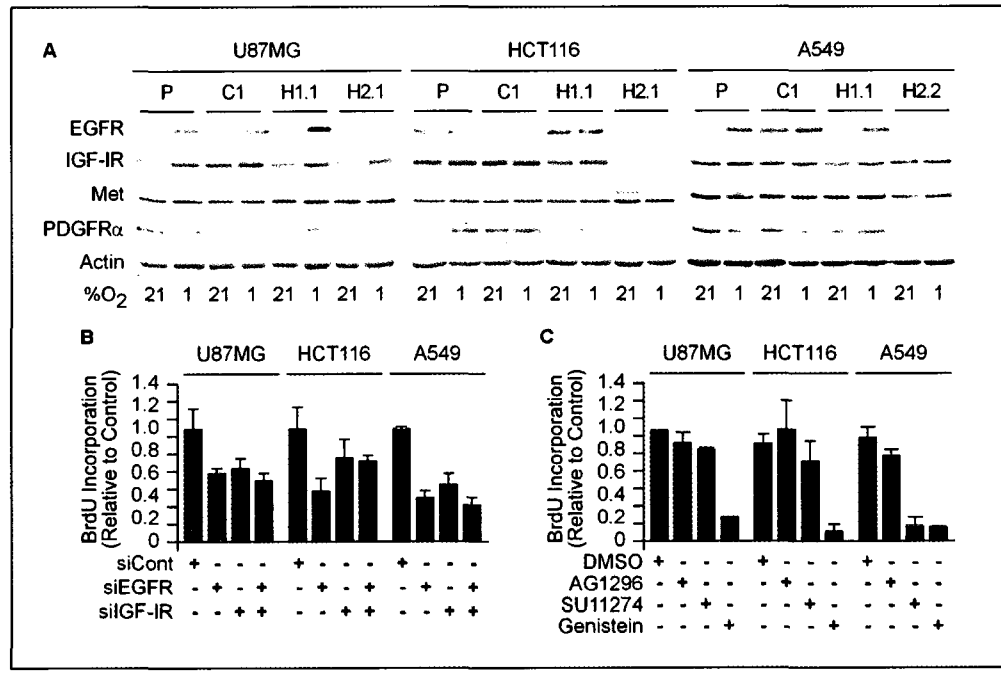
(Raval et al., 2005). Finally we examined the effect of silencing HIF-2α on the associated downstream signaling molecules. Predictably, cell lines lacking HIF-2α exhibited reduced ERK1/2 and/or Akt activation compared to the controls in normoxia and hypoxia (**Fig. 19B**). Altogether, these data demonstrate that HIF-2α is required for the efficient activation of the major RTK growth signaling pathways, albeit in a cell type-specific manner.

3.2.5. HIF-2α regulates the expression of RTKs required for growth autonomy.

The data presented here, and a recent report from another group, have revealed a role for HIF-2α in the amplification of EGFR signaling through its translational upregulation and stabilization (Wang et al., 2009b) (**Fig. 13**). We thus examined the effect of silencing HIF-2α on total RTK protein levels in order to establish whether their deactivation could be attributed to a decrease in receptor abundance. A reduction in total EGFR, IGF-IR and platelet-derived growth factor receptor-alpha (PDGFRα) levels was observed in normoxia and hypoxia, suggesting that HIF-2α can also regulate the expression and turnover of other RTKs (**Fig. 20A**). With the exception of the EGFR, and the IGF-IR in U87MG cells, the remaining RTKs did not appear to be hypoxia-inducible implying again that maximal HIF-2α activity can be achieved in normoxic conditions. Only a slight decrease in Met levels was noted in A549 cells, suggesting that HIF-2α may affect its activation (**Fig. 19A**) at the level of the ligand rather than the receptor (**Fig. 20A**).

The implications of blocking specific RTKs on cell growth autonomy were then explored using commercially-available siRNAs and inhibitors. We found that the EGFR and IGF-IR were both required for all three cell types to meet their full proliferative potential (**Fig.**

Figure 20. HIF-2α mediates the expression of RTKs involved in cell growth. (A) Western blot analysis of total cellular levels of various RTKs in serum-starved human cancer cell lines stably expressing control shRNA or shRNA targeted against the HIF-α subunits, after incubation in hypoxia for 24 hrs. Actin served as a loading control. **(B)** BrdU incorporation in serum-starved cells expressing siRNA (25 nM) against the EGFR, IGF-IR, or scrambled control siRNA for 72 hrs in normoxia. **(C)** BrdU incorporation in serum-starved human cancer cells treated with the platelet-derived growth factor receptor (PDGFR)-specific inhibitor AG1296 (10 μM), the c-Met-specific inhibitor SU11274 (1 μM), the general tyrosine kinase inhibitor Genistein (10 μM), or DMSO, as a vehicle control, for 72 hrs in normoxia. Data in B and C are presented as mean ± s.d. of triplicate experiments.


A
U87MG
HCT116
A549
P
C1
H1.1
H2.1
H2.2
EGFR
IGF-IR
Met
PDGFRα
Actin
%O2
21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1
B
U87MG
HCT116
A549
BrdU Incorporation (Relative to Control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
siCont
siEGFR
siIGF-IR
C
U87MG
HCT116
A549
BrdU Incorporation (Relative to Control)
DMSO
AG1296
SU11274
Genistein

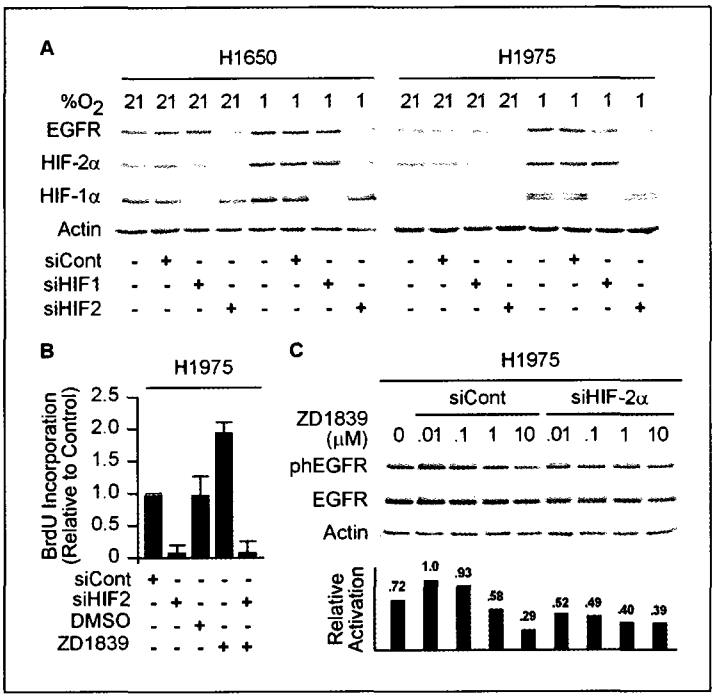
20B), while others were dispensable for cell growth (**Fig. 20C**). Interestingly, inhibition of Met signaling abrogated A549 cell growth; the only cell line in which silencing HIF-2α caused a decrease in total and activated forms of the receptor (**Fig. 19A, 20A,C**). These data argue that HIF-2α is competent to upregulate and/or activate discrete RTKs, instrumental to growth autonomy, in a cell-type specific manner.

3.2.6. HIF-2α regulates the expression of mutant forms of EGFR.

Since HIF-2α upregulates wild-type EGFR levels, we hypothesized that it would have a similar effect on mutant EGFR and could serve as an indirect method of targeting those that have acquired drug resistance mutations, a frequent occurrence and obstacle in the treatment of NSCLC (Kobayashi et al., 2005; Pao et al., 2005; Herbst et al., 2008). Mutant EGFR protein levels were indeed induced under hypoxic conditions in the H1650 (Δ exon 19; drug-sensitive mutant) and H1975 (L858R, T790M; drug-insensitive mutant) NSCLC cell lines (**Fig. 21A**), as was previously observed in several other cancer cell lines (**Fig. 10B, 20A**) (Pao et al., 2005). Consistently, transient silencing of HIF-2α, but not HIF-1α, constrained the normoxic expression and hypoxia inducibility of the mutant receptors (**Fig. 21A**).

We then examined the effect of inhibiting HIF-2α-mediated mutant EGFR upregulation on the normoxic growth of drug-resistant NSCLC cells. Silencing HIF-2α prevented the autonomous proliferation of H1975 cells both in the presence and absence of the EGFR tyrosine kinase inhibitor ZD1839 (**Fig. 21B**). Curiously, treatment with ZD1839 was not just ineffective in blocking the growth of these drug-resistant cells but actually enhanced it, indicating that its administration may prove to be entirely counterproductive from a

Figure 21. HIF-2α regulates the expression of mutant forms of EGFR. **(A)** Western blot analysis of mutant EGFR levels in serum-starved H1650 and H1975 non-small cell lung carcinoma cells transfected with siRNA targeted against HIF-1α or HIF-2α (50 nM) for 24 hrs, following incubation in normoxia or hypoxia for an additional 24 hrs. **(B)** BrdU incorporation in normoxic H1975 cells, as described in A, in the presence and absence of ZD1839 (1 μM) or DMSO as a vehicle control. Data are presented as mean ± s.d. of triplicate experiments. **(C)** Western blot analysis of phEGFR levels in hypoxic H1975 cells, as described in A, treated with increasing concentrations of ZD1839 for 3 hrs prior to stimulation with recombinant TGFα (20 ng/mL; 15 min). Bar graphs depict the relative levels of phosphorylated EGFR.


A
H1650
H1975
%O2
21 21 21 21 1 1 1 1 21 21 21 21 1 1 1 1
EGFR
HIF-2α
HIF-1α
Actin
siCont
siHIF1
siHIF2
B
H1975
BrdU Incorporation (Relative to Control)
2.5
2.0
1.5
1.0
0.5
0
siCont
siHIF2
DMSO
ZD1839
C
H1975
siCont
siHIF-2α
ZD1839 (μM)
0 .01 .1 1 10 .01 .1 1 10
phEGFR
EGFR
Actin
Relative Activation
.72
1.0
.93
.58
.29
.52
.49
.40
.39

therapeutic standpoint. Finally, we investigated the effect of inhibiting HIF-2α activity, and thereby reducing total EGFR levels, on receptor responsiveness to ZD1839. Somewhat surprisingly, while silencing HIF-2α resulted in a greater over-all decrease in EGFR phosphorylation the receptor was less sensitive to the actions of ZD1839 compared to the control cells (**Fig. 21C**), an effect which was not observed in *VHL*$^{+}$ RCC cells (**Fig. 15**) and may be peculiar to this EGFR mutant. This suggests that HIF-2α monotherapies may be the appropriate therapeutic route in the case of drug-resistant NSCLC and that no additional clinical benefit would be expected from combination therapies with RTK inhibitors. Together, these data suggest that HIF-2α drives the persistent proliferation of human cancers through the upregulation of various wild-type and mutant RTKs and endorse it as a therapeutic target to circumvent receptor resistance and redundancy issues.

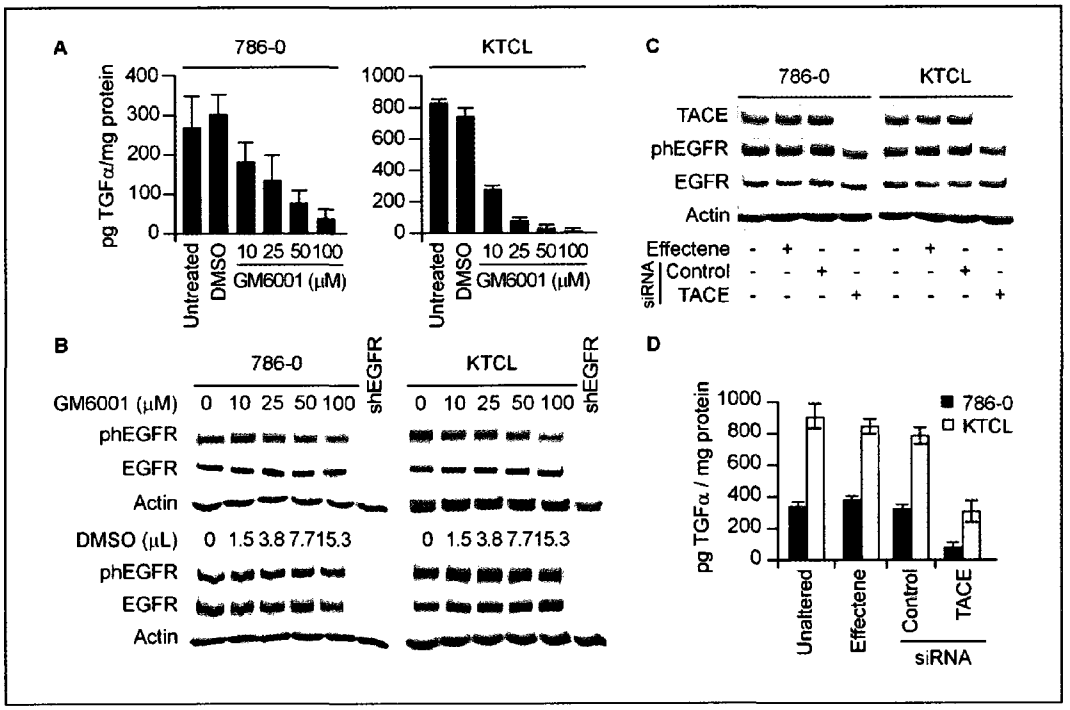
3.3. Targeting participants in the HIF-2α axis prevents RCC tumorigenesis

3.3.1. TGFα cleavage is required for efficient EGFR activation in RCC cells.

Self-sufficiency in growth signaling requires activation of an autonomous program that constitutively drives cell proliferation (Hanahan and Weinberg, 2000). A model that precisely exhibits characteristics of acquired growth autonomy by production of soluble growth factors is *VHL*$^{-/-}$ RCC (Pause et al., 1998; de Paulsen et al., 2001). Our previous work demonstrated that *VHL*$^{-/-}$ RCC, as a consequence of HIF-2α activation, engage in a classical TGFα/EGFR autocrine growth circuit required for RCC tumorigenesis (de Paulsen et al., 2001; Gunaratnam et al., 2003; Smith et al., 2005). TGFα is synthesized as a transmembrane precursor protein (pro-TGFα) that undergoes TACE-mediated

proteolytic cleavage to release the mature and presumably active ligand (Peschon et al., 1998; Sunnarborg et al., 2002). TACE is also involved in the ectodomain shedding of several membrane-bound ligands and cytokines and is a favored drug target in inflammatory disease conditions such as rheumatoid arthritis (Kenny, 2007). Given the number of potent TACE inhibitors currently in development and its prospective role in TGFα processing, we decided to examine its role in *VHL*$^{-/-}$ RCC; a human cancer model where participant molecules in this pathway, including TGFα, EGFR and TACE, are produced endogenously. To determine whether TGFα cleavage is a prerequisite for EGFR activation, two independent human sporadic *VHL*$^{-/-}$ RCC cell lines derived from primary renal tumors, 786-0 and KTCL, were treated for 48 hours with a general sheddase/matrix metalloprotease inhibitor, GM6001. The amount of TGFα secreted into the media by both *VHL*$^{-/-}$ RCC cell lines (**Fig. 22A**), and corresponding phosphorylation of the EGFR (**Fig. 22B**), decreased in a dose-dependent manner without affecting total EGFR protein levels (**Fig. 22B**). To confirm that the observed effect of the drug on the shedding of soluble TGFα and EGFR activation was not the result of the inhibition of other matrix metalloproteases or alternative growth pathways, TACE was transiently silenced using a commercially-available siRNA. TACE knockdown was confirmed by western blot analysis of cellular levels of the full-length TACE precursor protein (**Fig. 22C**). Predictably, the transient silencing of TACE inhibited TGFα ectodomain cleavage (**Fig. 22D**) and consequently in reduced EGFR phosphorylation (**Fig. 22C**). Notably, the decrease in receptor activation following the transient silencing of TACE only became evident after 96 hours, as additional time was required to observe these downstream

Figure 22. TGFα cleavage is required for efficient EGFR activation in RCC cells. **(A)** ELISA analysis of soluble TGFα levels in serum-starved *VHL*$^{-/-}$ RCC 786-0 and KTCL cell line conditioned media following a 48 hr treatment with the general matrix metalloprotease inhibitor, GM6001 (10-100 μM), or DMSO as a vehicle control. The amount of TGFα shed into the media was normalized to the total amount of protein in cell lysates. **(B)** Western blot analysis of total EGFR and phEGFR levels in cell lysates as described in A. Cell lines expressing shRNA targeting the EGFR (shEGFR) served as negative controls. Actin served as a loading control. **(C)** Western blot analysis of TACE, phEGFR, and total EGFR levels in 786-0 and KTCL cell lines transiently transfected with siRNA targeted against *TACE* mRNA (50 nM), scrambled control siRNA (Control) or effectene transfection reagent for 96 hrs. **(D)** ELISA analysis of TGFα levels in conditioned media of cell lines described in C. Data are presented as mean ± s.d. of at least three independent experiments performed in triplicate.

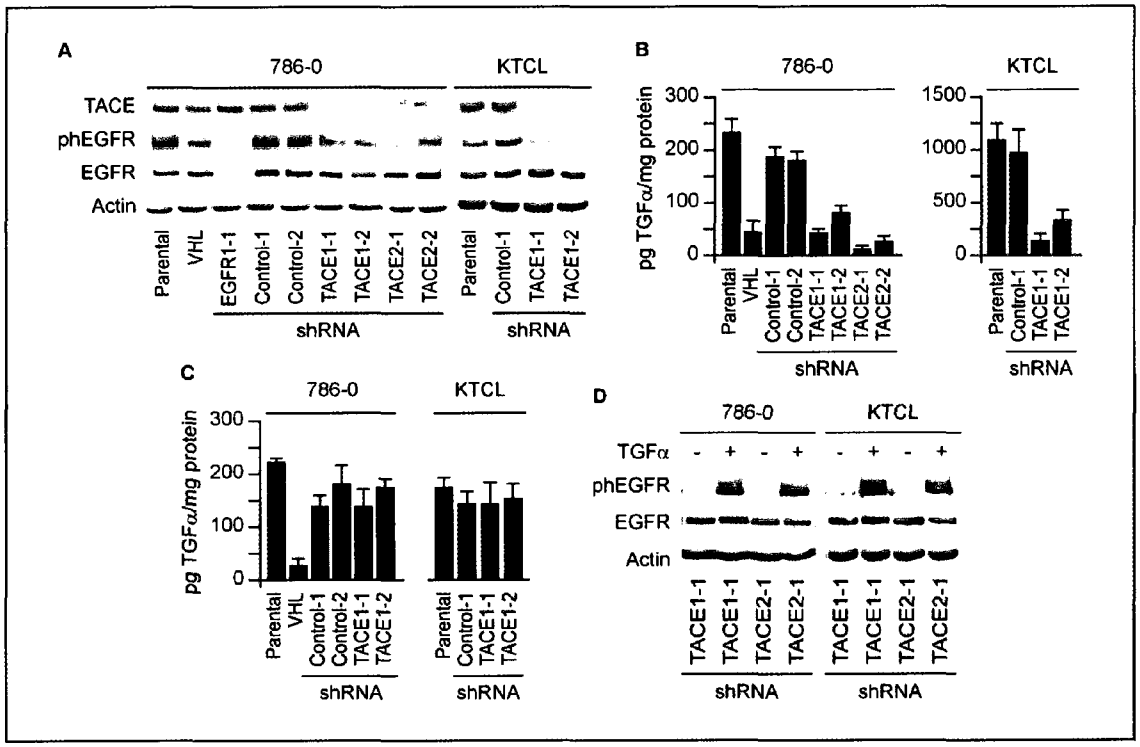

A
786-0
KTCL
pg TGFα/mg protein
400
300
200
100
0
1000
800
600
400
200
0
Untreated
DMSO
10 25 50 100
GM6001 (μM)
B
786-0
KTCL
shEGFR
GM6001 (μM) 0 10 25 50 100
phEGFR
EGFR
Actin
DMSO (μL) 0 1.5 3.8 7.7 15.3
C
786-0
KTCL
TACE
phEGFR
EGFR
Actin
Effectene
siRNA Control
TACE
D
pg TGFα / mg protein
786-0
KTCL
Unaltered
Effectene
Control
TACE
siRNA

effects. Nonetheless, these data indicate that TGFα ectodomain shedding is necessary for maximal EGFR activation and that this process is most likely mediated by TACE.

3.3.2. Silencing TACE inhibits TGFα shedding and EGFR activation.

To further examine the specific role of TACE in the establishment of the TGFα/EGFR autocrine signaling loop, we generated *VHL*$^{-/-}$ RCC cell lines with stably inactivated TACE using a shRNA strategy. Two independent shRNA sequences, including one which was previously described, targeted against *TACE* mRNA (shRNA TACE1 and TACE2) were used to silence its protein expression in the 786-0 and KTCL cell lines (Shao et al., 2003). Cell lines stably expressing either of the shRNA sequences exhibited a significant decrease in TACE protein levels when compared to parental cells and cells stably expressing scrambled control shRNA (**Fig. 23A**). Furthermore, silencing TACE inhibited the secretion of TGFα by 786-0 and KTCL cells compared with control cell lines suggesting that TACE is the major TGFα ectodomain sheddase in *VHL*$^{-/-}$ RCC (**Fig. 23B**). The observed decrease in secreted TGFα was not, however, accompanied by a measurable increase in cellular TGFα levels (**Fig. 23C**). This may be due to the fact that relatively little TGFα is released from the cell, such that any resulting increases in cell-associated TGFα would be undetectable at steady-state or alternatively it may reflect the disruption of a positive feedback loop that maintains cellular TGFα levels. Importantly, there was a concomitant reduction in phosphorylated EGFR levels, but not total EGFR levels, in shRNA-expressing cells (**Fig. 23A**). Also, addition of exogenous TGFα rescued EGFR phosphorylation indicating that silencing TACE did not affect the ability of the receptor to become activated in response to ligand stimulation (**Fig. 23D**). These

Figure 23. Silencing TACE inhibits TGFα shedding and EGFR activation. (A) Western blot analysis of TACE, phEGFR, and total EGFR levels in serum-starved 786-0 and KTCL cells stably transfected to express one of two shRNA sequences directed against *TACE* mRNA (TACE1 and TACE2) or a scrambled shRNA as a control. Cell lines re-expressing wild-type VHL (VHL) or shRNA directed against *EGFR* mRNA (EGFR1-1) were used as negative controls. Actin westerns served as loading controls. ELISA analysis of TGFα levels in **(B)** conditioned media and **(C)** cellular lysates of cell lines described in A following a 48 hr incubation period. Data are presented as mean ± s.d. of at least three independent experiments performed in triplicate. **(D)** Western blot analysis of phEGFR and total EGFR levels in cell lines described in A following stimulation with recombinant TGFα (20 ng/mL) for 15 min.


A
786-0
KTCL
TACE
phEGFR
EGFR
Actin
Parental
VHL
EGFR1-1
Control-1
Control-2
TACE1-1
TACE1-2
TACE2-1
TACE2-2
Parental
Control-1
TACE1-1
TACE1-2
shRNA
shRNA
B
786-0
KTCL
pg TGFα/mg protein
300
200
100
0
1500
1000
500
0
Parental
VHL
Control-1
Control-2
TACE1-1
TACE1-2
TACE2-1
TACE2-2
Parental
Control-1
TACE1-1
TACE1-2
shRNA
shRNA
C
786-0
KTCL
pg TGFα/mg protein
300
200
100
0
Parental
VHL
Control-1
Control-2
TACE1-1
TACE1-2
Parental
Control-1
TACE1-1
TACE1-2
shRNA
shRNA
D
786-0
KTCL
TGFα
- + - + - + - +
phEGFR
EGFR
Actin
TACE1-1
TACE1-1
TACE2-1
TACE2-1
TACE1-1
TACE1-1
TACE2-1
TACE2-1
shRNA
shRNA

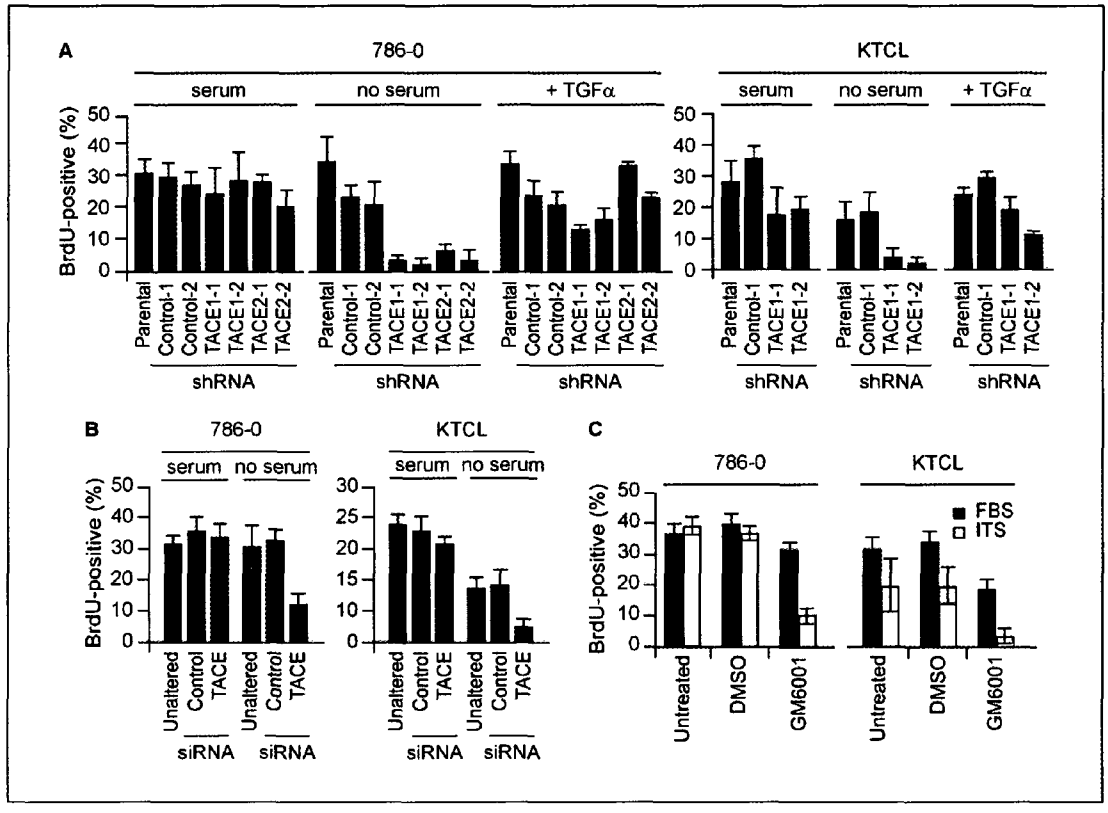
data demonstrate that TACE-mediated TGFα ectodomain shedding is a prerequisite for efficient EGFR activation in cells that produce endogenous ligand and receptor.

3.3.3. Inhibition of TACE-mediated TGFα shedding prevents RCC cell growth.

Based on the reduction in EGFR phosphorylation obtained upon inhibition of TACE activity, we decided to examine the effect of preventing TGFα cleavage on the ability of *VHL*$^{-/-}$ RCC to engage in autonomous proliferation upon serum withdrawal (Pause et al., 1998). The stable knockdown of TACE was sufficient to abrogate the ability of both 786-0 and KTCL cells to incorporate BrdU in the absence of serum growth factors (**Fig. 24A**). The degree to which silencing TACE repressed the autonomous proliferation of the *VHL*$^{-/-}$ RCC cell lines was comparable to the growth inhibition achieved upon EGFR silencing, suggesting that they both operate in the same growth pathway (Smith et al., 2005). Importantly, addition of exogenous TGFα to quiescent 786-0 and WT7 cells activated the EGFR (**Fig. 23D**) and re-initiated cell proliferation verifying that the loss of growth autonomy observed in shRNA-expressing cells was not due to a dominant effect on the cell cycle (**Fig. 24A**).

The transient silencing of TACE (**Fig. 24B**) and addition of GM6001 (**Fig. 24C**) similarly prevented 786-0 and KTCL cell growth in serum-free media, albeit to a lesser extent, indicating that the results obtained with stable silencing of TACE are not a consequence of fortuitous effects of shRNA expression or single cell clonal effects. Collectively, these data suggest that TACE-mediated processing of pro-TGFα into its soluble form is essential for EGFR phosphorylation and critical for the establishment of the TGFα/EGFR autonomous growth circuit in *VHL*$^{-/-}$ RCC cells.

Figure 24. Inhibition of TACE-mediated TGFα shedding prevents RCC cell growth. **(A)** BrdU incorporation in 786-0 and KTCL cell lines stably expressing shRNA targeted against *TACE* mRNA or control shRNA in the presence or absence of serum growth factors for 72 hrs. Cell growth in serum-free media was rescued by addition of recombinant TGFα (+TGFα; 20ng/mL) for an additional 24 hrs. **(B)** BrdU incorporation in 786-0 and KTCL cells transiently transfected to express siRNA targeted against *TACE* mRNA (50 nM), scrambled siRNA (Control) or effectene transfection reagent, in the presence or absence of serum for 72 hrs. **(C)** BrdU incorporation in cells following treatment with GM6001 (100 μM), or DMSO as a vehicle control, in the presence (FBS) or absence (ITS) of serum for 72 hrs. Data are presented as mean ± s.d. of at least three independent experiments performed in triplicate.


A
786-0
KTCL
serum
no serum
+ TGFα
BrdU-positive (%)
Parental
Control-1
Control-2
TACE1-1
TACE1-2
TACE2-1
TACE2-2
shRNA
B
786-0
KTCL
serum
no serum
BrdU-positive (%)
Unaltered
Control
TACE
siRNA
C
786-0
KTCL
FBS
ITS
BrdU-positive (%)
Untreated
DMSO
GM6001

3.3.4. TACE does not affect VHL-associated pro-angiogenic phenotypes.

The constitutive expression of pro-angiogenic HIF target genes and the inability of VHL to bind fibronectin and form a proper extracellular matrix are two key events believed to promote and maintain *VHL*$^{-/-}$ RCC tumorigenesis (Iliopoulos et al., 1996; Ohh et al., 1998; Stickle et al., 2004; Kurban et al., 2006). To assess whether TACE has functions unrelated to TGFα processing that may affect the well-established pathways involved in *VHL*$^{-/-}$ RCC tumorigenesis, we examined the effect of knocking down TACE on other defects associated with loss of VHL function. Silencing TACE did not alter HIF-2α protein expression (**Fig. 25A**) nor did it affect its induction of hypoxia-inducible mRNAs such as *GLUT1*, *VEGF* and *TGFA* (**Fig. 25B**). Additionally, silencing TACE did not restore the ability of RCC cells to deposit extracellular fibronectin matrix (**Fig. 25C**), a process that can be corrected by reintroduction of VHL (Ohh et al., 1998). Therefore, TACE function appears to be unrelated to some of the major pro-angiogenic traits associated with VHL loss of function and thus far has only affected *VHL*$^{-/-}$ RCC autocrine growth signaling.

3.3.5. Inhibition of TACE abolishes multiple acquired tumor capabilities *in vitro*.

While self-sufficiency in growth signaling is likely to be a critical initial step in cellular transformation, cancer cells must also acquire several other attributes for malignancy (Hanahan and Weinberg, 2000). First we examined the ability of *VHL*$^{-/-}$ 786-0 RCC cells stably expressing shRNA against TACE to proliferate in an avascular *in vitro* tumor spheroid assay used as a measure of the tumorigenic potential of cancer cells (Sutherland, 1988). In the absence of TACE the cells failed to form the highly dense spheroids characteristic of the parental cell line, but were not as sparse and loosely arranged as is

Figure 25. TACE does not affect pro-angiogenic VHL-loss phenotypes. (A) Western blot analysis of HIF-2α protein levels in 786-0 and KTCL cells stably expressing shRNA against TACE or scrambled control shRNA. 786-0 cells re-expressing wild-type VHL served as a negative control for HIF expression. Actin served as a loading control. **(B)** RT-PCR analysis of HIF target gene (*GLUT1*, *VEGF* and *TGFA*) expression in cell lines described in A. **(C)** Immunofluorescence detection of fibronectin matrix deposition in cells visualized at a magnification of 400x. Arrows indicate fibronectin deposits. Inset are corresponding images of Hoechst counter-stained nuclei.

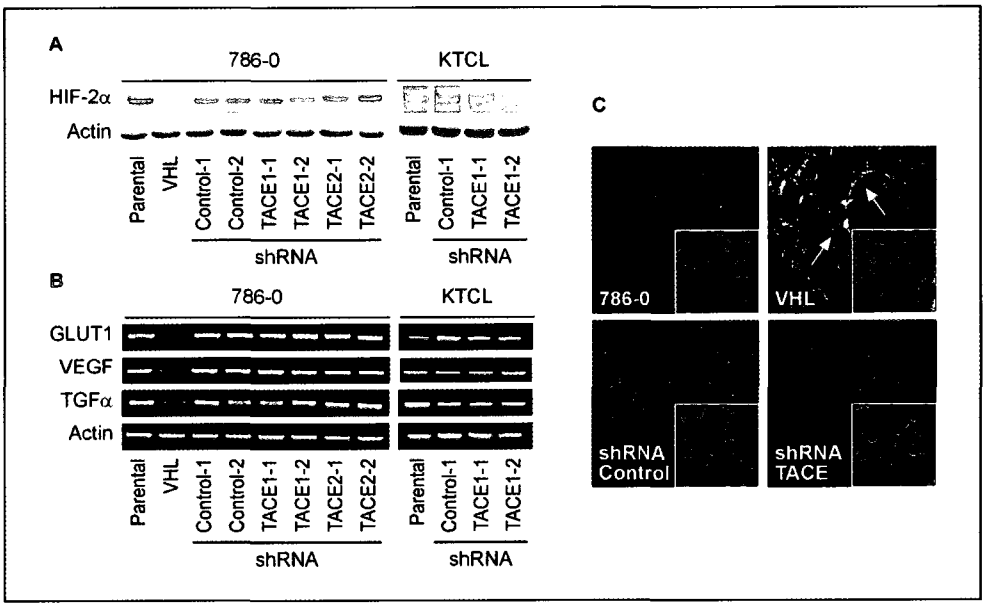
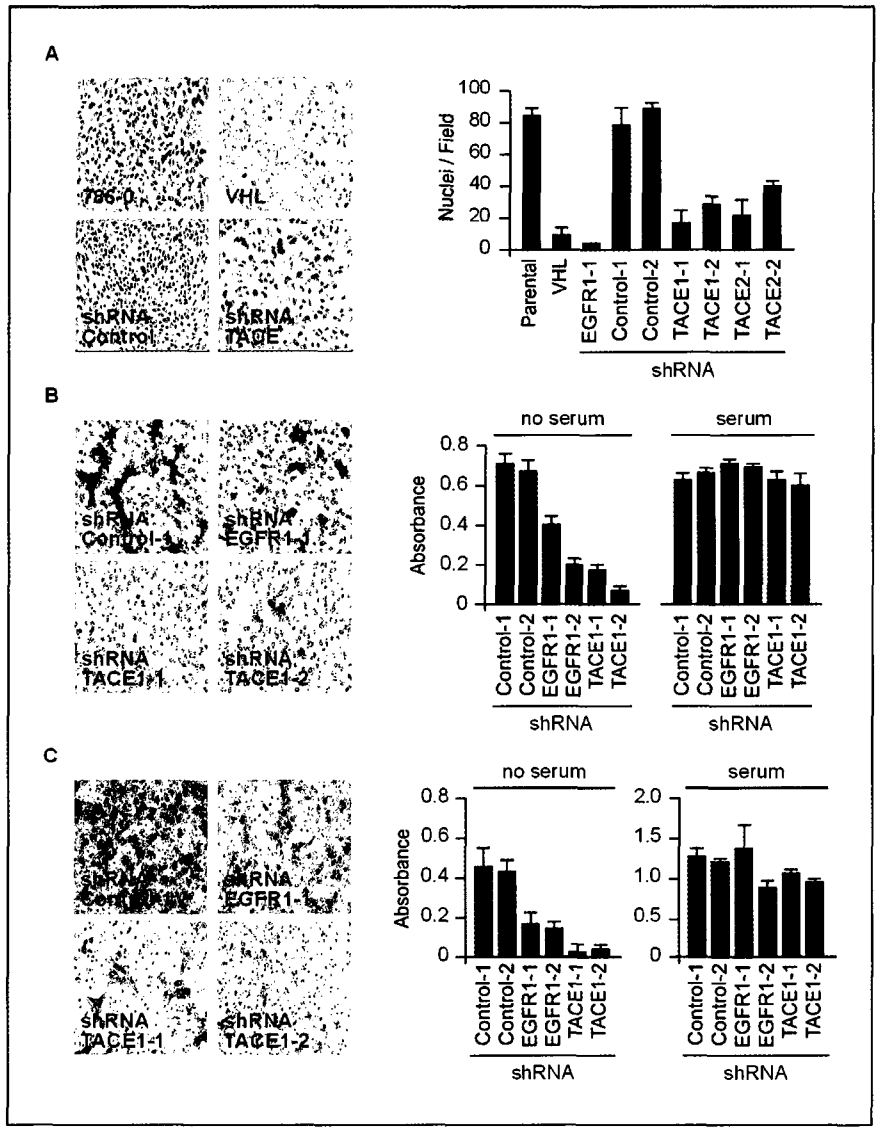

A
786-0
KTCL
HIF-2α
Actin
Parental
VHL
Control-1
Control-2
TACE1-1
TACE1-2
TACE2-1
TACE2-2
shRNA
B
GLUT1
VEGF
TGFα
C
786-0
VHL
shRNA Control
shRNA TACE

Figure 26. TACE inhibition abolishes multiple acquired tumor capabilities *in vitro*. **(A)** The ability of 786-0 cells stably expressing shRNA targeted against *EGFR* or *TACE* mRNA, or scrambled control shRNA, to form three-dimensional tumor spheroids *in vitro* was assessed. Histology was visualized at a magnification of 400x. Bar graph on right indicates quantitative analysis of spheroid density reported as nuclei/field. **(B)** The *in vitro* migration of cells through a Boyden chamber over a period of 16 hrs, in the presence and absence of chemoattractant (serum). **(C)** The *in vitro* invasion of cells into matrigel, over a period of 48 hrs, in the presence or absence of serum. Bar graphs on right indicate colorimetric quantitative analysis of migration and invasion as measured by absorbance readings at 574 nm. Representative images depict migration and invasion in a serum-free environment as visualized at a magnification of 200x. Data are presented as mean ± s.d. of triplicate experiments.

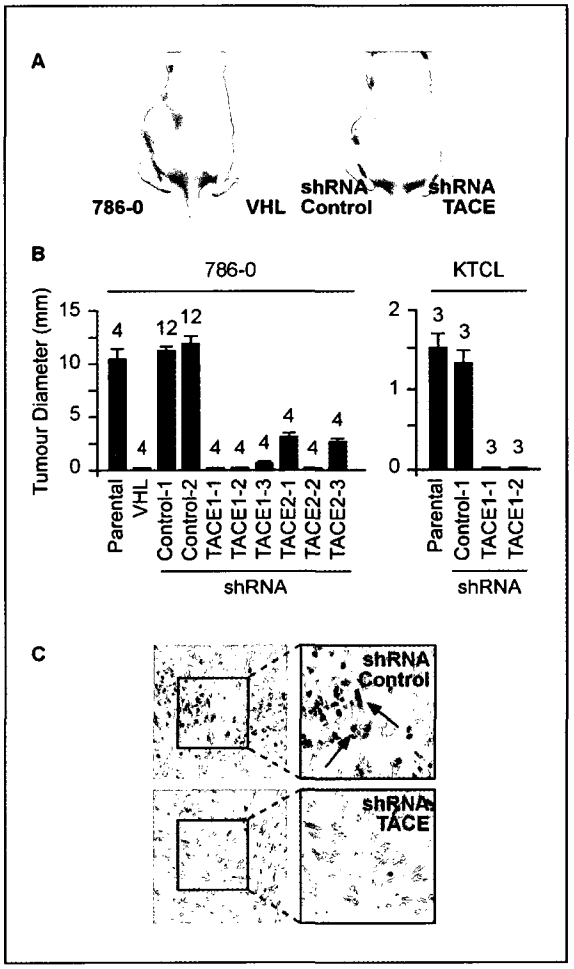

A
786-0
VHL
shRNA Control
shRNA TACE
Nuclei / Field
100
80
60
40
20
0
Parental
VHL
EGFR1-1
Control-1
Control-2
TACE1-1
TACE1-2
TACE2-1
TACE2-2
shRNA
B
shRNA Control-1
shRNA EGFR1-1
shRNA TACE1-1
shRNA TACE1-2
no serum
serum
Absorbance
0.8
0.6
0.4
0.2
0
Control-1
Control-2
EGFR1-1
EGFR1-2
TACE1-1
TACE1-2
shRNA
C
shRNA EGFR1-1
shRNA TACE1-1
shRNA TACE1-2
no serum
serum
Absorbance
0.8
0.6
0.4
0.2
0
2.0
1.5
1.0
0.5
0
Control-1
Control-2
EGFR1-1
EGFR1-2
TACE1-1
TACE1-2
shRNA

observed upon reintroduction of VHL or silencing of the EGFR (Lieubeau-Teillet et al., 1998; Smith et al., 2005) (**Fig. 26A**). This result may reflect the incomplete inhibition of EGFR signaling in cells expressing TACE shRNA or alternatively that EGFR has TGF-α-independent functions involved in anchorage-independent growth and the evasion of anoikis in these conditions. We next assessed the ability of the cells to migrate and invade basement membrane *in vitro*. The migratory ability of 786-0 cells expressing shRNA against TACE or the EGFR was severely compromised compared with parental and control cells in the absence of serum, which acts as a chemoattractant (**Fig. 26B**). Predictably, these cells likewise failed to invade *in vitro* reconstituted basement membrane, consisting mostly of laminin, collagen IV and heparin sulfate proteoglycans (**Fig. 26C**). Interestingly, silencing TACE had a greater negative effect on the cells migratory and invasive capacities compared to silencing the EGFR, suggesting that other TACE substrates influence these malignant phenotypes. Taken together these results demonstrate that TACE mediates the acquisition of multiple tumorigenic capabilities, required for overt malignancy, through EGFR-dependent and -independent mechanisms.

3.3.6. Silencing TACE is sufficient to suppress RCC tumorigenesis *in vivo*.

Finally, we examined the consequence of silencing TACE on $VHL^{-/-}$ RCC tumor formation *in vivo*. Parental and control $VHL^{-/-}$ 786-0 cells formed large subcutaneous tumors detectable 4-5 weeks post-injection (**Fig. 27A-B**). Reintroduction of a functional copy of VHL was sufficient to prevent tumor formation of $VHL^{-/-}$ RCC cells in nude mice, as expected (Iliopoulos et al., 1995). Somewhat surprisingly, cells stably expressing either one of the two shRNA sequences targeted against TACE failed to form tumors in the xenograft assay (n=11), unveiling a rate-limiting role for TACE in cancer

Figure 27. Silencing TACE is sufficient to suppress *VHL*$^{-/-}$ RCC tumorigenesis. (A) Representative images of endpoint tumors in nude mice injected with parental 786-0, 786-0 stably re-expressing wild-type VHL (VHL), scrambled shRNA (shRNA Control) or shRNA targeted against *TACE* mRNA (shRNA TACE). **(B)** Endpoint tumor diameters of cells described in A as observed 7-9 weeks post-injection. The number of injections per cell line is specified above the corresponding bar. Data are presented as mean ± s.e.m. **(C)** Representative images of endpoint tumor sections derived from 786-0 cells described in A, visualized at a magnification of 400x. Arrows indicate leukocytes, which appear as darkly stained nuclei.


A
786-0
VHL
shRNA Control
shRNA TACE
B
786-0
KTCL
Tumour Diameter (mm)
15
10
5
0
2
1
0
4
4
12
12
4
4
4
4
4
4
3
3
3
3
Parental
VHL
Control-1
Control-2
TACE1-1
TACE1-2
TACE1-3
TACE2-1
TACE2-2
TACE2-3
shRNA
Parental
Control-1
TACE1-1
TACE1-2
shRNA
C
shRNA Control
shRNA TACE

development (**Fig. 27A-B**). Similar data were obtained with the *VHL*$^{-/-}$ KTCL cell line expressing shRNA directed against *TACE* mRNA, though the size of the control tumors was significantly smaller compared to those derived from 786-0 cells (n=6) (**Fig. 27B**). Prolonged incubation times of 16 weeks resulted in the formation of very small tumors in only two of the 786-0 stable cell lines expressing shRNA TACE2, possibly due to partial silencing or loss of shRNA expression (**Fig. 27B**). Nonetheless, these results indicate that abolishing TACE function efficiently abrogates the tumorigenic potential of highly malignant *VHL*$^{-/-}$ RCC cells. Interestingly, analysis of H&E-stained sections of these small tumors revealed that the typical leukocyte infiltration, observed as darkly stained nuclei, in parental and scrambled control shRNA-expressing 786-0 tumors was discernibly less prominent when TACE was silenced (**Fig. 27C**). This effect is likely due to the involvement of TACE in the cleavage of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα) (Black et al., 1997). Thus in addition to suppressing various pro-tumorigenic traits, silencing TACE may also reduce tumor inflammation, a negative prognostic factor in human cancers (Ikemoto et al., 2003; Vakkila and Lotze, 2004). These data identify TACE inhibition as an effective means of blocking TGFα/EGFR autocrine signaling and the *in vivo* tumorigenesis of *VHL*$^{-/-}$ RCC cell lines, endorsing the clinical application of orally active anti-TACE drugs in the treatment of renal and perhaps other EGFR-dependent human cancers.

DISCUSSION

4. DISCUSSION

4.1. Human cancers converge at the HIF-2α oncogenic axis.

The current model of tumorigenesis is a genetic one whereby cancers are instigated by growth promoting mutational events and are maintained by ensuant adaptive genotypic-phenotypic alterations in response to environmental and physical barriers (Nowell, 1976; Fearon and Vogelstein, 1990; Gatenby and Gillies, 2008). The primary event in cancer development is hence the establishment of an actively proliferating population of cells. This inappropriate initiation of cell division hinges upon the external growth signal-independence of cancer cells, a critical hallmark trait thought to be acquired through the disruption of any number of oncogene or tumor suppressor gene pathways (Hanahan and Weinberg, 2000). Such genetic perturbations can amplify cell-autonomous growth signaling in several ways which include the overproduction of endogenous growth factors and their cognate receptors, the constitutive activation of these receptors or their downstream signaling molecules and the inactivation of negative regulatory mechanisms that restrain growth signaling (Vogelstein and Kinzler, 2004). To date, hundreds of genetic alterations affecting cell signaling cascades have been identified and reports that multiple, redundant RTK pathways can be activated within a single cancer type append an additional layer of complexity to these growth networks (Stommel et al., 2007; C.G.A.R.N., 2008; Jones et al., 2008; Parsons et al., 2008). It is therefore widely accepted that cancers evolve in a highly variable, but parallel, manner to attain growth autonomy.

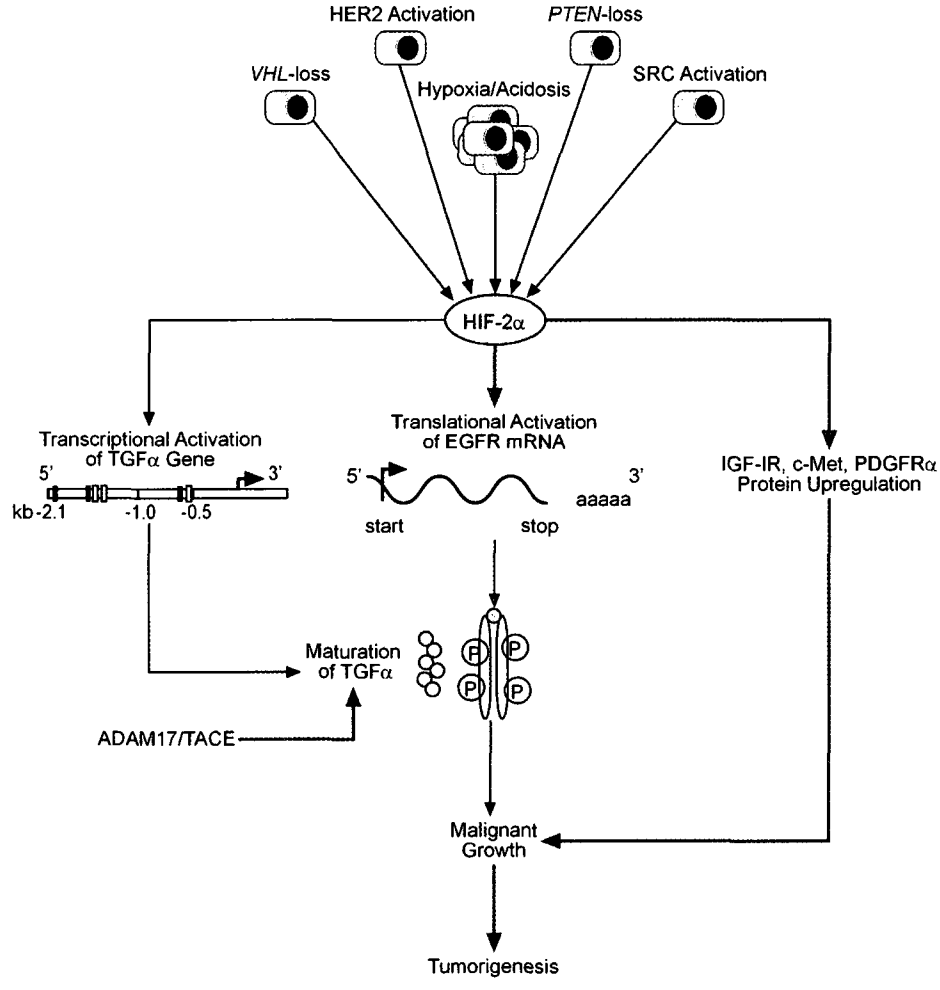
The contents of this thesis challenge the predominant stance that human cancers exploit mutually exclusive growth pathways subject to their mutational status. Our data suggest

that genetically diverse human cancers have evolved a common and obligatory growth stimulatory program, required for tumor formation, at the hub of which lies HIF-2α, an element of the oxygen sensing machinery (**Fig. 28**).

The oncogenic potential of HIF-2α was first revealed in pioneering studies of *VHL*$^{-/-}$ RCC development (Kondo et al., 2002; Mandriota et al., 2002; Maranchie et al., 2002; Kondo et al., 2003). Accounts of HIF-2α stabilization in solid tumors, as a consequence of mutational events and/or environmental cues, and its correlation with advanced disease led us to hypothesize that its oncogenic functions may reach beyond their well-described role in *VHL*$^{-/-}$ RCC tumorigenesis (Talks et al., 2000; Semenza, 2003; Qing and Simon, 2009). Indeed, we found that inhibition of HIF-2α suppresses the autonomous growth of a panel of cancer cell lines of varying tissues of origin (**Fig. 18**) and abolishes the *in vivo* tumorigenesis of highly malignant glioma, NSCLC, and colorectal carcinoma cells (**Fig. 16B**). In agreement with our findings, there is evidence that HIF-2α is preferentially induced in overt neuroblastoma and glioma stem cells, and at elevated oxygen tensions, conferring them with the aggressive phenotypes required for their tumorigenesis in xenograft and orthotopic cancer models (Holmquist-Mengelbier et al., 2006; Li et al., 2009). It has also been shown that the highly vascular hepatic tumors that arise in *VHL* knockout mice are likewise dependent on HIF-2α activation, highlighting its importance in an independent cancer model, albeit one that does not accurately depict the related human cancer syndrome (Maher and Kaelin, 1997; Rankin et al., 2008). More importantly, the conclusions of the aforementioned xenograft and transgenic studies are supported by analyses of glioblastoma, neuroblastoma, and renal cell carcinoma tumor specimens which clearly identify HIF-2α expression as a negative prognostic factor

Figure 28. Model of the HIF-2α oncogenic axis. We propose a model whereby HIF-2α, whose expression and activation is observed in the vast majority of solid tumors as a consequence of genetic alterations and/or environmental stressors, endows cells with the ability to engage in autonomous growth. The contents of this thesis (purple arrows) suggest that HIF-2α drives exogenous growth signal-independent cell proliferation and is, hence, required for the tumorigenesis of genetically diverse human cancers. This effect is likely mediated through the increased expression and activation of fundamental RTKs, including the EGFR, which we show here to be upregulated at the translational level by HIF-2α. This finding not only uncovers a novel role for HIF-2α in the control of protein synthesis, straying from its canonical role as a transcriptional regulator, but also provides a non-mutational explanation for the EGFR overexpression frequently observed and implicated in the pathogenesis of human cancers. We further demonstrate that inhibition of HIF-2α or elements of its pathway, such as the EGFR, IGF-IR or TACE, is sufficient to abrogate the *in vitro* growth of highly malignant cancer cells and their *in vivo* tumor formation, endorsing the use of therapies geared at obstructing this previously unappreciated oncogenic axis in the treatment of human cancers.

Model of HIF-2α Oncogenic Pathway



irrespective of clinical staging (Holmquist-Mengelbier et al., 2006; Gordan et al., 2008; Li et al., 2009). Collectively, these studies are evoking a strong interest in HIF-2α and are giving rise to the viewpoint that it may represent the Achilles heel of several human cancer types including, but as our data would suggest not restricted to, glioma, neuroblastoma and clear cell renal carcinoma.

4.2. HIF-2α is involved in RTK signaling and cell proliferation.

Cancer cells acquire growth autonomy, which encompasses insensitivity to anti-growth signals as well as self-sufficiency in growth signals, as a result of impaired cell cycle regulation or receptor tyrosine kinase signaling (Vogelstein and Kinzler, 2004). The serum responsiveness of the stable cell lines lacking HIF-2α suggests that their growth defect is rooted in the latter scenario (**Fig. 18D**). While several studies, including our own in *VHL*$^{-/-}$ RCC, have established a critical role for the EGFR in the pathogenesis of certain cancer types, its involvement in others has come into question due to the lackluster performance of EGFR antagonists in clinical trials (Smith et al., 2005; Halatsch et al., 2006; Herbst et al., 2008; Bernier et al., 2009). Furthermore, there is mounting evidence of RTK cross-talk and redundancy in individual tumors, such that it seems improbable that HIF-2α endorses growth autonomy through a single receptor pathway (Stommel et al., 2007). Accordingly, data presented here indicate that HIF-2α endows cells with the ability to proliferate autonomously by activating fundamental RTKs, such as the EGFR, IGF-IR and Met receptor, and their downstream signaling pathways (**Fig. 19-21, 28**). This effect seems to be imparted, in some cases, through upregulation of receptor expression levels (**Fig. 20A, 21A**). In contrast to the EGFR, whose expression and phosphorylation was exclusively HIF-2α-dependent, the IGF-IR and Met

receptor were differentially affected by HIF-2α inhibition from cell line to cell line (**Fig. 19A, 20A**). Significantly, HIF-2α was required for their expression only in the cell lines whose growth autonomy was contingent on their activation (**Fig. 19A, 20**). While we were unable to generate cell lines stably re-expressing HIF-2α to rescue xenograft formation, which has recently been shown to induce cell cycle arrest when overexpressed in culture, it would be interesting to attempt the same with the individual receptors or receptor combinations to examine their oncogenic potential in specific cancer types *in vivo* (Hackenbeck et al., 2009). Stable knockdown of the receptor(s) in the parental lines would similarly clarify their relative contributions in the development of genetically diverse cancers. Our results nonetheless suggest that HIF-2α is capable of activating the relevant mitogenic circuits in a context-specific manner, explaining how its inhibition can effectively prevent the tumorigenesis of genetically diverse cancers which are presumably reliant on highly convoluted growth signaling circuits (Stommel et al., 2007; C.G.A.R.N., 2008; Jones et al., 2008; Parsons et al., 2008) (**Fig. 28**). Taken together, these data argue that HIF-2α plays a central role in cell proliferation, orchestrating the overproduction and activation of discrete components of RTK signaling pathways.

4.3. HIF-2α upregulates *EGFR* mRNA translation in human cancers.

In line with its canonical role as a transcriptional activator, HIF-2α induces the production of a variety of diffusible signaling peptides, including TGFα and PDGF-B, which likely accounts for at least a portion of the enhanced receptor activation observed in its presence (Iliopoulos et al., 1996; Gunaratnam et al., 2003; Smith et al., 2005; Yoshida et al., 2006) (**Fig. 19A**). We reveal that HIF-2α activation has a two-fold effect

on this process, also initiating an oncogenic program that results in the translational upregulation of the EGFR proper (**Fig. 13**). This result offers an explanation for the general lack of *EGFR* mutations in cancers and demonstrates how such gene alterations would be futile in many settings where physiological/genetic mechanisms promoting HIF-2α activation and consequently EGFR overproduction, are already instated (Salomon et al., 1995; Paez et al., 2004; Kwak et al., 2005; Blehm et al., 2006; Halatsch et al., 2006; Spindler et al., 2006; Sharma et al., 2007). Importantly, we did confirm that this incessant production of EGFR protein allows for sustained receptor signaling and thus promotes the continuous serum-independent proliferation of cancer cells as would be predicted (**Fig. 14**). Furthermore, the ability of HIF-2α to induce RTK protein expression does not appear to be limited to the EGFR. Expression of the IGF-IR, PDGFRα and two independent EGFR mutants was hypoxia-inducible and/or HIF-2α-dependent (**Fig. 20-21**), although it has not been determined if this upregulation is instigated at the transcriptional or translational level. Although we did not observe this in the cell lines studied here, the Met receptor is reportedly upregulated under hypoxic conditions and is under HIF-1α transcriptional control, such that HIF-2α may similarly induce expression of the remaining RTKs directly (Pennacchietti et al., 2003). Notably, activation of redundant receptor pathways is thought to be a major mechanism of acquired resistance to specific RTK inhibitors (Stommel et al., 2007; Hopper-Borge et al., 2009). For example, amplification of Met causes gefitinib resistance by driving HER3-mediated PI(3)K signaling which is otherwise EGFR-dependent (Engelman et al., 2007). Heterodimerization of receptors, as is observed in the case of IGF-IR/EGFR pairs, and the resultant re-routing of the signaling circuitry serves as another mechanism by which

RTK antagonists are offset (Morgillo et al., 2006). Receptor co-expression is thus representative of a complementary means by which HIF-2α may bolster malignant growth signaling.

4.4. Therapeutic targeting of hallmark cancer traits.

In spite of their genetic and biochemical disparities, it is well-appreciated that cancer cells do ultimately utilize widespread mechanisms to acquire certain tumorigenic traits. An analogous, all-encompassing role, as we propose for HIF-2α in eliciting tumor cell proliferation, is seen in the ability of the HIF-α isoforms to simultaneously induce expression of multiple factors involved in the various stages of angiogenesis (Pugh and Ratcliffe, 2003). Antagonists of these systemic pathways are thus predicted to have greater clinical applicability compared to designer drugs directed at tumors displaying specific mutations or biomarkers (Kaiser, 2008). Fittingly, therapeutic agents targeting VEGF, a classical pro-angiogenic HIF target gene, have exhibited greater efficacy over other rational drugs in the treatment of several cancer types (Kerbel and Folkman, 2002; Ellis and Hicklin, 2008). Based on our data and in accordance with the oncogene addiction theory, inhibition of the HIF-2α growth promoting axis should render a comparable and perhaps improved response (Weinstein, 2002). Our findings provide a strong rationale for targeting HIF-2α in the treatment of cancers but also suggest that therapeutic strategies aimed at inhibiting HIF-1α may fall short of expectations. Silencing HIF-1α hindered tumor cell survival and/or vascularization (**Fig. 17B-C**), as previously described (Carmeliet et al., 1998; Ryan et al., 1998), yet it did not markedly affect the growth or tumorigenesis of any of the cancer cell lines examined here and even stimulated the proliferation of A549 lung carcinoma cells both *in vitro* and *in vivo* (**Fig.**

17A, 18B). In fact tumors derived from the latter cells displayed the highest degree of necrosis which clearly did not impede their progression (**Fig. 17B**). This paradoxical result can be explained by the failure of the immediate environment and the cells themselves to meet the increased energy demand of the actively proliferating population.

Consistently, the activation status of various RTKs and the downstream ERK1/2 and Akt kinases was impervious to HIF-1α knockdown for the most part; although silencing HIF-1α did enhance EGFR expression and phosphorylation in some cell lines implying that relief from a repressive force had been evoked (**Fig. 19A, 20A**). This is not an entirely unexpected outcome since the HIF isoforms have divergent and species-specific transcriptional activities and even play opposing roles in certain metabolic processes (Kondo et al., 2002; Acker et al., 2005; Raval et al., 2005; Gordan et al., 2007; Zhang et al., 2007). Moreover, similar results have been reported following the silencing of HIF-1α in human neuroblastoma, ovarian, breast, and renal carcinoma cell lines (Li et al., 2005; Holmquist-Mengelbier et al., 2006; Gordan et al., 2007; Favaro et al., 2008). Our data do not exclude the possibility that HIF-1α may promote cell proliferation in other cancer types (**Fig. 18E**), they do, however, strongly suggest that the isoforms are not functionally interchangeable with respect to tumor growth and should not be confused as so for drug design purposes.

4.5. Inhibition of EGFR signaling in VHL-loss RCC and other cancer types.

The studies outlined here have established a clear link between HIF-2α activation and EGFR protein overexpression in human cancers (**Fig. 11, 20A**). Moreover, our data validate the EGFR as a critical determinant in *VHL*$^{-/-}$ RCC tumorigenesis (Smith et al., 2005) and suggest that it has a similarly weighty role in the autonomous growth of other

cancer types, although its requirement *in vivo* remains to be formally demonstrated in those cases (**Fig. 20B**). The accessibility and approval of several EGFR inhibitors, which should theoretically abolish HIF-2α-dependent RCC and perhaps non-RCC tumor growth, makes them particularly convenient candidate drugs. The success of EGFR-based antagonists in clinical trials, however, has been highly variable with even the responsive tumors eventually becoming insensitive owing to intrinsic and/or acquired resistance to the drugs (Hopper-Borge et al., 2009). While there have been conflicting reports regarding the correlation between EGFR expression and the clinical benefits of RTK inhibitors, our finding that HIF-2α elicits the upregulation of EGFR protein synthesis led us to conjecture that obstruction of this process may ameliorate drug responsiveness (Arteaga, 2002). Our results suggest that wild-type EGFR overexpression does not affect intrinsic receptor sensitivity to tyrosine kinase inhibition at the biochemical level. We did, however, observe an increased IC50 on a per cell basis in HIF-2α-expressing cells proportional to their elevated EGFR levels (**Fig. 15E-F**), indicating that the continuous administration of higher drug concentrations would likely be required to efficiently compete this relentless receptor production.

Surprisingly, a drug-resistant, constitutively active mutant EGFR became more resistant to the actions of gefitinib in a NSCLC cell line upon silencing of HIF-2α (Pao et al., 2005) (**Fig. 21C**). HIF-2α inhibition did, however, cause a greater over-all decrease in receptor expression and phosphorylation at the tested drug concentrations and abolished the autonomous growth of these cells *in vitro*, suggesting that targeting HIF-2α alone would be more advantageous in this situation (**Fig. 21B-C**). Independent corroboration in additional cancer cell lines would be necessary to confirm if HIF-2α does indeed affect

mutant EGFR drug sensitivity. Nonetheless, we propose that inhibition of HIF-2α may provide a means of preventing receptor upregulation in the first place, given the lack of such agents, one approach that is of appeal is the simultaneous inhibition of mTOR, which promotes HIF-2α synthesis (Hudson et al., 2002; Thomas et al., 2006; Toschi et al., 2008), and EGFR pathways (**Fig. 11F**). Indeed, this therapeutic strategy has rendered promising results in recent preclinical studies involving *VHL*$^{-/-}$ RCC and non-RCC cancers alike (Gemmill et al., 2005; Wang et al., 2006; Bianco et al., 2008).

4.6. Alternative approaches of obstructing the HIF-2α oncogenic axis in RCC.

Based on our data, it is reasonable to speculate that monotherapies directed at HIF-2α would simultaneously inhibit several RTK signaling pathways thereby circumventing receptor resistance and redundancy issues (Morgillo et al., 2006; Engelman et al., 2007; Stommel et al., 2007). Due to the unavailability of HIF-2α-based agents and the second-rate clinical performance of RTK inhibitors, we investigated the therapeutic potential of another participant in the HIF-2α oncogenic axis. Specifically, the feasibility of targeting upstream TGFα maturation events prior to EGFR activation was assessed in *VHL*$^{-/-}$ RCC cells. Blocking TGFα ectodomain shedding by stably silencing TACE suppressed EGFR phosphorylation in two human *VHL*$^{-/-}$ RCC cell lines (**Fig. 23**). Furthermore, silencing TACE restored dependence on exogenous growth factors for proliferation, suppressed *in vitro* cell migration and invasion, and prevented the *in vivo* tumor formation of the renal cancer cells (**Fig. 24, 26-27**), providing the first demonstration that ligand processing is a prerequisite for tumorigenesis in an endogenous setting. Importantly, cell proliferation in the absence of serum was rescued by addition of recombinant TGFα, contending that the

obstruction of EGFR signaling is the root cause of the inability of the TACE-deficient *VHL*$^{-/-}$ RCC cells to form tumors in nude mice (**Fig. 24**).

Our data also provide evidence that TACE may have EGFR-independent functions pertaining to metastatic tumor cell capabilities. It is well-established that TGFα and other EGFR ligands, most of which are TACE substrates, can promote the migration of a multitude of cell types, ranging from mammary epithelial to ovarian cancer cells (Barrandon and Green, 1987; Maheshwari et al., 2001; Sewell et al., 2005). We found, however, that inhibition of TACE function was more efficient than silencing the EGFR in eradicating *in vitro* cell migration and invasion (**Fig. 26B-C**). TACE has previously been linked to the regulation of cell-matrix interactions through adhesion molecules such as CD44 and L1, and is thought to modulate cell migration by its interaction with various integrins (Beer et al., 1999; Bax et al., 2004; Nagano et al., 2004; Huang et al., 2005). Notably the ErbB and integrin signaling pathways have been implicated, and likely function synergistically, in focal adhesion kinase (FAK)-mediated cell migration (Sieg et al., 2000; Wang et al., 2009a). At any rate it is evident that TACE may modulate cell migration *via* EGFR- and integrin-dependent mechanisms that warrant additional examination.

4.7. TACE as a novel target in EGFR-dependent human cancers.

TACE represents a particularly attractive target as several orally active inhibitors are already in existence; developed for the treatment of inflammatory conditions such as rheumatoid arthritis (Smolen and Steiner, 2003). Human trials have confirmed the efficacy of such drugs in healthy subjects, as evidenced by decreased blood levels of various TACE substrates, and that they are well-tolerated at greater doses than required

for pharmacological activity (Beck et al., 2002; Liu et al., 2006). Our finding that silencing TACE is sufficient to abrogate multiple acquired tumor cell capabilities and *VHL*$^{-/-}$ RCC tumorigenesis *in vivo,* define a clear clinical context where these agents would likely be of avail (**Fig. 26-27**). We suggest that TACE may also represent a promising therapeutic target in the treatment in other HIF-2α/EGFR-dependent cancers. In line with this notion, several laboratories have recently provided compelling data implicating TACE in the pathogenesis of other cancers. It has been reported that small molecule and siRNA-mediated TACE inhibition is sufficient to revert malignant phenotypes in a three-dimensional culture model of breast cancer progression (Kenny and Bissell, 2007). The authors also revealed that TACE and TGFα are enriched in triple (estrogen receptor (ER), progersterone receptor (PR) and HER2)-negative breast carcinomas, which is of particular clinical interest since this basal subset is typically unresponsive to the available hormone- and HER2-based therapies (Fadare and Tavassoli, 2008). This is in accordance with a previous report that TACE is consistently overexpressed in breast tumors compared with adjacent normal tissues (Borrell-Pages et al., 2003). Additional studies involving a novel small molecule inhibitor of TACE, INC3619, have demonstrated its efficacy in reducing the levels of circulating TGFα, HB-EGF, amphiregulin, and heregulin and, more importantly, in preventing the *in vivo* tumorigenesis of several breast and NSCLC cell lines (Zhou et al., 2006; Fridman et al., 2007). Not only did this inhibition abolish tumor growth *in vivo* but it also enhanced the sensitivity of HER3-dependent NSCLC cancer cells to gefitinib (Zhou et al., 2006). This suggests that the concomitant inhibition of TACE and EGFR should improve patient responses and increase survival. Together, the results of these studies highlight TACE

inhibition as an alternative to traditional EGFR-based therapies, and demonstrate at the conceptual level that silencing one biologically relevant enzyme in the HIF-2α oncogenic axis is sufficient to abrogate tumorigenesis of *VHL*$^{-/-}$ RCC and other human cancers.

4.8. Future directions I: Investigation of HIF-2α gene regulation.

The challenge now lies in further unraveling the molecular mechanisms at play in the HIF-2α growth promoting axis such that superior therapeutic targets can be identified. A fundamental question that remains unanswered is how HIF-2α itself becomes activated in solid tumors. While HIF-1α is ubiquitously expressed, HIF-2α mRNA expression is generally confined to more specific cell types, involved in the systemic delivery of oxygen, including endothelial cells, type II pneumocytes, cardiomyocytes, kidney fibroblasts, interstitial cells of the pancreas and duodenum, and hepatocytes (Patel and Simon, 2008). It has recently been shown that basal HIF-2α mRNA synthesis is preferentially elevated in glial stem cells compared with other neural progenitor or glioma cell lines; an effect which is further induced under hypoxic conditions (Li et al., 2009). These cell lines would provide an interesting model system in elucidating the upstream transcriptional effector proteins and/or chromatin remodeling factors that dictate HIF-2α expression patterns. Comparison of HIF-2α gene modifications and DNA-associated proteins in non-expressing renal epithelial versus HIF-2α-dependent *VHL*$^{-/-}$ RCC cells may likewise prove to be informative in this regard. If our model that genetically diverse human cancers ultimately exploit the HIF-2α oncogenic pathway as a means of attaining growth autonomy is correct (**Fig. 28**), this would suggest that one of the main objectives of known oncogene and tumor suppressor pathways is to endorse

HIF-2α activity. If such is the case, one could speculate that HIF-2α expression is a requirement for cellular and viral oncogene-induced transformation and tumorigenesis. Expression of the IGF-IR for example, for which we provide evidence is under HIF-2α control (**Fig. 19A, 20A**), is required for Ha-ras, c-Src, SV40, and EGFR-induced transformation of mouse embryo fibroblasts (Sell et al., 1993; Coppola et al., 1994; Sell et al., 1994; Valentinis et al., 1997). Inhibition of IGF-IR similarly prevents the tumorigenesis of K-ras-dependent basal-like breast cancers in mouse and human tumor models (Klinakis et al., 2009). While the concurrent expression of HIF-2α or its knock-in at the HIF-1α locus has been shown to enhance Ras/c-Myc-mediated colony formation and tumorigenesis of primary mouse embryonic stem cells, respectively, it would be interesting to determine if its knock-out would cause the cells to become resistant to transformation (Covello et al., 2005; Gordan et al., 2007).

4.9. Future directions II: Identification of molecular allies in the HIF-2α axis.

Additional, and perhaps more clinically relevant, participants in the HIF-2α oncogenic axis may include co-conspirators, such as members of the ETS family of transcription factors which confer target gene specificity to HIF-2α, as well as downstream growth stimulatory/regulatory molecules (Elvert et al., 2003; Aprelikova et al., 2006; Hu et al., 2007; Le Bras et al., 2007). As discussed above, verification of the individual contribution of various RTKs, identified here as being HIF-2α-dependent (**Fig. 19A, 20A**), to tumorigenesis will be a critical aspect in this process. To date HIF-2α oncogenic functions have been studied mainly in *VHL*$^{-/-}$ RCC. In this setting, HIF-2α drives the growth and tumorigenesis of *VHL*$^{-/-}$ RCC cells by promoting TGF-α/EGFR

autocrine signaling through the transcriptional activation of the ligand and, as demonstrated here, the translational activation of the receptor (Gunaratnam et al., 2003; Smith et al., 2005) (**Fig. 13**). We similarly found that the EGFR was consistently deactivated in the absence of HIF-2α and had the greatest impact on cell growth autonomy in the other cancer types studied here (**Fig. 19-20**). It will thus be of interest to delineate the precise mechanism by which HIF-2α mediates the upregulation of EGFR protein synthesis and if it accounts for the HIF-2α-dependent expression and/or hypoxic induction of other RTKs, such as the IGF-IR and PDGFRα. The bidirectional signaling effects observed in NSCLC cells *in vitro*, where enhanced mTOR-mediated EGFR protein synthesis occurs as a consequence of IGF-IR activation, is certainly worth pursuing and seems a logical avenue by which HIF-2α may impact receptor upregulation (Morgillo et al., 2006). Alternatively, HIF-2α may induce the expression of an activator of EGFR translation or the repression of a negative regulator of receptor synthesis. The Usp18 deubiquitinase and miRNAs-7 and -128b are recently identified candidates that appear to have such positive and negative effects on EGFR translation, respectively (Kefas et al., 2008; Weiss et al., 2008; Duex and Sorkin, 2009). Hypoxia, and more specifically HIF-α activation, is also known to modulate the expression and cellular localization of several integrins (Koike et al., 2004; Kong et al., 2004; Cowden Dahl et al., 2005). Given that $\alpha_6\beta_4$ integrin-dependent HER2 translational control has been reported, it is reasonable to speculate that a similar mechanism may contribute to the upregulation of the EGFR, a related receptor (Yoon et al., 2006). A more provocative possibility, supported by the rapid kinetics of EGFR protein induction in hypoxic conditions (**Fig. 10C**), is that HIF-2α may directly act as a translation factor capable of

binding HRE-like RNA elements. Irrespective of whether HIF-2α mediates the upregulation of the above-mentioned receptors through direct or indirect mechanisms, further studies would provide great insight into RTK expression dynamics which, needless to say, is of utmost importance in cancer biology.

4.10. Concluding Statement.

In conclusion, our data argue that, despite their genetic and molecular diversity, human cancers are unified by an underlying program that confers them with autonomous growth capabilities. We propose a model whereby various oncogene and tumor suppressor pathways endorse the upregulation and stabilization of HIF-2α, which in contrast to overt carcinomas is rarely expressed in normal tissues, with the goal of attaining growth signal autonomy (**Fig. 28**). This effect is likely amplified by the tumor microenvironment enabling HIF-2α to activate central and context-specific growth signaling pathways, explaining why its inhibition overrides RTK redundancy and can effectively abolish the tumorigenesis of various human cancer cell lines. We further provide evidence that HIF-2α activation mediates the translational upregulation of the EGFR, and likely other RTKs, providing a non-mutational explanation for the receptor overexpression so commonly observed in human cancers in the absence of gene alterations. Finally, we demonstrate at the conceptual level that targeting key elements downstream of HIF-2α, such as TACE, is sufficient to abrogate multiple malignant phenotypes and *in vivo* tumor formation. Given its systemic and rate-limiting nature, we propose that HIF-2α and participants in its oncogenic axis should be investigated as prime therapeutic targets, particularly in epithelial cancers with yet unidentified proliferative mechanisms.

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APPENDICES

6. APPENDICES

6.1. Appendix A – First Author Publications

Multiple Acquired Renal Carcinoma Tumor Capabilities Abolished upon Silencing of ADAM17

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Abstract

Malignancy is a manifestation of acquired defects in regulatory circuits that direct normal cell proliferation and homeostasis. Most of these circuits operate through cell autonomous pathways, whereas others potentially involve the neighboring microenvironment. We report that the metalloprotease ADAM17 plays a pivotal role in several acquired tumor cell capabilities by mediating the availability of soluble transforming growth factor-α, an epidermal growth factor receptor (EGFR) ligand, and thus the establishment of a key autocrine signaling pathway. Silencing of ADAM17 in human renal carcinoma cell lines corrects critical features associated with cancer cells, including growth autonomy, tumor inflammation, and tissue invasion. Highly malignant renal carcinoma cancer cells fail to form *in vivo* tumors in the absence of ADAM17, confirming the essential function of this molecule in tumorigenesis. These data show that ligand shedding is a crucial step in endogenous EGFR activation and endorse prospective therapeutic strategies targeting ADAM17 in human cancer. (Cancer Res 2006; 66(16): 8083-90)

Introduction

Cancer cells share numerous defects in distinct regulatory circuits that govern cell proliferation and homeostasis; these defects are often called hallmarks of cancer (1). Through successive genomic alterations, transformed cells develop intricate programs that drive tumor-associated phenotypes, including growth autonomy, resistance to apoptosis, and tissue invasion. These common, yet complex, acquired tumor cell capabilities are thought to define malignancy of the majority of neoplasia and have thus been exploited in anticancer therapeutic strategies. It is thought that these acquired capabilities arise from the alterations of functionally distinct genes and, as a consequence, unrelated regulatory circuits (2, 3). Whether these alterations occur during the earliest steps of transformation or follow a linear model of chronological events remains debatable. Certainly, an overwhelming number of independent pathways have been implicated in the process of tumorigenesis. Nonetheless, it is reasonable to speculate that self-sufficiency in growth signaling is the initial acquired capability during the process of cellular transformation and perhaps the most critical aspect of neoplasia. Normal cells require exogenous growth stimulatory cues to engage in cellular division. This is reflected by their ability to withdraw from the cell cycle and enter quiescence in the absence of exogenous growth factors (4). This tightly regulated growth control is lost in essentially all cancer cells, often as a consequence of the constitutive production of endogenous growth factors (1, 5, 6). Self-sufficiency in growth signaling can be triggered by the aberrant and constitutive production of growth factors in a process called an autocrine loop. A suitable example of acquired autocrine loop capacity is observed in clear cell renal carcinoma cells. Renal epithelia require exogenous growth factors to exit quiescence and enter the cell cycle (7, 8). In stark contrast, renal carcinoma cells are able to constitutively produce the soluble transforming growth factor-α (TGF-α), a mitogen of renal epithelial cells and a ligand of the epidermal growth factor receptor (EGFR; refs. 9–13). Inhibition of the TGF-α/EGFR growth stimulatory pathway is sufficient to prevent self-sufficiency in growth of renal carcinoma cells in culture and, as a result, tumor formation *in vivo* (9, 14, 15). In several systems, TGF-α undergoes ectodomain shedding by ADAM17, a metalloprotease also called TACE [tumor necrosis factor-α (TNF-α) converting enzyme; refs. 16, 17]. However, it remains unclear whether this process is a universal requirement for EGFR signaling and tumorigenesis (18, 19). ADAM17 is involved in the ectodomain shedding of a wide variety of membrane-bound ligands and cytokines that are implicated in diverse biological processes, including growth and inflammation (20). Recent preclinical studies have yielded encouraging results as to the potency of specific ADAM17 inhibitors in the treatment of inflammatory diseases, such as rheumatoid arthritis and ischemic stroke (21, 22). Given the number of anti-ADAM17 drugs that are currently in development and its prospective role in TGF-α processing and renal carcinoma tumorigenesis, we decided to examine the function of this specific sheddase in a model of human cancer for therapeutic purposes.

We report that blocking TGF-α shedding by stably silencing ADAM17 in two independent human renal carcinoma cell lines, 786-0 and KTCL, is sufficient to suppress EGFR activation and multiple unrelated acquired tumor cell capabilities. Silencing of ADAM17 restored dependence on exogenous growth factor signaling, suppressed acquired pathways involved in tissue invasion, and prevented *in vivo* tumor formation of highly malignant renal cancer cells. These data show that the frequently altered regulatory circuits implicated in the process of tumorigenesis intersect at ADAM17, providing compelling evidence to target this particular metalloprotease in anticancer therapy.

Materials and Methods

Cell culture and reagents. The human sporadic von Hippel-Lindau(−/−) [VHL(−/−)] renal carcinoma cell line 786-0 was obtained from the American Type Culture Collection (Rockville, MD). The 786-0 cells stably transfected with hemagglutinin-tagged VHL (786-0 + VHL) were a kind gift from Dr. W.G.

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Kaelin (Harvard University, Boston, MA). The KTCL cell line was a kind gift from Dr. Peter Ratcliffe (University of Oxford, Oxford, United Kingdom). Cell lines were incubated at 37°C under a 5% CO_2 environment. Cell lines were maintained in serum-containing medium consisting of DMEM with 5% fetal bovine serum (FBS). Serum-free medium consisted of DMEM supplemented with 1% insulin-transferrin-selenium (ITS; Invitrogen, Burlington, Ontario, Canada).

ADAM17 RNA interference. For transient silencing of ADAM17, the VHL(−/−) renal carcinoma cell lines 786-0 and KTCL were transfected with commercially available double-stranded 21-nucleotide-long small interfering RNA (siRNA) targeting ADAM17 or a control siRNA (Ambion, Austin, TX). The cell lines were also stably transfected to express one of two independent short-hairpin RNA (shRNA) sequences targeting ADAM17 with Effectene reagent (Qiagen, Valencia, CA). For each sequence, two complimentary ssDNA oligonucleotides designed with overhangs encoding *Bam*HI/*Hin*dIII restriction enzyme sites were synthesized and subsequently annealed with 1× DNA Annealing Solution according to the protocol of the manufacturer (Ambion). The annealed inserts were subsequently ligated into a pSilencer 3.1-H1 neo vector (Ambion). Sequence 1 (5′-3′): shRNA ADAM17-1 forward GGAUGUAAUUGAACGAUUU and ADAM17-1 reverse AAAUCGUUCAAUUACAUCC (Ambion, siRNA ID: 12917). Sequence 2 (5′-3′): shRNA ADAM17-2 forward AAGCTTGATTCTTTGCTCTCA and shRNA ADAM17-2 reverse AATGAGAGCAAAGAATCAAGC (23). All constructs were verified by standard DNA sequencing. A pSilencer 3.1-H1 neo vector encoding scramble shRNA was purchased and served as a negative control. Positive clones were selected and maintained in neomycin-containing medium. The 786-0 cell lines stably transfected with shRNA-targeting EGFR were generated by our group as previously described (15).

Measurement of TGF-α levels. An equal number of cells were plated and incubated for indicated times in DMEM supplemented with 5% FBS. In experiments involving matrix metalloprotease inhibitors, cells were treated with 10 to 100 μmol/L GM6001 (Calbiochem, San Diego, CA) or corresponding volumes of DMSO as a vehicle control. The cell lysates and conditioned medium were collected and TGF-α levels were analyzed according to ELISA kit instructions (Oncogene, Boston, MA).

Western blot analysis. Cells were washed with PBS and harvested in 4% SDS in PBS. Protein concentrations were quantified using a BCA protein assay kit and samples (50 μg protein) were separated on a denaturing polyacrylamide gel containing SDS and transferred to a methanol-activated polyvinylidene difluoride membrane (NEN, Boston, MA). Before immunodetection, membranes were blocked with 5% (w/v) skim milk powder in a 0.2% Tween-PBS solution. Membranes were then incubated with anti-actin (Sigma, St. Louis, MO), anti-HIF2α (Novus, Littleton, CO), anti-ADAM17 precursor (Santa Cruz Biotechnology, Santa Cruz, CA), anti-EGFR (LabVision, Fremont, CA), or anti-py-EGFR (Santa Cruz Biotechnology) primary antibody overnight at 4°C followed by incubation with horseradish peroxidase (HRP)–conjugated anti-mouse (Amersham Biosciences, Piscataway, NJ) or anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) secondary antibody for 1 hour at room temperature. The bands were detected by use of a chemiluminescent HRP substrate (Pierce, Rockford, IL).

Measurement of cell proliferation. Cells were plated at low density on glass coverslips and incubated overnight in DMEM supplemented with 5% FBS. At the start of experiments, cells were washed and supplemented with fresh serum-containing or serum-free medium. Following indicated time periods/treatments, cells were labeled with 5-bromo-2′-deoxyuridine (BrdUrd), fixed, and immunostained according to the protocol of the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). The coverslips were counterstained with Hoechst reagent (Hoechst 33258; Sigma) and the percentage of BrdUrd incorporation was assessed using a Zeiss Axiovert S100TV microscope (Thornwood, NY).

RNA isolation and reverse transcription-PCR analysis. Total RNA was collected using TriPure Isolation Reagent (Roche Molecular Biochemicals) according to the protocol of the manufacturer. Reverse transcription-PCR (RT-PCR) was done on 1 μg RNA using the One-Step Superscript RT Platinum TaqRT-PCR kit (Invitrogen) and 0.6 μmol/L of each primer. All primers and cycle details for RT-PCR analysis of vascular endothelial growth factor (VEGF), Glut-1, TGF-α, and actin mRNA levels were described elsewhere (14). Products were analyzed with gel electrophoresis and ethidium bromide staining, and visualized using a Kodak Digital Science IC440 system.

Immunofluorescence. Cells were plated at low density on glass coverslips and incubated for 6 days in DMEM supplemented with 8% FBS. Cells were fixed in 95% ethanol for 30 minutes at −20°C. The ethanol was aspirated and coverslips were allowed to air dry at 4°C. Cells were immunostained with antifibronectin antibody (Abcam, Inc., Cambridge, MA) and fibronectin deposition was assessed as previously described (24).

***In vitro* tumor spheroids.** Multicellular spheroids were prepared as previously described (15, 25). Briefly, 24-well plates were coated with preheated 1% Seaplaque agarose (Cambrex, Rockland, ME) in serum-free medium. One hundred thousand of the indicated cells were plated per 1 mL

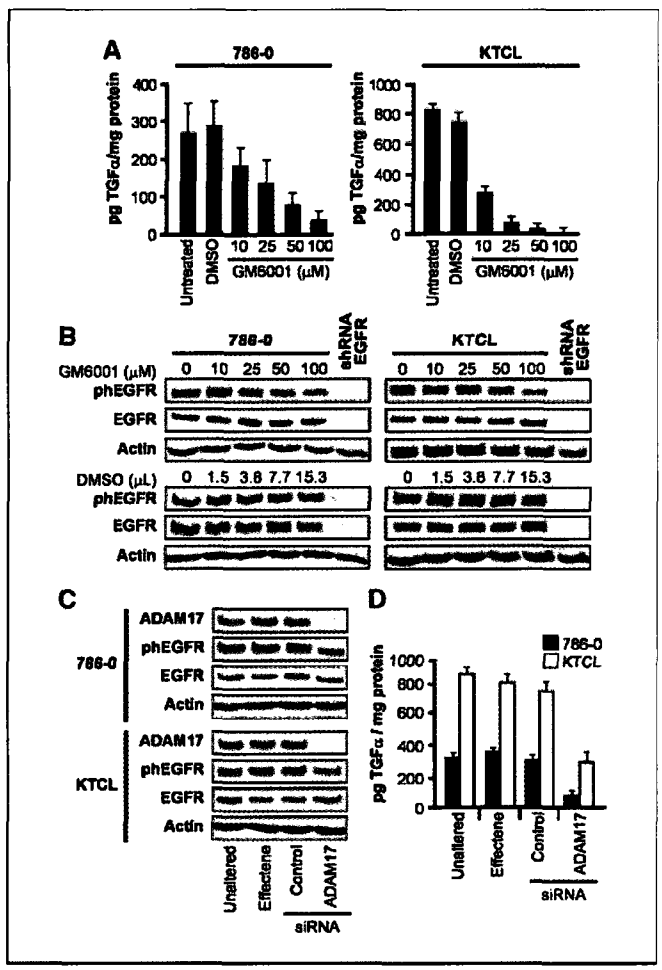

Figure 1. TGF-α ectodomain cleavage is required for efficient EGFR activation. *A*, the effect of inhibiting TGF-α shedding with general matrix metalloprotease inhibitor, GM6001, on EGFR activation in renal carcinoma cell lines was examined. ELISA analysis of soluble TGF-α levels in serum-starved VHL(−/−) renal carcinoma 786-0 and KTCL cell line conditioned medium following a 48-hour treatment with GM6001 (10-100 μmol/L) or an equivalent volume of DMSO as a vehicle control. *B*, Western blot analysis of total (*EGFR*) and phosphorylated EGFR (*phEGFR*) levels in cell lysates from (*A*). Cell lines expressing shRNA directed against EGFR (*shRNA EGFR*) were used as negative controls. Actin Westerns were done as loading controls. *C*, the effect of transiently silencing ADAM17, the major TGF-α sheddase, on TGF-α shedding and EGFR activation in renal carcinoma cell lines was examined. Western blot analysis of ADAM17, phEGFR, and total EGFR levels in 786-0 and KTCL cell lines transiently transfected with 50 nmol/L siRNA against ADAM17, 50 nmol/L scramble siRNA (*Control*), or an equivalent volume of Effectene transfection reagent for 96 hours. *D*, ELISA analysis of TGF-α levels in conditioned medium of cell lines described in (*C*). *Bars (A and D)*, SD of at least three independent experiments done in triplicate.

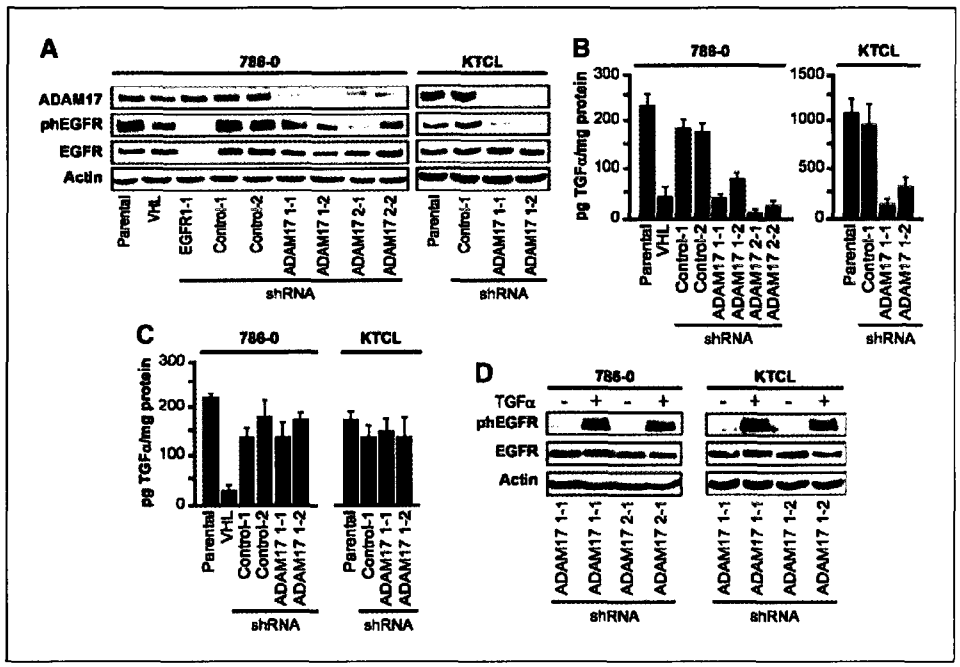

Figure 2. Stable inhibition of ADAM17 inhibits TGF-α shedding and EGFR activation. *A,* shRNA directed against ADAM17 mRNA suppresses ADAM17 protein levels. Western blot analysis of ADAM17, phEGFR, and total EGFR levels in VHL(−/−) renal carcinoma, 786-0 and KTCL, cell lines stably transfected with pSilencer expressing scramble shRNA (*Control*), or expressing one of two shRNA sequences directed against ADAM17 (*shRNA ADAM17-1* or *shRNA ADAM17-2*). Cell lines expressing wild-type VHL (*VHL*) or shRNA directed against EGFR (*shRNA EGFR1*) were used as controls. Actin Westerns were done to ensure equal loading. *B,* stable silencing of ADAM17 inhibits shedding of soluble TGF-α. ELISA analysis of TGF-α levels in conditioned medium of cell lines described in (*A*) following a 48-hour incubation period. *C,* stable silencing of ADAM17 does not alter total cellular TGF-α levels. ELISA analysis of TGF-α levels in cellular lysates of cell lines described in (*A*). *Bars (B and C),* SD of at least three independent experiments done in triplicate. *D,* stable silencing of ADAM17 does not disrupt the ability of the EGFR to be activated upon addition of exogenous ligand. Western blot analysis of phEGFR and total EGFR levels following a 15-minute stimulation with 20 ng/mL recombinant TGF-α.

of medium per well. To promote cell-to-cell adhesion, the plates were gently swirled 30 minutes after plating. Spheroids were grown for 6 days at 37°C under 5% CO_2 in serum-containing medium. Spheroids were then collected and fixed in 10% formaldehyde, embedded in paraffin, sectioned, mounted on slides, and stained with H&E.

Migration and invasion assays. Colorimetric cell migration assays (Chemicon, Temecula, CA) were done according to the protocol of the manufacturer. Briefly, 5.0×10^4 cells were plated in Boyden chambers. Cells were allowed to migrate for 16 hours toward serum-containing (10% FBS) or serum-free (5% bovine serum albumin) medium. The chambers were then removed and placed in staining reagent provided in the kit. The dye was solubilized and the absorbance was measured on a 96-well microplate reader at 574 nm. Colorimetric cell invasion assays were done on BD BioCoat Matrigel invasion chambers (BD Biosciences, San Jose, CA). Cells (1.5×10^5) were plated in the chambers in the presence (10% FBS) or absence (1% ITS) of serum and incubated for 48 hours. The chambers were removed and stained with crystal violet. The dye was solubilized with 10% acetic acid and its absorbance was read at 574 nm.

Nude mouse xenograft assays. Nude mouse xenograft assays were done as previously described (15, 26). Briefly, female nude mice (Charles River,

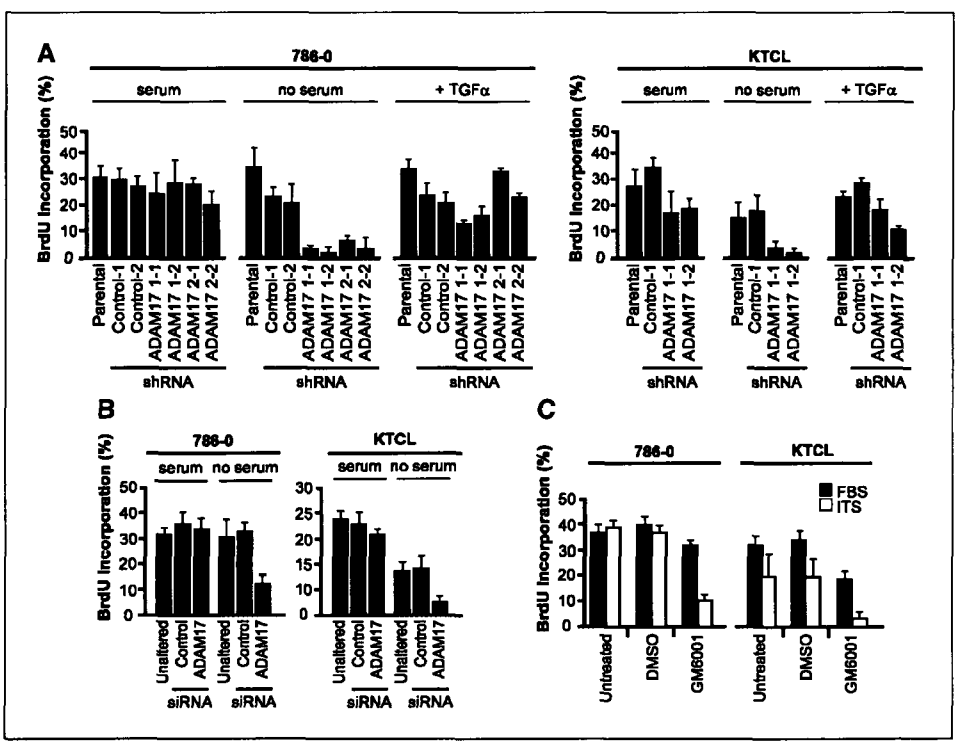

Figure 3. Inhibition of ADAM17-mediated TGF-α shedding abolishes the ability of renal carcinoma to engage in autonomous growth. The ability of VHL(−/−) renal carcinoma, 786-0 and KTCL, cell lines to engage in autonomous proliferation in the absence of serum growth factors was assessed as a function of their ability to incorporate BrdUrd. *A,* stable silencing of ADAM17 prevents the autonomous growth of renal carcinoma. 786-0 and KTCL cell lines stably transfected with shRNA against ADAM17 or scramble shRNA (*Control*) were cultured in 5% FBS or serum-free medium for 72 hours followed by addition of 20 ng/mL recombinant TGF-α (+*TGF-α*) for 24 hours. *B,* transient silencing of ADAM17 prevents the autonomous growth of renal carcinoma. 786-0 and KTCL cell lines were transiently transfected with 50 nmol/L siRNA targeting ADAM17, 50 nmol/L scramble siRNA (*Control*), or an equivalent volume of Effectene transfection reagent, in DMEM supplemented with 5% FBS or serum-free medium for 72 hours. *C,* inhibition of TGF-α shedding with a general matrix metalloprotease inhibitor prevents the autonomous growth of renal carcinoma. 786-0 and KTCL cell lines were treated with 100 μmol/L GM6001 or an equivalent volume of DMSO, in DMEM supplemented with 5% FBS or serum-free medium for 72 hours. *Bars,* SD of at least three independent experiments done in triplicate.

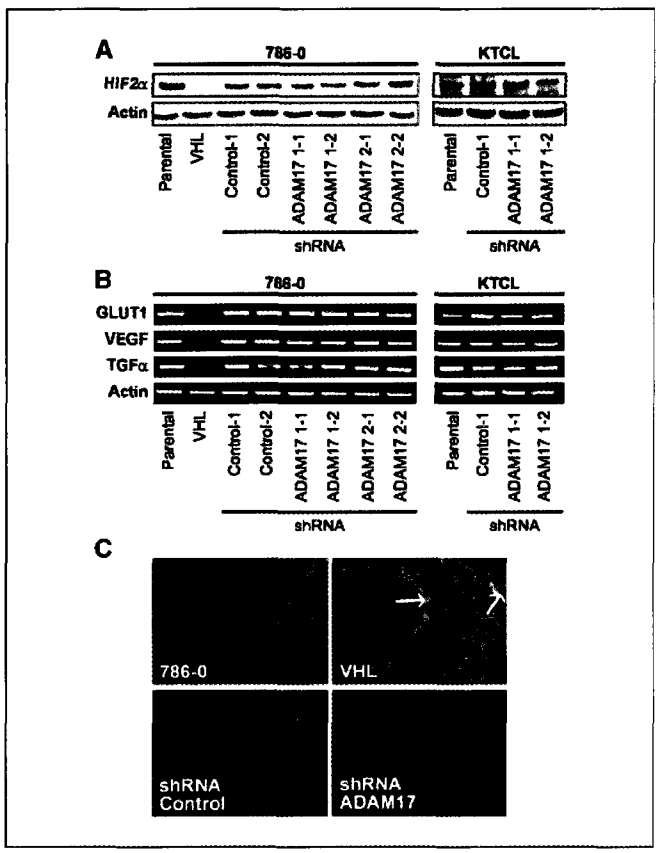

Figure 4. *Silencing ADAM17 does not affect proangiogenic phenotypes* characteristic of VHL-loss renal carcinoma. Expression of proangiogenic factors associated with the highly vascular nature of VHL-loss renal carcinoma was examined in VHL(−/−)renal carcinoma 786-0 cells, VHL(+)renal carcinoma cells (*VHL*), and 786-0 cells stably expressing shRNA (*Control, EGFR,* and *ADAM17*). *A,* stable silencing of ADAM17 does not affect HIF2α expression. Western blot analysis of HIF2α levels in various cell lines. Actin was used as a loading control. *B,* stable silencing of ADAM17 does not affect expression of hypoxia-inducible mRNAs. RT-PCR analysis of mRNA levels of HIF target genes, *GLUT1, TGF-α,* and *VEGF,* in various cell lines. *C,* stable silencing of ADAM17 does not restore the ability of VHL(−/−) renal carcinoma to deposit extracellular fibronectin matrix. Immunofluorescence detection of fibronectin matrix deposition was visualized at a magnification of ×400.

Wilmington, MA) were injected in the flanks with 10^7 control (parental or scramble shRNA) and ADAM17 shRNA-expressing cells. Mice were sacrificed 7 to 9 weeks postinjection according to facility protocol (University of Ottawa). Tumor size was measured weekly and the tumors were excised and weighed at the time of sacrifice. Experiments were done double blinded.

Results

TGF-α ectodomain cleavage is required for efficient EGFR activation in renal carcinoma cell lines. Self-sufficiency in growth signaling requires activation of an autonomous program that constitutively drives cell proliferation. A model that precisely exhibits characteristics of acquired growth autonomy by production of soluble growth factors is renal carcinoma. Human renal carcinoma often harbor defective alleles of the *VHL* tumor suppressor and, as a consequence, overproduce hypoxia-inducible factor (HIF), a transcription factor that activates an array of genes involved in oxygen homeostasis (27, 28). One HIF target gene of particular interest with respect to tumor formation is *TGF-α*. We recently showed that renal carcinoma engage in a classic TGF-α/EGFR autocrine circuit required for autonomous proliferation and *in vivo* tumorigenesis (9, 14, 15). TGF-α is synthesized as a transmembrane precursor protein (pro-TGF-α) that undergoes ADAM17-mediated proteolytic cleavage to release the mature ligand (16). There have been conflicting accounts in the literature regarding the requirement for TGF-α shedding in EGFR activation. Although recent reports suggest that ADAM17-dependent shedding of EGFR ligands is a requirement for EGFR activation, it has also been reported that the proteolytic cleavage of TGF-α is defective in many cancer cells leading to the accumulation of membrane-bound forms and enhanced EGFR activation (18, 19). We therefore decided to examine the role of ADAM17 in a model system of human renal cancer where participant molecules in this pathway, including TGF-α, EGFR, and ADAM17, are produced endogenously. To determine whether TGF-α cleavage is necessary for EGFR activation, two independent sporadic renal carcinoma cell lines derived from human primary renal tumors with clear cell type histology and harboring loss of function mutations in the *VHL* tumor suppressor gene, 786-0 and KTCL, were treated for 48 hours with a general sheddase/matrix metalloprotease inhibitor, GM6001. The amount of TGF-α secreted into the medium by both renal carcinoma cell lines (Fig. 1*A*), and corresponding phosphorylation of the EGFR, decreased in a dose-dependent manner (Fig. 1*B*) without affecting total levels of cellular TGF-α or EGFR (Fig. 1*B* and data not shown). To confirm that the observed effect of the drug on TGF-α shedding and EGFR phosphorylation was not a result of the inhibition of other matrix metalloproteases or alternative pathways, ADAM17 was transiently silenced using small-interfering RNA (siRNA) technology. ADAM17 knockdown was confirmed by Western blot analysis of cellular levels of the full-length ADAM17 precursor protein (Fig. 1*C*). Predictably, the transient silencing of ADAM17 inhibited TGF-α ectodomain cleavage (Fig. 1*D*) and resulted in reduced EGFR phosphorylation (Fig. 1*C*). The decrease in EGFR activation following transient silencing of ADAM17 only became evident after 96 hours, as it required additional time following the efficient silencing of ADAM17 to observe these downstream effects. Nonetheless, the data indicate that TGF-α shedding is required for maximal EGFR activation and that this process is most likely mediated by ADAM17.

Stable inhibition of ADAM17 inhibits TGF-α shedding and EGFR activation in renal carcinoma cell lines. To further examine the specific role of ADAM17 in the establishment of the TGF-α/EGFR autocrine signaling loop, we generated renal carcinoma cell lines with stably inactivated ADAM17 using a shRNA strategy. Two independent shRNA sequences targeting ADAM17, called shRNA ADAM17-1 and ADAM17-2, were used to silence ADAM17 protein expression in the 786-0 and KTCL cell lines. Renal carcinoma stably expressing either of the shRNA sequences against ADAM17 exhibited a significant decrease in ADAM17 protein levels compared with parental cells and cells stably expressing control scrambled shRNA (Fig. 2*A*). Stable expression of shRNA against ADAM17 inhibited the secretion of TGF-α by 786-0 and KTCL cells compared with parental and control cell lines suggesting that ADAM17 is the major TGF-α ectodomain sheddase in renal carcinoma (Fig. 2*B*). The observed decrease in secreted TGF-α was not accompanied by a measurable increase in cellular TGF-α levels (Figs. 2*C* and 4*B*). The parental renal carcinoma cell lines shed low levels of TGF-α over time, such that any resulting increases in cell-associated TGF-α were not

detectable at steady state in the cell lines where ADAM17 was silenced. Importantly, there was a concomitant reduction in phosphorylated EGFR levels, but not total EGFR levels, in shRNA-expressing cells (Fig. 2*A*). Addition of exogenous TGF-α rescued EGFR phosphorylation, indicating that silencing ADAM17 did not affect the ability of the receptor to become activated in response to ligand stimulation (Fig. 2*D*). These data show that ADAM17-mediated TGF-α ectodomain shedding is a prerequisite for efficient EGFR activation in cells that produce endogenous ligand and receptor.

Inhibition of ADAM17-mediated TGF-α shedding abolishes the ability of renal carcinoma to engage in autonomous growth. Based on the data shown in Fig. 2, we decided to examine the effect of preventing TGF-α cleavage on the ability of renal carcinoma to engage in autonomous proliferation upon serum withdrawal (29). Stable silencing of ADAM17 was sufficient to abrogate the ability of both 786-0 and KTCL cells to incorporate BrdUrd in serum-free medium (Fig. 3*A*) to levels similar to those observed in renal carcinoma cells upon stable silencing of EGFR (15). After the cells had been cultured for 72 hours in the absence of serum, addition of exogenous TGF-α activated the EGFR (Fig. 2*D*) and reinitiated cell proliferation demonstrating that loss of growth autonomy observed in shRNA-expressing cells was not due to a dominant effect on the cell cycle (Fig. 3*A*). Similar data were also obtained with transient silencing of ADAM17 (Fig. 3*B*) and the treatment of cells with GM6001 (Fig. 3*C*), indicating that the results obtained with stable silencing of ADAM17 are not a consequence of fortuitous effects of shRNA expression or single cell clonal effects. These data suggest that ADAM17-mediated processing of pro-TGF-α into its soluble form is essential for EGFR phosphorylation and critical for the establishment of the TGF-α/EGFR autonomous growth circuit observed in renal carcinoma cells.

Silencing ADAM17 does not affect proangiogenic phenotypes characteristic of VHL-loss renal carcinoma. Secretion of proangiogenic factors, such as the VEGF, in conjunction with disruption of extracellular matrix deposition has been shown to promote and maintain renal carcinoma tumor angiogenesis (30). To assess whether ADAM17 has functions unrelated to TGF-α processing that may affect the well-established pathways involved in renal carcinoma tumorigenesis, we examined the effect of ADAM17 knockdown on other defects associated with VHL-loss. Silencing ADAM17 did not alter the expression of HIF2α protein (Fig. 4*A*) nor the expression of classic hypoxia-inducible mRNAs, such as glucose transporter-1 (GLUT1) or VEGF (Fig. 4*B*). Additionally, silencing ADAM17 did not restore the ability of renal carcinoma cells to deposit extracellular fibronectin matrix (Fig. 4*C*), a process that can be corrected by reintroduction of VHL (31). Thus, ADAM17 function is unrelated to the major proangiogenic characteristics associated with VHL-loss.

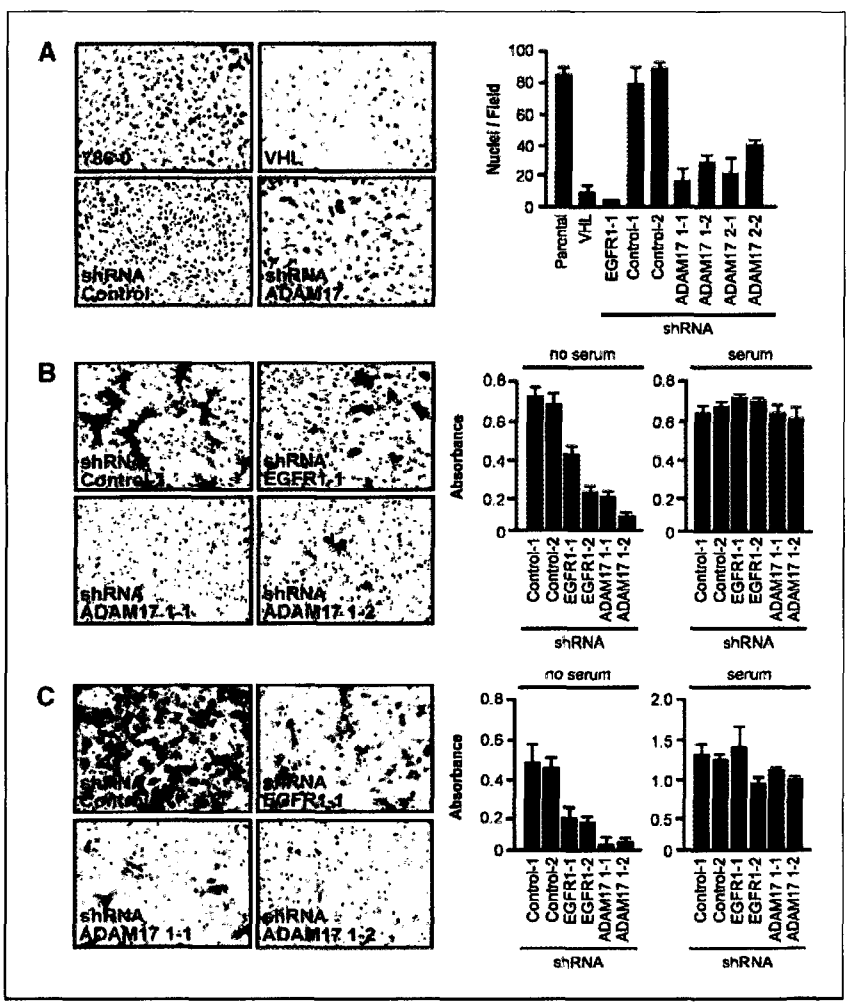

Figure 5. Inhibition of ADAM17 abolishes multiple acquired tumor capabilities *in vitro*. The ability of VHL(−/−) renal carcinoma 786-0 cells, VHL(+) renal carcinoma cells (*VHL*), and 786-0 cells stably expressing shRNA (*Control, EGFR*, and *ADAM17*) to grow in an anchorage-independent manner, migrate toward chemoattractant, and invade basement membrane was assessed *in vitro*. *A*, stable silencing of ADAM17 hinders the ability of renal carcinoma to grow as tumor spheroids. Cells were grown as three-dimensional tumor spheroids for 6 days *in vitro*. Histology from spheroids is visualized at a magnification of ×400. Spheroid density was measured at a magnification of ×100 in at least three independent spheroid sections and reported as nuclei/field. *B*, stable silencing of ADAM17 abolishes the ability of renal carcinoma to migrate. Cells were plated in Boyden chambers to assess their ability to migrate through a porous membrane in the presence or absence of chemoattractant (serum) over a 16-hour period. Photographs depict migration in a serum-free environment (×200). *C*, stable silencing of ADAM17 abolishes the ability of renal carcinoma to invade basement membrane. Invasion of Matrigel in the presence or absence of serum over a 48-hour incubation period. Photographs depict invasion in a serum-free environment (×200). Absorbance readings were taken at 574 nm. *Bars*, SD of at least three independent experiments done in triplicate.

Inhibition of ADAM17 abolishes multiple acquired tumor capabilities *in vitro*. Although the self-sufficiency in growth is likely to be a critical initial step in cellular transformation, cancer cells must also acquire several other characteristics for malignancy. Silencing of ADAM17 prevented the formation of highly dense spheroids (Fig. 5*A*) in an avascular *in vitro* tumor assay that measures the tumorigenic potential of cancer cells (25). No difference in the number of apoptotic cells, as determined by propidium iodide exclusion and nuclear morphology assays, was observed in renal carcinoma cells expressing shRNA against ADAM17 or EGFR compared with parental renal carcinoma (data not shown). However, the migratory ability of renal carcinoma cells expressing shRNA against ADAM17 or EGFR was severely compromised compared with parental and control cells (Fig. 5*B*), and hence they failed to invade basement membrane *in vitro* (Fig. 5*C*). Taken together, these results show that ADAM17 mediates multiple acquired tumor capabilities required for overt malignancy.

Silencing of ADAM17 is sufficient to suppress renal carcinoma tumor formation *in vivo*. Finally, we examined the consequence of silencing ADAM17 on renal carcinoma tumor formation *in vivo*. Parental and control VHL-defective 786-0 renal carcinoma cells formed large tumors detectable 4 to 5 weeks after injection. Reintroduction of a functional copy of VHL was sufficient to prevent tumor formation of VHL-defective renal carcinoma cells, as expected (26). 786-0 cells expressing shRNA ADAM17-1 or ADAM17-2 failed to form tumors in the xenograft mouse assay (Fig. 6*A* and *B*). Similar data were obtained with the VHL-defective KTCL cell line expressing shRNA directed against ADAM17 mRNA although the size of the control tumors was smaller compared with 786-0 renal carcinoma (Fig. 6*B*). Prolonged incubation time resulted in the formation of very small tumors in only two of the 786-0 renal carcinoma cell lines expressing shRNA ADAM17-2, indicating that abolishing ADAM17 function efficiently abrogated the tumorigenic potential of highly malignant renal carcinoma cells. Interestingly, analysis of H&E-stained sections of these small tumors revealed that the typical leukocyte infiltration, observed as darkly stained nuclei, in parental and control renal carcinoma tumors, was significantly less evident when ADAM17 was silenced (Fig. 6*C*). This effect is likely due to the involvement of ADAM17 in the cleavage of the proinflammatory cytokine TNF-α (32). Thus, in addition to its role in suppressing cell proliferation and tumorigenesis, silencing ADAM17 may reduce tumor inflammation, a negative prognostic factor in renal carcinoma (33). Therefore, silencing of ADAM17 is sufficient to abolish *in vivo* tumor formation of highly malignant cell lines derived from human primary renal tumors.

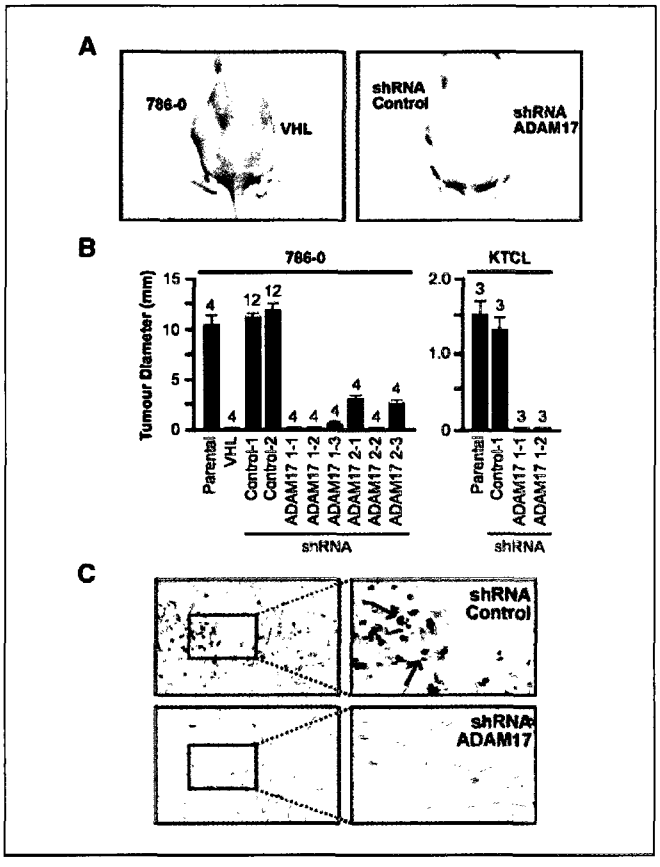

Figure 6. Silencing of ADAM17 is sufficient to suppress renal carcinoma tumor formation *in vivo*. VHL(−/−) renal carcinoma 786-0 and KTCL cells expressing scramble shRNA or one of two shRNA sequences targeting ADAM17 were injected in the flanks of female nude mice. *A*, renal carcinoma stably expressing shRNA targeting ADAM17 fail to form tumors in nude mouse xenograft assays. Representative nude mice injected with parental 786-0, 786-0 expressing wild-type VHL (*VHL*), scramble shRNA (*shRNA Control*), or shRNA against ADAM17 (*shRNA ADAM17*) 7 to 9 weeks postinjection. *B*, average size of tumors observed after 7 to 9 weeks. Number of injections per cell line is indicated. *Columns*, mean; *bars*, SE. *C*, renal carcinoma tumors stably expressing shRNA against ADAM17 exhibit reduced leukocyte infiltration compared with control tumors. Histology from 786-0 tumors is visualized at a magnification of ×400. *Arrows*, leukocytes, which appear as darkly stained nuclei. Experiments were done double-blinded.

Discussion

The primary event leading up to the development of cancer is the establishment of an actively proliferating population of cells. This inappropriate initiation of cell division hinges upon the ability of cancer cells to engage in autonomous proliferation through self-induced signaling cascades, such as the TGF-α/EGFR growth stimulatory circuit (6). Uncontrolled cellular proliferation alone is not sufficient, however, for the progression and persistence of a malignancy. Tumors would not survive without the oxygen and nutrients supplied by the circulatory system (34), nor would they metastasize to distal organs, the major cause of cancer-associated death, without the means to compromise and invade the extracellular matrix (35). The endurance of tumor cells is dependent on five additional acquired traits that bestow them with fundamental growth and survival advantages. In contrast to their normal counterparts, cancer cells are able to resist growth-inhibiting signals and programmed cell death, induce angiogenesis, migrate and invade surrounding tissues, and replicate limitlessly (1).

Together, the results of this study show that silencing one biologically relevant enzyme, ADAM17, is sufficient to abrogate cancer cell growth autonomy, migration and invasion, and likely tumor inflammation. ADAM17 function was analyzed in a model system with endogenous participants, eliminating the possibility of interference due to overproduction of molecules. In this setting, ADAM17-mediated shedding of membrane-bound TGF-α is required for EGFR activation and its ability to drive autonomous proliferation. The ability of TGF-α to promote cell migration has been shown in a multitude of cell types, ranging from mammary epithelial cells to ovarian cancer cell lines (36–38). Furthermore, inhibition of EGFR signaling with receptor tyrosine kinase inhibitors is sufficient to prevent migration of glioblastomas in

an orthotopic nude mouse model (39). Although it is likely that the well-characterized role of ADAM17 as an EGFR ligand sheddase is at the root of the observed phenotypes, further investigation of alternative functions of ADAM17 is required. ADAM17 has previously been linked to the regulation cell-matrix interactions through adhesion molecules such as CD44 and L1, and is known to modulate cell migration by its interaction with various integrins through its disintegrin domain (40–43). Notably, the integrin signaling pathway has also been implicated in focal adhesion kinase–mediated cell migration (44). Although a full-scale study of ADAM17-associated integrins has not yet been conducted, ADAM17 has an emerging role as a modulator of integrin-mediated migration that warrants additional examination (42, 43).

Although silencing of ADAM17 was sufficient to block tumor formation *in vivo* by eradicating at least three of the acquired capabilities characteristic of cancer cells, the angiogenic potential and resistance to apoptosis of the cells seemed to be unaffected by the knockdown. Our results suggest that there is no correlation between ADAM17 expression and HIF2α activation and resistance to cell detachment–induced apoptosis. Based on our observations, inhibition of cell proliferation, rather than enhancement of cell death or blockade of angiogenesis, is sufficient to prevent *in vivo* renal carcinoma tumor formation.

The results shown here argue that ADAM17-mediated shedding of TGF-α is required for renal carcinoma growth autonomy and *in vivo* tumor formation. The data support the notion that ADAM17-mediated ectodomain cleavage of TGF-α is required for EGFR activation and tumor formation in a biologically relevant human cancer model system that expresses endogenous ADAM17, ligand, and receptor. The central role of EGFR signaling in the development of human cancer is well documented and therapeutic agents directed at inhibiting ligand-dependent receptor activation, such as monoclonal antibodies targeting the EGFR and receptor tyrosine kinase inhibitors, have been in clinical trials for a number of years. However, such therapeutic strategies have yielded poor response rates in patients and effective therapies remain elusive (45–48). In light of the fact that ADAM17 plays a central role in acquired tumor cell capabilities, such as tumor cell growth autonomy, inflammation, migration, and invasion, we suggest that ADAM17 is an ideal therapeutic target in the treatment of VHL-defective renal carcinoma and other EGFR autocrine signaling-dependent human cancers.

Acknowledgments

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Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer

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Overexpression of the EGF receptor (EGFR) is a recurrent theme in human cancer and is thought to cause aggressive phenotypes and resistance to standard therapy. There has, thus, been a concerted effort in identifying EGFR gene mutations to explain misregulation of EGFR expression as well as differential sensitivity to anti-EGFR drugs. However, such genetic alterations have proven to be rare occurrences in most types of cancer, suggesting the existence of a more general physiological trigger for aberrant EGFR expression. Here, we provide evidence that overexpression of wild-type EGFR can be induced by the hypoxic microenvironment and activation of hypoxia-inducible factor 2-α (HIF2α) in the core of solid tumors. Our data suggest that hypoxia/HIF2α activation represents a common mechanism for EGFR overexpression by increasing EGFR mRNA translation, thereby diminishing the necessity for gene mutations. This allows for the accumulation of elevated EGFR levels, increasing its availability for the autocrine signaling required for tumor cell growth autonomy. Taken together, our findings provide a nonmutational explanation for EGFR overexpression in human tumors and highlight a role for HIF2α activation in the regulation of EGFR protein synthesis.

hypoxia inducible factor | receptor tyrosine kinase signaling | tumor microenvironment | VHL

Amplification of EGF receptor (EGFR) expression and signaling is a common feature in a variety of human cancers including renal, breast, glioma, ovarian, non-small-cell lung, prostate, pancreatic, and head and neck cancers (1). Ligand-induced activation of the EGFR, a receptor tyrosine kinase, can instigate a wide range of cellular responses such as growth, differentiation, migration, and survival through various signaling pathways (2). Accordingly, it has been shown that persistent activation of the EGFR enables cancer cells to engage in autonomous proliferation, which is the first and debatably the most critical hallmark of cancer (3). Moreover, EGFR expression has long been recognized as a prognostic marker of advanced tumor stage, resistance to standard therapeutic approaches, and reduced patient survival (4). The dependence of certain cancer cells on the EGFR for growth and survival combined with the above-mentioned factors has directed much attention to the EGFR, which is currently a central target for cancer therapy (5).

Despite the plethora of studies aimed at divulging mechanisms by which deregulation of EGFR expression occurs, the cause of EGFR overexpression in human cancer remains unresolved. Aberrant EGFR expression in tumors can arise as a result of amplification of the *EGFR* gene, receptor-activating mutations, or evasion from negative regulatory mechanisms (6, 7). With the exception of a few cancers such as glioblastoma multiforme or non-small-cell lung cancer, that display the highest incidence of *EGFR* gene amplifications and mutations (20–40%), these oncogenic phenomena are scarcely observed in other tumor types (8–13). It would, therefore, be reasonable to speculate that the widespread overexpression of the EGFR in human cancer occurs as a consequence of a common physiological event in tumors in lieu of gene mutations. Solid tumors do share certain universal properties attributable to cellular adaptations to the tumor microenvironment. Abnormal cellular expansion creates intermittent regions of hypoxia as tumor cells distance themselves from blood vessels (14). One compensatory mechanism for this reduced oxygen supply is increased aerobic glycolysis, that results in acidification of the microenvironment or the "Warburg effect" (15). Thus features such as tumor hypoxia, acidosis, and nutrient deprivation are observed in the vast majority of human malignancies. A key regulatory protein involved in the response to such cellular conditions is the hypoxia inducible factor (HIF) (16). HIF is a transcription factor that drives expression of a multitude of genes involved in the control of anaerobic metabolism, neovascularization, pH, and survival (17). Importantly, tumor hypoxia, like EGFR expression, is predictive of tumor progression and poor clinical outcome, and a correlation between the two has been reported (18, 19).

In this study, we show that EGFR protein levels are up-regulated in response to a physiological trigger, and are not due to mutational events, in a panel of human cancer cell lines. We demonstrate that tumor hypoxia/HIF2α activation elicits an increase in EGFR protein synthesis, and hence receptor expression, that is required for tumor cell growth autonomy. Our data provide evidence of a common pathway whereby wild-type receptor is overexpressed in human cancers as a result of tumor hypoxia/HIF2α activation and not necessarily because of genetic alterations.

Results

The Hypoxic Tumor Microenvironment Triggers EGFR Expression. The EGFR has been implicated in the pathogenesis of many human cancers. The widespread overexpression of the receptor in such cancers remains unexplained because mutations of the *EGFR* gene are seldom observed. We hypothesized that this overexpression of the EGFR is the result of common physiological stressors observed in the tumor microenvironment, rather than genetic alterations. We first examined whether factors in the tumor microenvironment can in fact lead to the accumulation of EGFR using a 3D multicellular tumor spheroid model to mimic such conditions (20). We focused on the U87MG glioma cell line because it expresses low levels of wild-type EGFR, and yet amplification of the EGFR is believed to be a fundamental event in the development of glioblastoma multiforme, a highly malignant form of brain cancer (8). Western blot analysis of lysates from U87MG spheroids revealed significantly

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Abbreviations: EGFR, EGF receptor; HIF, hypoxia inducible factor.

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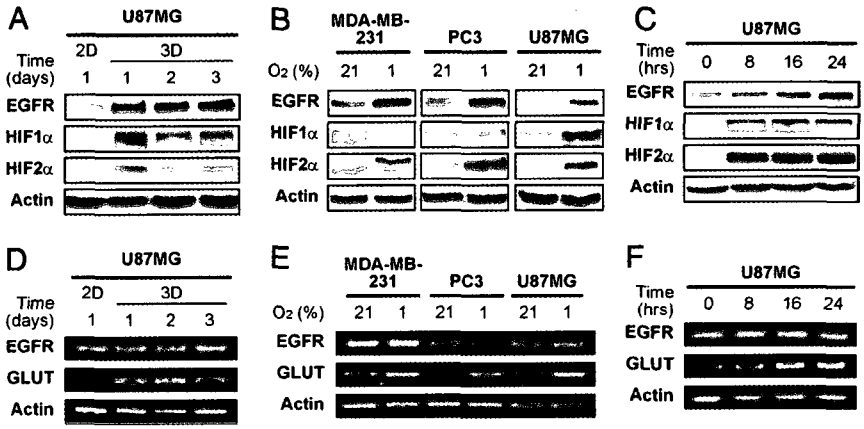

Fig. 1. The hypoxic tumor microenvironment triggers EGFR expression. (*A*) Western blot analysis of total EGFR and HIFα levels in U87MG glioma cells grown as 3D spheroids for 1–3 days. Control cells were grown in 2D culture. (*B*) Western blot analysis of EGFR and HIFα levels in U87MG, MDA-MB-231 breast, and PC3 prostate cancer cells exposed to normoxia (21% O_2) or physiological hypoxia (1% O_2) for 24 h. (*C*) Western blot analysis of EGFR and HIFα levels in U87MG cells exposed to hypoxia for the indicated times. (*D–F*) RT-PCR analysis of EGFR and GLUT mRNA levels in cells described in *A–C*. Actin served as a loading control in *A–F*.

higher levels of EGFR protein compared with control cells grown in 2D culture (Fig. 1*A*). These results suggest that EGFR protein levels can be up-regulated because of inherent features of the tumor microenvironment. It has been well established that the core of tumors is hypoxic, and that this hypoxic milieu is sufficient to induce activation of HIF and expression of hypoxia inducible genes that promote tumor growth and invasiveness (21). Fittingly, we observed stabilization of the two isoforms of the α-subunit of HIF, HIF1α and HIF2α, in the U87MG spheroids (Fig. 1*A*). This was in contrast to cells cultured in standard growth conditions that failed to express detectable levels of either HIFα isoform. We thus reasoned that the elevated EGFR levels observed in human cancers might be the result of tumor hypoxia. To assess whether the EGFR is hypoxia inducible, we measured EGFR levels in three independent human cancer cell lines after exposure to physiological hypoxia (1% O_2). U87MG cells, MDA-MB-231 breast cancer and PC3 prostate cancer cells all expressed increased EGFR protein levels after exposure to hypoxia for 24 h (Fig. 1*B*). Similar results were obtained with ovarian (OVCAR8) and lung (HOP62) cancer cell lines (data not shown). The HIF2α isoform was strongly up-regulated in all three cell lines, whereas there was variability in HIF1α stabilization (Fig. 1*B*). This is in accordance with a recent study that showed that HIF2α expression is more persistent over time in certain cell types and is associated with a more aggressive tumor phenotype (22). To better examine the kinetics involved in the hypoxic up-regulation of EGFR protein levels, we conducted a time-course experiment using the U87MG cell line. There was a measurable increase in EGFR protein levels within 8 h of hypoxic exposure (Fig. 1*C*). To determine whether this increase in EGFR protein was a consequence of transcriptional up-regulation, we examined EGFR mRNA levels in tumor spheroids (Fig. 1*D*) and in hypoxic cancer cells (Fig. 1 *E* and *F*). As expected, there was hypoxic induction of glucose transporter-1 (GLUT) mRNA, a known HIF target gene, in both settings (16). An analogous increase in EGFR mRNA levels was not observed, indicating that the EGFR is not induced at the transcriptional level under hypoxic conditions (Fig. 1 *D–F*). These data suggest a general mechanism by which the hypoxic tumor microenvironment elicits the up-regulation of EGFR protein, but not mRNA, levels in human cancer cells.

HIF2α Activation Results in Induction of EGFR Protein Levels. We next examined whether the observed induction of EGFR protein in tumor spheroids and in hypoxic cells was a function of HIF activity. U87MG cells were infected with adenovirus expressing a dominant-negative form of HIF (dnHIF) or flag-tagged GFP as a viral control, and cultured as 3D spheroids (23, 24). Expression of dnHIF diminished GLUT levels, as expected, and EGFR up-regulation in U87MG spheroids, demonstrating that the increased EGFR levels observed in this model system of the tumor microenvironment is the result of HIF activation (Fig. 2*A*). Similarly, inhibition of HIF activity by dnHIF prevented EGFR up-regulation in U87MG and MDA-MB-231 cells exposed to hypoxia, indicating that HIF activity is also required for the hypoxic induction of EGFR protein (Fig. 2*B*). Next, we evaluated the individual contributions of the two HIFα isoforms in the up-regulation of EGFR protein expression. To this end we attempted to recapitulate EGFR up-regulation under normoxic conditions by infecting U87MG cells with adenoviruses expressing HIF1α or HIF2α variants that are stable in the presence of oxygen (25, 26). Expression of HIF2α, but not HIF1α, was sufficient to induce an increase in EGFR protein under normoxic conditions (Fig. 2*C*). Although expression of either HIF1α or HIF2α resulted in an induction of GLUT mRNA, verifying that both variants are transcriptionally active, neither isoform promoted the accumulation of EGFR mRNA (Fig. 2*D*). Consistent with these observations, siRNA-mediated silencing of HIF2α was sufficient to abolish EGFR induction under hypoxic conditions (Fig. 2*E*). No such effect was observed when cells were transfected with siRNA against HIF1α or control scramble siRNA. Finally, we examined the role of the mTOR (mammalian target of rapamycin) pathway, which is involved in HIFα mRNA translation, in the up-regulation of EGFR protein levels (27). Predictably, pretreatment of U87MG cells with rapamycin prevented accumulation of HIF2α and hence EGFR in hypoxia (Fig. 2*F*). Thus activation of HIF, and more specifically of the HIF2α isoform, is required and sufficient for the up-regulation of EGFR protein in hypoxic cancer cells.

HIF2α Activation Results in Increased EGFR mRNA Translation. We next wanted to determine at what level HIF2α activation affects EGFR protein expression. To address this question, we exploited VHL-loss clear cell renal carcinoma cells ($VHL^{-/-}$ RCC), that express only HIF2α and irrespectively of oxygen tension, as a model system for studying EGFR metabolism in the presence of endogenous HIF2α activity (28, 29). The $VHL^{-/-}$ RCC 786-0 cell line expresses significantly higher levels of EGFR protein, but not mRNA, compared with 786-0 cells stably expressing wild-type VHL (VHL^{+} RCC), which prevents the normoxic accumulation of HIF2α, consistent with the data in Figs. 1 and 2 and reports by another group (Fig. 3*A*) (30). Moreover, similar results were obtained with 786-0 cells infected with adenovirus expressing

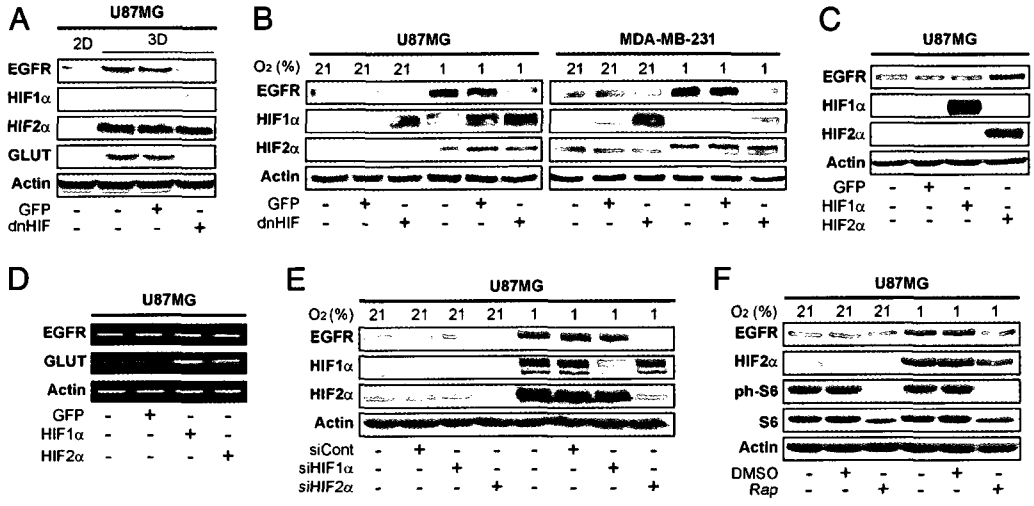

Fig. 2. HIF2α activation results in induction of EGFR protein levels. (*A*) Western blot analysis of EGFR, HIFα, and GLUT levels in U87MG cells infected with adenovirus expressing flag-tagged GFP or a dominant-negative form of HIF (dnHIF). Cells were grown as 3D spheroids for 3 days. Control cells were grown in 2D culture for the same period. (*B*) Western blot analysis of EGFR and HIFα levels in U87MG and MDA-MB-231 cells infected with GFP or dnHIF for 24 h. before exposure to normoxia or hypoxia for an additional 24 h. (*C* and *D*) Western blot (*C*) and RT-PCR (*D*) analysis of EGFR levels in U87MG cells infected to express GFP or HIFα variants (HIF1α and HIF2α) for 72 h in normoxia. (*E*) Western blot analysis of EGFR and HIFα levels in U87MG transiently transfected with siRNA (50 nM) targeting HIF1α (siHIF1α), HIF2α (siHIF2α), or a scramble sequence (siCont) for 48 h before exposure to hypoxia for an additional 24 h. (*F*) Western blot analysis of EGFR and HIF2α levels in U87MG pretreated with 10 nM rapamycin (Rap), or DMSO as a vehicle control, for 1 h and then exposed to normoxia or hypoxia for an additional 16 h. The phosphorylation status of the S6 ribosomal protein (ph-S6) served as a control for drug activity. Actin served as a loading control in *A–F*.

GFP-tagged VHL as well as an additional 786-0 cell line stably expressing GFP-tagged VHL, indicating that the reduction in EGFR protein upon reintroduction of VHL is not due to acquired clonal variation [see supporting information (SI) Fig. 5 *A–D*]. To confirm that the elevated EGFR protein levels in 786-0 cells were a result of HIF2α activation rather than loss of VHL, we infected 786-0 cells with adenovirus expressing GFP or dnHIF for up to 4 days. Inhibition of endogenous HIF2α activity with dnHIF resulted in a time-dependent decrease in total EGFR protein levels indicating that HIF2α activity is required for the high levels of EGFR protein in $VHL^{-/-}$ RCC as well (Fig. 3*B*). Expression of dnHIF prevented induction of GLUT mRNA, controlling for inhibition of HIF activity, but failed to affect EGFR mRNA levels (Fig. 3*B Right*). Finally, we examined the roles of the two HIFα isoforms in the up-regulation of EGFR protein expression in $VHL^{-/-}$ RCC. Once again, expression of HIF2α, but not HIF1α, resulted in an increase in EGFR protein (Fig. 3*C*), but not mRNA (data not shown), in VHL^{+} RCC cells under normoxic conditions, corroborating the pivotal role of this HIF isoform in the regulation of EGFR protein levels.

Given that there was no difference in EGFR mRNA levels in the presence and absence of HIF, we hypothesized that the elevated levels of EGFR in HIF2α-expressing cells could be caused by a greater rate of translation relative to other cells. To better ascertain the intricacies of EGFR metabolism in $VHL^{-/-}$ RCC cells compared with their VHL-positive counterparts, the rate of EGFR protein synthesis was assessed by using metabolic labeling techniques. The 786-0 $VHL^{-/-}$ RCC and VHL^{+} RCC cell lines were labeled with [^{35}S]methionine ([^{35}S]Met) for different lengths of time, lysed and immunoprecipitated with an antibody specific to the EGFR (Fig. 3*D*). There was a marked and consistent increase in [^{35}S]Met incorporation in the 786-0 cells compared with the VHL-positive cells even within very short labeling periods (Fig. 3*D*). This increase was abolished upon adenoviral infection of 786-0 cells with dnHIF, demonstrating that the increased rate of EGFR protein synthesis is HIF2α-dependent (Fig. 3*E*). Radiolabel incorporation during short pulses generally reflects rates of protein synthesis rather than protein turnover. To further ensure that the difference in [^{35}S]Met incorporation (Fig. 3*D*) was not due to very rapid receptor turnover in VHL-positive cells, a pulse–chase experiment was conducted with $VHL^{-/-}$ RCC and VHL^{+} RCC cells. No receptor degradation was observed within 8 h of chase in either cell line, in line with studies that have reported receptor half-lives of 15–30 h in the absence of exogenous ligand, such that the observed difference in [^{35}S]Met incorporation could not be attributed to enhanced receptor degradation in VHL^{+} RCC (Fig. 3*F*) (31, 32). The increase in radiolabel incorporation at 4 h of chase is in accordance with other reports that have shown posttranslational modification of the receptor (31, 32). Similarly, a reduction in total receptor levels was not observed upon inhibition of protein synthesis within a period of 4 h. (SI Fig. 6*A*) and addition of several proteasomal and lysosomal inhibitors had no demonstrable effect on EGFR protein levels, regardless of VHL and HIF status in RCC, glioma and breast cancer cell lines (SI Fig. 6 *C* and *D* and data not shown). Consistent with the [^{35}S]Met labeling data, inhibition of HIF2α activity in 786-0 cells with dnHIF resulted in a shift of the EGFR mRNA to monosomes from actively translating polysomes, as assessed by RT-PCR analysis (Fig. 3*G* and SI Fig. 7 *A–C*). These data provide evidence that EGFR protein synthesis is up-regulated under hypoxic conditions and uncover a previously uncharacterized role for HIF2α activation in the translational control of the EGFR.

HIF2α Activation and EGFR Overexpression Are Required for Growth Autonomy. Overproduction of transforming growth factor-α (TGFα) is thought to be required for the growth autonomy of a variety of human cancers (1). Both soluble TGFα and EGFR expression are necessary for autocrine growth signaling and VHL-loss RCC tumor formation (24, 25, 33, 34). It is thus of interest to distinguish whether HIF2α-mediated production of TGFα is sufficient or whether the above-described boost in EGFR protein synthesis is also required for RCC growth autonomy. VHL^{+} RCC stimulated with exogenous TGFα failed to grow in the absence of serum, as measured by BrdU incorporation, indicating that overproduction of ligand alone is not sufficient to sustain growth autonomy (Fig. 4*A*). Conversely, adenoviral expression of HIF2α was sufficient to promote serum-independent growth of VHL^{+} RCC, indicating that HIF2α has a function other than the induction of TGFα that is required for autonomous growth (Fig. 4*A*). As

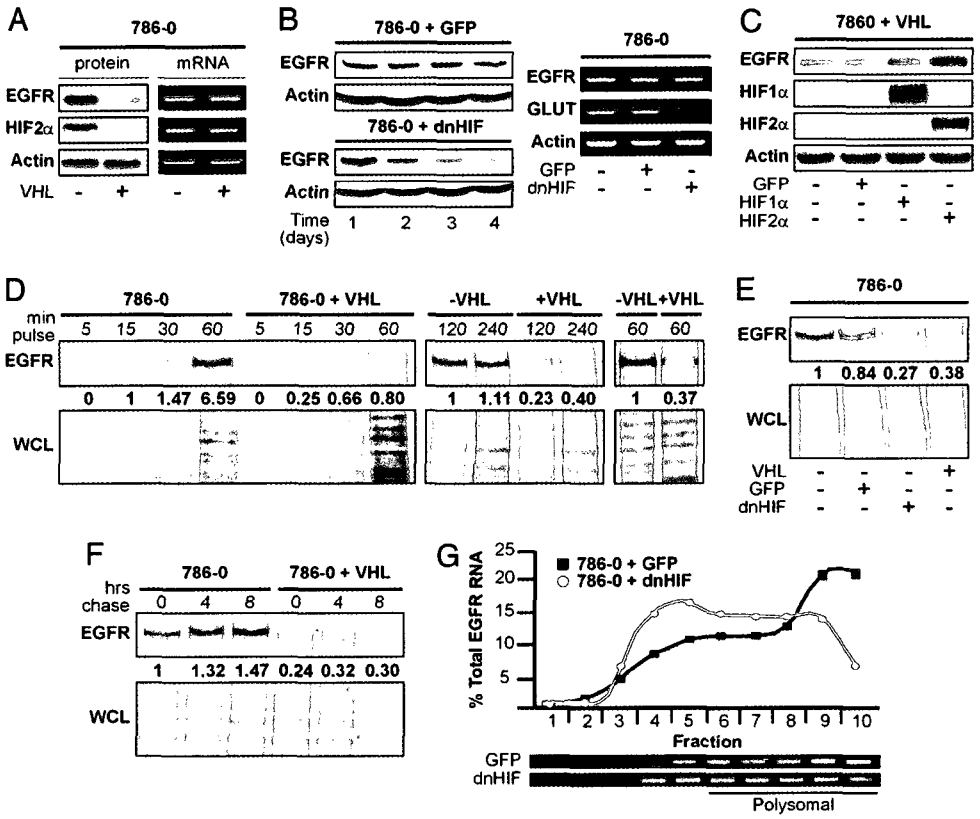

Fig. 3. HIF2α activation results in increased EGFR mRNA translation. (*A*) Western blot and RT-PCR analysis of EGFR protein and mRNA levels in the VHL$^{-/-}$ RCC, 786-0, cell line (786-0), and 786-0 cells stably expressing wild-type VHL (786-0 + VHL). (*B*) Western blot (*Left*) and RT-PCR (*Right*) analysis of EGFR protein and mRNA levels in 786-0 cells infected with adenovirus expressing GFP or dnHIF for 24–96 h. (*C*) Western blot analysis of EGFR protein levels in 786-0 + VHL cells infected with GFP, HIF1α or HIF2α for 72 h. Actin served as a loading control in *A–C*. (*D*) EGFR radiolabel incorporation in cells pulsed for the indicated times with [^{35}S]methionine ([^{35}S]Met) and immunoprecipitated with an anti-EGFR antibody. Cells were also labeled and immunoprecipitated with a second EGFR antibody as a specificity control (*Right*). (*E*) EGFR [^{35}S]Met incorporation in 786-0 cells infected with GFP or dnHIF for 72 h and labeled for 1 h. (*F*) EGFR [^{35}S]Met incorporation in cells labeled for 2 h and cold-chased for indicated times. Whole-cell lysates (WCL) served as radiolabel incorporation and loading controls in *D–F*. (*G*) RT-PCR analysis of EGFR mRNA levels in polysomal fractions of 786-0 cells infected with GFP or dnHIF for 48 h and subjected to sucrose gradient fractionation. The percentage of total EGFR mRNA in each fraction is plotted.

expected, inhibition of HIF2α activity by adenoviral dnHIF expression in VHL$^{-/-}$ RCC abolished the ability of the cells to incorporate BrdU in the absence of serum (Fig. 4*B*). Although stimulation with excess ligand instigated a slight rise in the number of proliferating GFP-infected control cells, it failed to rescue autonomous growth in the absence of HIF2α activity (Fig. 4*B*). We did, however, note a transient growth spurt in the latter cells, demonstrating that there is an immediate proliferative response to exogenous TGFα that is not sustained over time in cells expressing minimal EGFR likely because of negative regulatory mechanisms (35). Finally, we wanted to directly examine the effect of varying EGFR levels on the ability of VHL$^{-/-}$ RCC to engage in autonomous proliferation. EGFR protein levels were diminished in a dose-dependent manner by transient silencing of the receptor with various concentrations of siRNA (Fig. 4*C*). There was a corresponding decrease in the serum-independent growth of cells expressing siRNA targeting the EGFR, indicating that the ability of cells to engage in autonomous growth depends on the amount of receptor expressed (Fig. 4*D*). HIF2α-dependent overproduction of the EGFR did not, however, affect its inherent susceptibility to the actions of receptor tyrosine kinase inhibitors (Fig. 4*E* and SI Fig. 8 *A–F*). Notably, the IC_{50} of drug for inhibition of kinase activity was a function of the amount of receptor on a per cell basis in the presence and absence of HIF2α activity. These results emphasize the importance of HIF2α in maintaining adequate EGFR expression and availability for the autocrine ligand action that enables autonomous proliferation of tumor cells and endorse targeting the EGFR for therapeutic purposes in HIF2α-expressing cancers.

Discussion

Here, we report that hypoxia initiates an oncogenic program that results in the translational up-regulation of the EGFR, in a HIF2α-dependent manner. Our results provide an explanation for the lack of receptor mutations in the majority of human tumors that overexpress EGFR protein and demonstrate that alterations of the *EGFR* gene are not required because cancer cells have evolved a physiological mechanism by which the receptor can be up-regulated. Furthermore, we show that the incessant production of EGFR protein allows for sustained receptor signaling and thus promotes the autonomous growth of cancer cells. These findings reveal an important link between tumor hypoxia and up-regulation of the EGFR in the bulk of human cancers that do not display genetic alterations of the receptor. As such, we propose an alternative working model by suggesting that tumor hypoxia may represent the common denominator for the aberrant EGFR expression observed in solid tumors.

It will be of great interest to delineate the precise mechanism by which EGFR protein synthesis is induced under hypoxic conditions. Hypoxia/HIF2α activation may result in the expression of a hypoxia-inducible activator of EGFR translation or alternatively may inhibit a negative regulator of receptor synthesis. Identification of participating molecules could lead to the discovery of novel

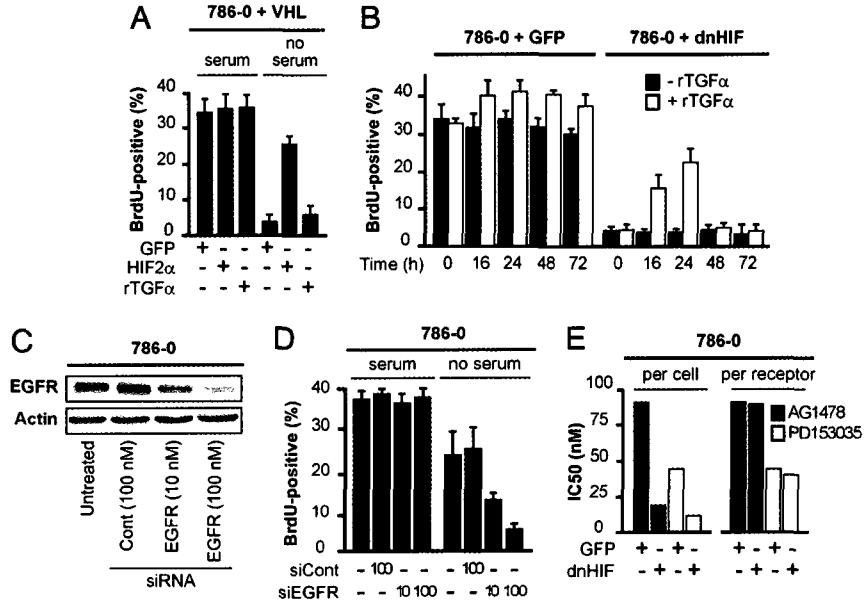

Fig. 4. Overexpression of EGFR results in cancer cell growth autonomy but does not affect sensitivity to anti-EGFR drugs. (*A*) VHL-competent RCC cells (786-0 + VHL) were infected with adenovirus expressing GFP or HIF2α or treated with 20 ng/ml recombinant TGFα (rTGFα) and then cultured in the absence or presence of serum for 72 h before labeling with BrdU. (*B*) BrdU incorporation in VHL-deficient 786-0 cells infected with GFP or dnHIF after addition of rTGFα for the indicated times in serum-free media. (*C*) Western blot analysis of EGFR levels in 786-0 cells transiently transfected with the indicated amounts of siRNA (10–100 nM) targeting the EGFR or a scramble control sequence for 72 h. Actin served as a loading control. (*D*) BrdU incorporation in cells described in *C*. Bars represent standard deviation of at least three independent experiments in *A*, *B*, and *D*. (*E*) IC_{50} concentrations of receptor tyrosine kinase inhibitors, AG1478 and PD153035, for inhibition of EGFR kinase activity in 786-0 cells infected with GFP or dnHIF for 48 h.

oncogenes or tumor suppressors, respectively. It would be tempting to speculate that direct genetic alteration of such molecules could contribute to receptor up-regulation in the absence of tumor hypoxia or HIF2α activity. Further studies would provide great insight into EGFR expression dynamics and could potentially reveal novel and perhaps more effective therapeutic targets in the treatment of EGFR-related cancers.

Clinical responses to EGFR-based treatments have been largely disappointing owing to intrinsic and/or acquired resistance to such drugs (8, 36–38). Although there have been conflicting reports regarding the correlation between EGFR expression and clinical responses to receptor tyrosine kinase inhibitors, elevated receptor levels have been associated with drug sensitivity and improved patient survival. Our results suggest that EGFR overexpression does not affect receptor sensitivity to such inhibitors at the biochemical level. We did, however, observe an increased IC_{50} on a per cell basis in HIF2α-expressing cells proportional to their elevated EGFR levels, such that the continuous administration of higher drug concentrations would likely be required to efficiently compete constitutive receptor production clinically. We suggest that direct inhibition of HIF2α may provide a means of circumventing EGFR resistance issues by preventing receptor up-regulation in the first place. Although no such inhibitors are currently in existence, one approach that is of appeal and certainly warrants further examination is the simultaneous inhibition of the mTOR and EGFR pathways, which has rendered promising results in recent studies (30, 39).

In conclusion, we provide evidence that hypoxia/HIF2α activation mediates the up-regulation of EGFR protein levels, providing a nonmutational explanation for the receptor overexpression so commonly observed in human cancers. The data presented in this contribution also introduce the intriguing possibility that the tumor microenvironment may act as a universal oncogenic trigger that drives the autonomous growth of tumor cells. We suggest that future studies should focus on targeting HIF2α activity as a means of preventing not only angiogenesis but also tumor proliferation.

Materials and Methods

Cell Lines. The VHL-deficient, 786-0 RCC cells were purchased from the American Type Culture Collection (Manassas, VA). The 786-0 cells stably transfected with hemagglutinin-tagged VHL (WT7) were a kind gift from W. G. Kaelin (Harvard University, Boston, MA). The MDA-MB-231, PC3, and U87MG human cancer cell lines were kind gifts from John Bell and Ian Lorimer (Ottawa Regional Cancer Center, Ottawa, ON, Canada). Normoxic cells were maintained in DMEM supplemented with 5% FBS at 37°C in a 5% CO_2 environment. Hypoxic cells were incubated in a hypoxia chamber at 37°C in a 1% O_2, 5% CO_2, and N_2-balanced atmosphere. Serum-free medium consisted of DMEM supplemented with 1% insulin–transferrin–selenium (ITS; Invitrogen, Burlington, ON, Canada).

***In Vitro* Tumor Spheroids.** Multicellular spheroids were prepared as described (20, 25). Briefly, cells (10^5) were plated in 24-well plates precoated with 250 μl of 1% Seaplaque agarose (Cambrex, Rockland, ME). To promote cell–cell adhesion, plates were gently swirled 30 min after plating. Spheroids were grown for indicated times, harvested, and lysed with 4% SDS in PBS before immunoblotting.

Western Blot Analysis. Cells were harvested and lysed in 4% SDS in PBS. Samples were separated by SDS/PAGE and transferred to a PVDF membrane. Membranes were blocked in 5% wt/vol milk before incubation with primary antibody. Monoclonal antibodies were used to detect EGFR (Ab-12; LabVision, Fremont, CA) and HIF1α (BD Transduction Laboratories, Lexington, KY). Polyclonal antibodies were used to detect HIF2α (Novus, Littleton, CO), phospho-S6 ribosomal protein (Ser-235/236; Cell Signaling Technology, Danvers, MA), total S6 ribosomal protein (Cell Signaling Technology), phosphorylated EGFR (Tyr-1173; Santa Cruz Biotechnology, Santa Cruz, CA), and actin (Sigma, St. Louis, MO). Membranes were then blotted with HRP-conjugated anti-mouse

(Amersham Biosciences, Piscataway, NJ) or anti-rabbit (Jackson ImmunoResearch, West Grove, PA) secondary antibodies. Bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL).

RT-PCR Analysis. Total RNA was collected by using TriPure isolation reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. RT-PCR was performed on 1 μg of RNA by using the One-Step SuperScript RT Platinum TaqRT-PCR kit (Invitrogen). The primer sequences used are as follows: EGFR forward: 5′-ACCTGCGTGAAGAAGTGTCC-3′; EGFR reverse: 5′-CACATCTCCATCACTTATCTCC-3′; HIF2α forward: 5′-CGGAGAGGAGGAAGGA GAAG-3′; and HIF2α reverse: 5′-GCCATTCATGAAGAAGTCCC-3′. All other primer and cycle details were as described (24). Products were analyzed by gel electrophoresis and ethidium bromide staining with a Digital Science IC440 system (Kodak, Rochester, NY).

Adenoviral Infections. Adenoviruses encoding GFP, dnHIF, and the HIFα variants were generated as described (24, 25). Vectors encoding mutated HIF1α (P405A and P531A) and HIF2α (P402A and P406A) subunits were a kind gift from W. G. Kaelin. Cells were infected with equal multiplicity of infection of each virus in all experiments.

Radioisotope Labeling. Cells were grown in 10-cm plates for 72 h in serum-free medium, incubated for 30 min in glutamine-, methionine, and cysteine-free DMEM and then labeled with [^{35}S]Met [50 μCi/ml (1 Ci = 37 GBq)] for indicated times. For pulse–chase experiments, cells were labeled with [^{35}S]Met for 2 h, washed with PBS and cold-chased in DMEM supplemented with 1% ITS for indicated times. Semiquantitative analysis of radiolabel incorporation was performed by measurement of band intensities, less background readings for equivalent areas, as determined by using Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

Immunoprecipitation. Cells were washed with PBS and lysed with modified RIPA buffer [Tris·HCl (pH 7.4)/1% Nonidet P-40/0.25% sodium deoxycholate/150 mM NaCl/1 mM EDTA/1 mM PMSF/1 μg/ml aprotinin/leupeptin/pepstatin/1 mM Na_3VO_4/1 mM NaF] by rocking for 30 min at 4°C. The cell lysates were clarified by centrifugation at 14,000 × g for 15 min, and supernatants were collected and incubated overnight at 4°C with an agarose-conjugated EGFR antibody (Santa Cruz Biotechnology) or with an anti-EGFR antibody (Ab-12; LabVision) coupled with Protein A/G PLUS Agarose (Santa Cruz Biotechnology) while mixing on an orbital rocker. Immunoprecipitates on beads were washed three times with modified RIPA by centrifugation at 1,000 × g, dissolved in electrophoresis sample buffer, and boiled for 5 min. Samples were separated on a 6% SDS-polyacrylamide gel. After electrophoresis, the gels were dried at 80°C and exposed to autoradiography film (BioMax MS Film; Kodak) overnight.

Polysomal Analysis. See *SI Methods* for details.

BrdU Labeling. BrdU incorporation assays were performed as described (34). Briefly, cells were plated on glass coverslips and incubated for indicated times in DMEM supplemented with 5% FBS or 1% ITS. Cells were labeled with BrdU, fixed, and stained according to the manufacturer's protocol (Roche Molecular Biochemicals). Coverslips were counterstained with Hoechst reagent (Hoechst 33258; Sigma), and the percentage BrdU incorporation was assessed by fluorescence microscopy.

RNA Interference. Commercially available double-stranded 21-nucleotide-long siRNA targeting the EGFR, HIF1α, HIF2α, and negative control siRNA were obtained from Ambion (Austin, TX). HIF1α Sequence (5′-3′) sense: GGGUAAAGAACAAAACACA; HIF1α antisense: UGUGUUUUGUUCUUUACCC (siRNA ID # 42840); HIF2α Sequence (5′-3′) sense: GGUUUUGUUGCUAGCCCUU; HIF2α antisense: AAGGGCUAGCAACAAAACC (siRNA ID no. 106447). The siRNA sequence targeting the EGFR was described (25). Cells (10^5) were transiently transfected with siRNA for 48–72 h in serum-free media by using Effectene reagent (Qiagen, Valencia, CA).

Receptor Tyrosine Kinase Inhibitor Assays. See *SI Methods* for details.

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Human cancers converge at the HIF-2α oncogenic axis

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Cancer development is a multistep process, driven by a series of genetic and environmental alterations, that endows cells with a set of hallmark traits required for tumorigenesis. It is broadly accepted that growth signal autonomy, the first hallmark of malignancies, can be acquired through multiple genetic mutations that activate an array of complex, cancer-specific growth circuits [Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57–70; Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. *Nat Med* 10:789–799]. The superfluous nature of these pathways is thought to severely limit therapeutic approaches targeting tumor proliferation, and it has been suggested that this strategy be abandoned in favor of inhibiting more systemic hallmarks, including angiogenesis (Ellis LM, Hicklin DJ (2008) VEGF-targeted therapy: Mechanisms of anti-tumor activity. *Nat Rev Cancer* 8:579–591; Stommel JM, et al. (2007) Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science* 318:287–290; Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2:727–739; Kaiser J (2008) Cancer genetics: A detailed genetic portrait of the deadliest human cancers. *Science* 321:1280–1281]. Here, we report the unexpected observation that genetically diverse cancers converge at a common and obligatory growth axis instigated by HIF-2α, an element of the oxygen-sensing machinery. Inhibition of HIF-2α prevents the in vivo growth and tumorigenesis of highly aggressive glioblastoma, colorectal, and non–small-cell lung carcinomas and the in vitro autonomous proliferation of several others, regardless of their mutational status and tissue of origin. The concomitant deactivation of select receptor tyrosine kinases, including the EGFR and IGF1R, as well as downstream ERK/Akt signaling, suggests that HIF-2α exerts its proliferative effects by endorsing these major pathways. Consistently, silencing these receptors phenocopies the loss of HIF-2α oncogenic activity, abrogating the serum-independent growth of human cancer cells in culture. Based on these data, we propose an alternative to the predominant view that cancers exploit independent autonomous growth pathways and reveal HIF-2α as a potentially universal culprit in promoting the persistent proliferation of neoplastic cells.

epidermal growth factor receptor | growth signaling | hypoxia-inducible factor | insulin-like growth factor receptor | oncogene

Cancer is caused by a succession of genotypic changes that confer cells with six rate-limiting traits, coined the hallmarks of cancer, required for tumorigenesis (1). These hallmarks include the ability to proliferate in a growth signal-independent manner, evade anti-growth and proapoptotic signals, induce new blood vessel formation, and invade surrounding tissues. The latter attributes essential for tumor progression are largely dependent on physiological parameters and thus involve more widespread mechanisms. In contrast, cell autonomous proliferative capability, the first hallmark of cancers, is acquired through the genetic activation of any number of dominant oncogenes or inactivation of tumor suppressor genes (2). These complexities are amplified by emerging evidence that multiple redundant signaling pathways can be activated within a single cancer (3, 4). As such, the current belief is that cancer cells evolve in a parallel manner to attain growth autonomy, and any attempts at antagonizing these pathways would be restricted to cancers with defined mutational profiles.

The phenomena referred to as oncogene addiction and tumor suppressor gene hypersensitivity further substantiate that various genetic alterations can confer a selective growth advantage to mutant cells. The oncogene addiction theory contends that, despite the myriad of genetic aberrations observed in an individual cancer, disruption of a central oncogenic pathway would cause growth inhibition and likely tumor regression (5). It has, for instance, been demonstrated that continued expression of *KRAS* and *MYC* are required for maintenance of the tumorigenic state in lung tumors and osteogenic sarcomas induced by the corresponding oncogenes (6, 7). The efficacy of agents targeting BCR/ABL and HER-2 in patients with chronic myeloid leukemia and breast carcinomas, respectively, similarly provides important clinical evidence that human cancers may rely wholly on a single gene, and the specific pathways it impinges on, to sustain tumor growth (8, 9).

Restoration of tumor suppressor function has also been shown to inhibit cancer cell growth. A classic example of this is the reintroduction of a wild-type copy of the von Hippel-Lindau (VHL) tumor suppressor gene in clear cell renal carcinoma (RCC) (10). In this model system, loss of VHL results in the constitutive stabilization of the hypoxia-inducible factor (HIF) and its subsequent activation of the circuits that drive RCC tumorigenesis (11–14). The HIF-2α isoform in particular promotes autocrine growth signaling and cell cycle progression via epidermal growth factor receptor (EGFR) and c-Myc-dependent mechanisms (15, 16). Expression of HIF is not, of course, unique to RCC and is observed in the vast majority of overt carcinomas (17). In addition to being the primary cellular response to hypoxia, HIF activation is endorsed by many oncogene and tumor suppressor gene pathways that increase its synthesis or stability (18). Given that virtually all cancers exploit HIF to attain the angiogenic phenotype, we hypothesized that they might funnel through the HIF-2α pathway as a systemic means of acquiring growth autonomy in an analogous manner.

Here, we show that silencing HIF-2α abrogates the in vivo proliferation and tumorigenesis of a panel of genetically diverse human cancers. We provide mechanistic evidence that this effect can be attributed to the activation of key receptor tyrosine kinases, including EGFR and IGF1R, and their major downstream signaling pathways. Given the catalog of genetic mutations observed in human cancers, obstructing more general processes such as angiogenesis has been favored over the specific targeting of oncogenic pathways (4, 19, 20). We propose that HIF-2α inhibition constitutes a method of targeting the autonomic growth capabilities of tumor cells and may be of broad clinical interest in the treatment of cancers with variable genetic profiles and tissue distributions.

Results

Inhibition of HIF-2α Prevents the Tumorigenesis of Genetically Diverse Human Cancers. The unique ability of HIF-2α to drive VHL-loss RCC growth autonomy and tumorigenesis is well-documented (11, 13, 16, 21). Since HIF-2α is frequently expressed in the core of human tumors we reasoned that it may also activate autonomous

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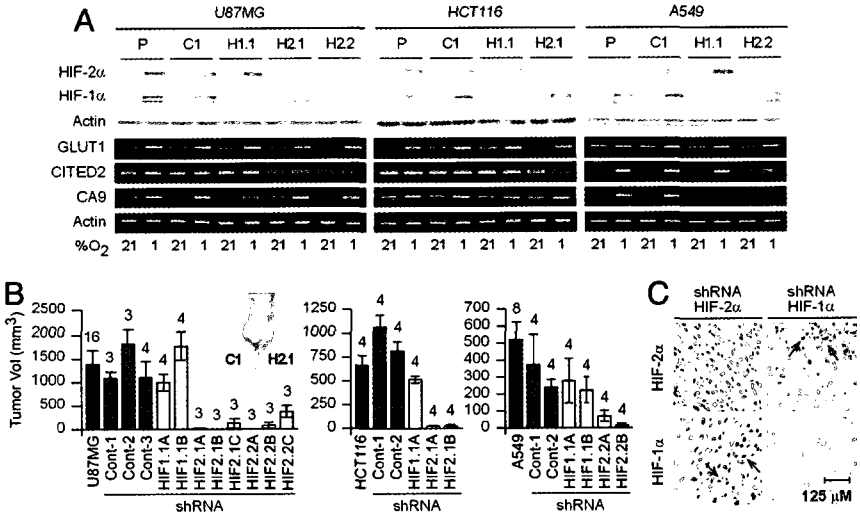

Fig. 1. Inhibition of HIF-2α prevents the tumorigenesis of genetically diverse human cancers. (*A*) Western blot (*Upper*) and RT-PCR (*Lower*) analysis of HIF-α subunit and target gene expression in serum-starved U87MG glioblastoma, HCT116 colorectal and A549 lung carcinoma cells stably expressing scramble control (C), HIF-1α (H1), or HIF-2α (H2.1 and H2.2) shRNA following incubation in normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. (*B*) Endpoint tumor volumes observed in nude mouse xenograft assays performed with the stable cell lines described in *A*. The number of injections per cell line is specified above the corresponding bar. Data are presented as mean ± SEM. (*C*) Representative images of HIF-2α and HIF-1α stained initial mass tumor sections, excised 1-week post-injection, derived from U87MG cells expressing HIF-1α or HIF-2α shRNA. Immunostaining was visualized at magnification, ×400.

growth pathway(s) and contribute to the development of other cancer types. To address this prospect, we selected the U87MG glioblastoma, HCT116 colorectal, and A549 lung carcinoma cell lines, which differ considerably both genetically and histopathologically, the former being *PTEN*-null and the latter two harboring activating *KRAS* mutations (22–24). HIF-2α was stably silenced using one of two shRNA sequences, achieving an 85–90% reduction in protein levels in all three cell lines (Fig. 1*A*). Stable knockdown of HIF-2α restricted the hypoxic expression of *CITED2*, a HIF-2α-specific target gene, but not common HIF (glucose transporter-1; *GLUT1*) and HIF-1α-specific (carbonic anhydrase-9; *CA9*) target genes (Fig. 1*A*) (25, 26). Notably, *CITED2* was often maximally expressed in normoxia, indicating that basal HIF-2α levels are sufficient for target gene induction (27, 28). Next, we examined the effect of silencing HIF-2α on the tumorigenic capacity of the cells. Parental and control cells formed large xenograft tumors within 4 weeks of injection, reflecting the particularly aggressive nature of these cancer types (Fig. 1*B*). Remarkably, silencing HIF-2α abolished, or significantly impeded (≥ 5-fold reduction in tumor volume), the ability of these highly malignant cell lines to form tumors in nude mice ($n = 34$) (Fig. 1*B*). Silencing HIF-1α did not, however, dramatically affect tumorigenesis ($n = 20$) (Fig. 1*B*), in line with previous studies conducted with human cancer cell lines (29–31). Importantly, extensive or complete knockdown of HIF-1α protein and target gene induction was confirmed in vitro and/or in vivo (Fig. 1 *A* and *C*, and Fig. S1). Residual target gene expression in the U87MG and HCT116 cell lines was found to be HIF-independent suggesting that functional inhibition of HIF-1α was attained (Fig. S2). This result underscores a distinction between the HIF isoforms in tumor biology and reveals HIF-2α as a rate-limiting molecule in the development of genetically diverse human cancers.

Inhibition of HIF-2α Prevents the Proliferation of Human Cancer Cells in Vivo. Given that cells expressing HIF-2α shRNA failed to form palpable tumors, we extracted the initial cell mass, 1 week post-injection, to ascertain if this outcome stemmed from their inability to proliferate in vivo or if other hallmark traits were affected. Silencing HIF-2α inhibited tumor cell division considerably, as denoted by a marked reduction (50–90%) in Ki-67 staining compared to sections derived from control cells (Fig. 2*A*). Silencing HIF-1α did not measurably affect tumor cell proliferation and even resulted in a slight increase in Ki-67 staining in A549 cells, an effect that was recapitulated in vitro (Figs. 2*A* and 3*B*). This result highlights the unique ability of HIF-2α to drive cell proliferation, providing a mechanistic explanation for the divergent tumorigenic potentials of the HIF isoforms. Since silencing the individual HIF isoforms did not affect the expression of the remaining one in vivo, the induction of common HIF target genes should not be compromised and should not account for this effect (Fig. 1*C* and Fig. S1). Next, TUNEL- and H&E-stained initial mass tumor sections were visually inspected to determine if silencing HIF-2α had an effect on tumor cell death and vascularization. Tumors derived from cells expressing HIF-2α shRNA did in some cases display increased cell death (Fig. 2*B* and Fig. S3) and decreased blood vessel density (Fig. 2*C* and Fig. S3), yet no gross differences were observed when compared to those lacking HIF-1α. It is thus unlikely that silencing HIF-2α inhibits in vivo tumor formation by negatively affecting proangiogenic or anti-apoptotic pathways alone. Taken together, these data suggest that HIF-2α plays a central role in promoting the persistent proliferation and tumorigenesis of these cancers of variable genetic backgrounds and tissue distributions.

Silencing HIF-2α Prevents the Autonomous Growth of Human Cancer Cells in Vitro. In view of the fact that silencing HIF-2α led to a defect in tumor vascularization it was desirable to uncouple its role in tumor cell growth from its proangiogenic function. The ability of the stable cell lines to form avascular, 3-D spheroids in vitro was thus considered. Several layers of actively proliferating cells, expressing Ki-67, were detected at the periphery of control spheroids (Fig. 3*A*). Fewer cells in the HIF-2α knockdown spheroids stained positive for Ki-67, substantiating its direct involvement in cell proliferation (Fig. 3*A*). Unlike their normal counterparts cancer cells possess inherent mechanisms that allow them to proliferate in the absence of external growth cues (1). To better distinguish if HIF-2α had a deleterious effect on cell growth autonomy per se, we assessed the ability of the cells to proliferate in the absence of serum growth factors in vitro; the standard approach to measuring this capability (33). In contrast to the controls, cells expressing HIF-2α shRNA did not exhibit sustained growth upon serum withdrawal in hypoxia (Fig. 3*B*) or normoxia (Fig. 3*C*), as measured by BrdU incorporation. Silencing HIF-2α resulted in a reduction in normoxic (30–50%) and hypoxic (25–40%) cell growth relative to the control cells. Silencing HIF-1α, in contrast, either had no effect on or stimulated the autonomous proliferation of the cells. Similar conclusions were drawn based on serial cell counts in normoxia and hypoxia over time in our serum-free system (Fig. S4). These results corroborate that HIF-2α is functional in normoxia (Fig. 1*A*) and confers cells with their autonomic growth capabilities (Fig. 3 *B* and *C*) (27, 28). Importantly, cell doubling times in the presence of serum were unaffected indicating that no massive defects in cell cycle progression were incurred upon shRNA introduction and that

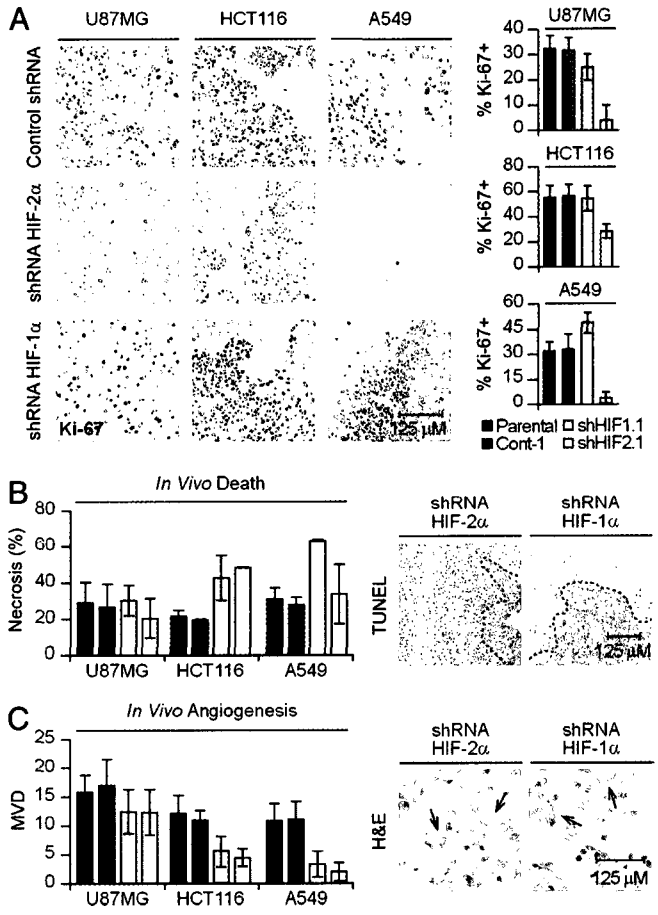

Fig. 2. Inhibition of HIF-2α prevents the proliferation of human cancer cells in vivo. (*A*) Representative images of Ki-67-stained initial mass tumor sections, derived from human cancer cell lines stably expressing scramble control, HIF-1α or HIF-2α shRNA, excised 1 week post-injection and visualized at magnification, ×200. Bar graph on the right indicates quantitative analysis of the percentage of Ki-67 positive cells in tumor sections. (*B*) Percentage of necrotic areas in initial mass tumor sections as assessed by TUNEL-staining and histological analysis. (*C*) Initial mass tumor vascularization expressed as a function of microvessel density (MVD) per field. Representative images of TUNEL- and H&E-stained sections derived from U87MG cells described in *A*, visualized at magnification, ×100 and ×400, respectively, are on the right in *B* and *C*. Dashed lines segregate necrotic and viable regions and arrows indicate the presence of blood vessels. Data in *A–C* are presented as mean ± SD in at least triplicate tumor sections.

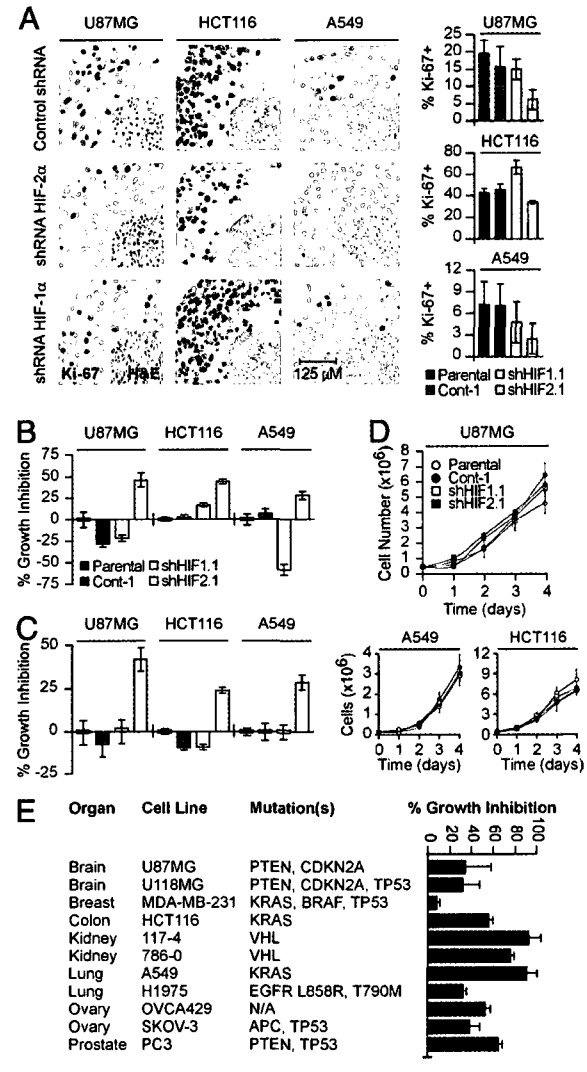

Fig. 3. Silencing HIF-2α prevents the autonomous growth of human cancer cells in vitro. (*A*) The ability of human cancer cell lines stably expressing scramble control, HIF-1α or HIF-2α shRNA to form multicellular spheroids and proliferate in an avascular setting was assessed by Ki-67-staining. Spheroids were grown for 5–7 days and representative images were visualized at magnification, ×400. Bar graph on the right indicates quantitative analysis of the percentage of Ki-67 positive cells. Data are presented as mean ± SD of triplicate spheroid sections. The ability of cells described in *A* to proliferate in the absence of serum growth factors was measured by BrdU labeling following incubation in (*B*) hypoxia or (*C*) normoxia for 48 h. The percentage of BrdU-positive cells was determined at magnification, ×100 in at least three independent fields per experiment and is expressed as the percentage decrease in BrdU incorporation (growth repression) relative to parental cell lines. (*D*) Cell proliferation under growth (10% FBS) conditions in normoxia was measured by serial cell counts over time. (*E*) BrdU labeling in a panel of serum-starved human cancer cell lines infected with adenovirus to express a dominant-negative form of HIF-2α (DN-HIF) for 48 h in normoxia. Data are expressed as the percentage growth repression relative to GFP-expressing controls. Data in *B–E* are presented as mean ± SD of triplicate experiments.

cell growth could be rescued by exogenous growth factors (Fig. 3*D*). We then extended our studies and examined the effect of abrogating HIF function on the autonomous growth of a panel of human cancer cell lines harboring various genetic mutations. Expression of a dominant-negative form of HIF, which competes binding to HIF-β and hypoxia response promoter elements, greatly inhibited the serum-independent proliferation of most of the cell lines tested, including prostate and ovarian carcinomas (Fig. 3*E*) (33, 34). Collectively, these data suggest that human cancers converge at a growth axis driven by the HIF system, challenging the notion that cancers use mutually exclusive pathways to gain a selective proliferative advantage.

HIF-2α Activates the EGFR and IGF1R Tyrosine Kinases and Their Downstream Signaling Molecules. Since exogenous growth factors can rescue the growth of cells lacking HIF-2α this suggests that their inability to proliferate autonomously is a consequence of defective receptor tyrosine kinase (RTK) signaling as opposed to cell cycle gene expression. Whereas EGFR signaling is pivotal in the pathogenesis of RCC, there is evidence that multiple RTKs are coactivated in other cancers to support tumorigenic phenotypes (3, 15, 21). We thus assessed the global effect of silencing HIF-2α on endogenous RTK activation in serum-starved cells using an antibody array. Surprisingly few RTKs were detected with the EGFR, insulin-like growth factor receptor type I (IGF1R) and insulin

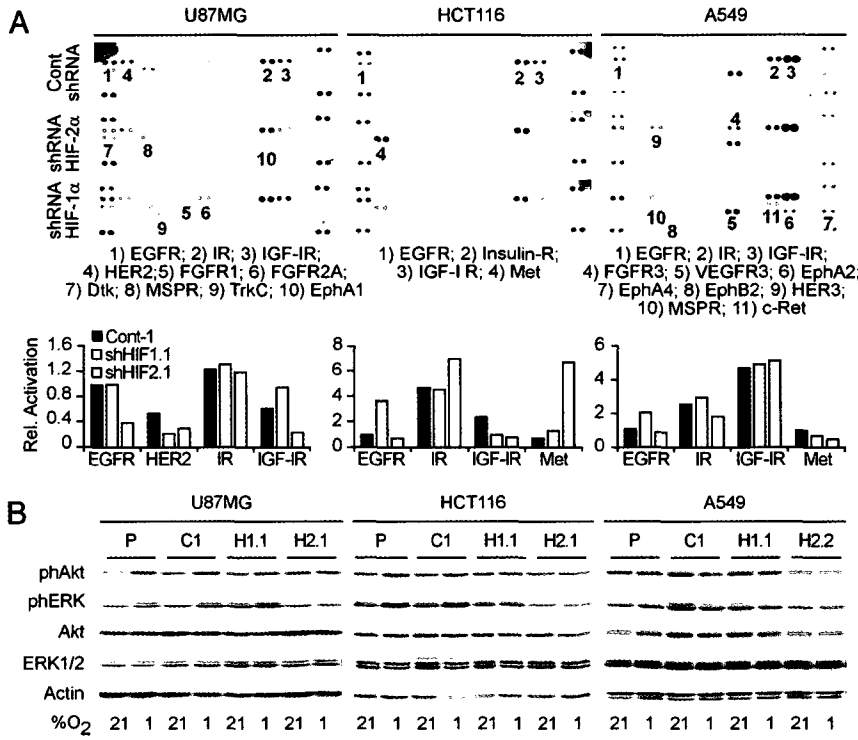

Fig. 4. HIF-2α activates the EGFR and IGF1R tyrosine kinases and their downstream signaling molecules. (*A*) Relative levels of RTK phosphorylation in serum-starved human cancer cell lines stably expressing scramble control, HIF-1α or HIF-2α shRNA after incubation in hypoxia for 24 h, as assessed using an antibody array. Numbers identify the specific RTK represented by duplicate spots. Spots in upper and lower corners are phosphorylation (positive) controls. Bar graphs depict the relative differences in activation of the most highly expressed RTKs in each cell line. Data are presented as the mean of duplicate samples. (*B*) Western blot analysis of ERK1/2 and Akt phosphorylation (phERK and phAkt) in cells as described in *A*. Actin served as a loading control.

receptor (IR) being the most highly and consistently activated (Fig. 4*A*). Phosphorylation of the EGFR and IGF1R was HIF-2α-dependent in the cell lines, with the exception of the IGF1R in A549 cells (Fig. 4*A*). The remaining RTKs displayed cell type-specific alterations in activation status when the HIF-α subunits were silenced. Silencing HIF-1α, on the other hand, increased EGFR activation and/or expression in all of the cell lines (Figs. 4*A* and 5*A*) and enhanced A549 cell growth in vitro and in vivo (Figs. 2*A* and 3*B*). The HIF-α isoforms often display reciprocally suppressive interactions, hence it is conceivable that these positive effects on RTK signaling and cell proliferation might be due to the derepression of HIF-2α (26). Finally we examined the effect of silencing HIF-2α on the associated downstream signaling molecules. Cell lines lacking HIF-2α exhibited reduced ERK1/2 or Akt activation compared to the controls in normoxia and hypoxia, whereas silencing HIF-1α had no such effect on either signaling pathway (Fig. 4*B*). These data demonstrate that HIF-2α is required for the efficient activation of major RTK growth signaling pathways, albeit in a cell-type specific manner.

HIF-2α Regulates the Expression of Wild-Type and Mutant RTKs Required for Cell Growth Autonomy. Recent reports have revealed a role for HIF-2α in the amplification of EGFR signaling through its translational up-regulation and stabilization (15, 35). We examined the effect of silencing HIF-2α on total RTK protein levels to establish whether their deactivation could be attributed to a decrease in receptor abundance. Consistent with the array data a reduction in total EGFR and IGF1R levels was observed, suggesting that HIF-2α can also regulate the expression and turnover of other RTKs (Fig. 5*A*). Coincidentally, these effects are unlikely to be mTOR-dependent as activation of this pathway was unaffected by silencing HIF-2α (Fig. S5). The implications of blocking specific

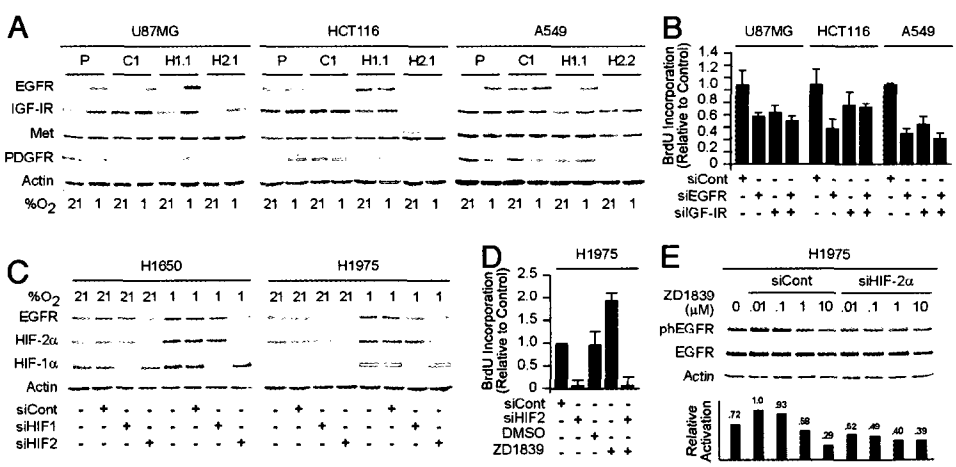

Fig. 5. HIF-2α regulates the expression of wild-type and mutant RTKs required for cell growth autonomy. (*A*) Western blot analysis of total cellular levels of various RTKs in human cancer cell lines stably expressing scramble control, HIF-1α, or HIF-2α shRNA after incubation in normoxia or hypoxia for 24 h. Actin served as a loading control. (*B*) BrdU incorporation in serum-starved cells expressing siRNA (25 nM) against the EGFR, IGF1R, or control siRNA for 72 h in normoxia. (*C*) Western blot analysis of EGFR levels in serum-starved H1650 and H1975 lung carcinoma cells transfected with siRNA (50 nM) against HIF-1α or HIF-2α for 24 h, following incubation in normoxia or hypoxia for an additional 24 h. (*D*) BrdU incorporation in normoxic H1975 cells, as described in *C*, in the presence and absence of the EGFR inhibitor ZD1839 (1 μM). Data in *B* and *D* are presented as mean ± SD of triplicate experiments. (*E*) Western blot analysis of phosphorylated EGFR (phEGFR) in hypoxic H1975 cells, as described in *C*, following treatment with increasing concentrations of ZD1839 and stimulation with rTGFα. Bar graphs depict the relative levels of phosphorylated receptor.

RTKs on cell growth autonomy were then explored using commercially available siRNAs and inhibitors. We found that the EGFR and IGF1R were both required for all three cell types to meet their full proliferative potential (Fig. 5*B*), while others were dispensable for cell growth (Fig. S6). Since HIF-2α up-regulates wild-type EGFR levels, we hypothesized that it would have a similar effect on mutant EGFR and could serve as an indirect method of targeting those that have acquired drug resistance mutations, a frequent occurrence in non–small-cell lung carcinomas (NSCLC) (36). Indeed, transient silencing of HIF-2α constrained the normoxic expression and hypoxic induction of mutant EGFR in H1650 (drug-sensitive) and H1975 (drug-resistant) NSCLC cells (Fig. 5*C*). Moreover, inhibition of HIF-2α prevented the autonomous growth of H1975 cells in vitro (Fig. 5*D*) and improved receptor sensitivity to the EGFR-specific inhibitor ZD1839 (Fig. 5*E*). These data suggest that HIF-2α drives the persistent proliferation of human cancers through the up-regulation of various wild-type and mutant RTKs and endorse its therapeutic targeting to circumvent receptor resistance and redundancy issues.

Discussion

The Darwinian model of carcinogenesis depicts a transformation process whereby genetic alterations induce growth promoting phenotypic changes and subsequent physical barriers force additional adaptations that support tumor expansion (37). With this study we offer an alternative to the prevailing view that cancers exploit distinct growth pathways subject to their mutational status. We show that genetically diverse human cancers have evolved a common and obligatory growth stimulatory program, required for tumor formation, at the hub of which lies HIF-2α. Consistently, HIF-2α stabilization is observed in the vast majority of solid tumors, as a consequence of both mutational events and environmental cues, and is associated with aggressive tumorigenic behaviors (17, 18, 38). Our data further suggest that HIF-2α endows cells with the ability to proliferate autonomously by activating fundamental RTKs, including EGFR and IGF1R, and their downstream signaling pathways.

Despite their genetic and biochemical disparities, it is well-appreciated that cells use widespread mechanisms to acquire certain tumorigenic traits including the angiogenic phenotype. Antagonists of these pathways are thus predicted to have greater clinical applicability compared to designer drugs directed at tumors displaying specific mutations or biomarkers (4). Fittingly, therapeutic agents targeting VEGF, a proangiogenic HIF target gene, have exhibited greater efficacy over other rational drugs in the treatment of several cancer types (19, 20). Based on our data and in accordance with the oncogene addiction theory, inhibition of the HIF-2α autonomic growth axis should render a comparable and perhaps improved response. While our findings provide a rationale for targeting HIF-2α in the treatment of cancers, they also suggest that therapeutic strategies aimed at inhibiting HIF-1α may fall short of expectations. Silencing HIF-1α hindered tumor cell survival and vascularization as would be predicted (Fig. 2 *B* and *C*), yet it did not markedly affect the in vivo proliferation or tumorigenesis of any of the cancer cell lines examined here (Figs. 1*B* and 2*A*). This is in line with reports that HIF-1α knockdown has either no effect or even boosts human neuroblastoma, breast, ovarian, and renal carcinoma tumor growth (29–31). This is not an entirely unexpected outcome since the HIF isoforms have divergent and species-specific transcriptional activities and even play opposing roles in certain metabolic processes (11, 16, 26, 39, 40). Our data do not preclude the possibility that HIF-1α may promote cell proliferation in other cancer types (Fig. 3*E*), but do strongly suggest that the isoforms are not functionally interchangeable with respect to tumor cell growth autonomy.

The challenge now lies in unraveling the molecular mechanisms at play in this process such that clinically relevant participants in HIF-2α growth axis can be identified. These elements may include upstream coconspirators, such as ETS family transcription factors which are thought to confer target gene specificity to HIF-2α, as well as downstream growth regulatory molecules (25, 41). To date HIF-2α oncogenic functions have been studied mainly in VHL-loss RCC. In this system, HIF-2α drives the growth autonomy and tumorigenesis of RCC cells by allowing the establishment of the TGF-α/EGFR autocrine signaling loop (15, 21). We similarly found that the EGFR was consistently deactivated in the absence of HIF-2α and had the greatest effect on the autonomous proliferation of the cancers studied here (Figs. 4*A* and 5*A–D*). It has recently been inferred that HIF-2α cooperates with c-Myc to promote cell cycle progression through the activation of cyclin-D2 and E2F1 (16). Consistent with this report, we did observe HIF-dependent effects on c-Myc target gene expression in our serum-free growth model. Silencing HIF-2α resulted in decreased *E2F1* and increased *P27* mRNA expression in hypoxic HCT116 cells while silencing HIF-1α had the reverse effect (Fig. S7) (16). Knockdown of the HIF isoforms differentially impacted c-Myc target gene expression across the cell lines; an outcome that can probably be explained by variations in the relative levels of c-Myc, HIF-1α and HIF-2α protein (16). Based on these findings, one could speculate that HIF-2α serves to endorse cancer cell proliferation through both the activation of RTKs and the modified expression of downstream mediators of cell cycle progression. Monotherapies directed at HIF-2α should thus simultaneously inhibit several RTK signaling pathways and correct inherent defects in cell cycle regulation, thereby circumventing drug resistance issues at both levels.

In conclusion, our data suggest that despite their genetic and molecular diversity cancers share an underlying program that confers them with autonomous growth capabilities. We propose a model whereby various oncogene and tumor suppressor pathways endorse the up-regulation and stabilization of HIF-2α, which in contrast to overt carcinomas is rarely expressed in normal tissues, with the goal of attaining growth signal autonomy (18, 19, 27). This effect is likely amplified by the tumor microenvironment enabling HIF-2α to activate central growth signaling pathways, explaining why inhibition of HIF-2α overrides RTK redundancy. Given its systemic and rate-limiting nature, we propose that participants in the HIF-2α growth pathway should be investigated as prime therapeutic targets, particularly in cancers with yet unidentified proliferative mechanisms.

Materials and Methods

Cell Culture. The U87MG glioma and A549 lung carcinoma cell lines, kind gifts from Ian Lorimer (Ottawa Regional Cancer Center, Ottawa, ON), were maintained in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 environment. All other cell lines were obtained from the American Type Culture Collection and propagated as suggested. Hypoxic cells were incubated in a chamber at 37 °C in a 1% O_2, 5% CO_2, and N_2-balanced atmosphere. Serum-free medium consisted of base medium supplemented with 1% insulin-transferrin-selenium (ITS; Invitrogen).

RNA Interference. Cells were transiently transfected with small-interfering RNA (siRNA) targeting the EGFR (ID no. 4833), HIF-1α (ID no. 42840), HIF-2α (ID no. 106447), or negative control siRNA (Ambion Inc.). ON-TARGETplus SMARTpool siRNA targeting the IGF1R was obtained from Dharmacon Inc. Cells were also stably transfected to express short hairpin RNA (shRNA) sequences targeting HIF-2α (42) or HIF-1α (43). For each sequence, cDNA oligonucleotides with *BamHI*/*HindIII* site overhangs were synthesized and annealed with 1× DNA Annealing Solution (Ambion). The annealed inserts were ligated into a pSilencer 3.1-H1 neo vector (Ambion). The pSilencer 3.1-H1 neo vector encoding nontargeting shRNA served as a negative control. Positive clones were selected and maintained in neomycin-containing medium.

Immunoblotting. Cell lysates (25–50 μg) were separated by SDS/PAGE and transferred to a PVDF membrane. Monoclonal antibodies were used to detect EGFR (Ab-12; LabVision), HIF-1α (BD Transduction Laboratories) and Met (Cell Signaling Technology Inc.). Polyclonal antibodies were used to detect Akt (Cell Signaling), ERK1/2 (Promega), HIF-2α (Novus), IGF1R (Cell Signaling), PDGFRα (Cell Signaling), phospho-Akt (Thr-308; Cell Signaling), phospho-EGFR (Y1068; Abcam Inc.), phos-

pho-ERK (T202/Y204; Cell Signaling), total and phospho-4E-BP1 (S65; Cell Signaling), total and phospho-S6 ribosomal protein (S235/236; Cell Signaling), and actin (Sigma). Membranes were then blotted with HRP-conjugated anti-mouse (Amersham Biosciences) or anti-rabbit (Jackson ImmunoResearch Laboratories Inc.) secondary antibodies. Bands were detected by enhanced chemiluminescence (Pierce).

RT-PCR Analysis. Total RNA was extracted using TriPure Isolation Reagent (Roche Molecular Biochemicals). RT-PCR analysis was performed on 1 μg RNA using the AccessQuick RT-PCR System (Promega). Products were resolved by agarose gel electrophoresis and ethidium bromide staining was visualized with a Kodak Digital Science IC440 system. See *SI Text* for primer and cycle details.

Xenograft Tumors. Female CD-1 nude mice (Charles River) were injected s.c. in their flanks with 10^7 cells diluted in 200 μL sterile 1× PBS. Mice were killed 6–8 weeks post-injection according to facility protocols (University of Ottawa) or earlier in cases of significant morbidity. Tumor dimensions were recorded weekly and final volumes measured at the time of kill. All experiments were performed double-blinded.

Histology and Immunohistochemistry. See *SI Text* for details.

Proliferation Assays. Autonomous growth of serum-starved cells was measured using a 5-bromo-2′-deoxy-uridine (BrdU) Labeling and Detection kit I (Roche) as described in ref. 34. The percentage of BrdU-labeled cells vs. Hoechst-stained nuclei (Hoechst 33258; Sigma) was assessed by fluorescence microscopy. To determine cell doubling times, cells were plated in 6-cm dishes and incubated in media supplemented with 10% FBS or 1% ITS. For each time point, plates were trypsinized and Trypan blue-excluding cells were counted in a hemocytometer.

In Vitro Spheroids. See *SI Text* for details.

Adenoviruses. See *SI Text* for details.

Phospho-RTK Array. Cells were lysed in Nonidet P-40 buffer [1% Nonidet P-40, 20 mM Tris·HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, and 10 μg/mL aprotinin/leupeptin] by rotating for 30 min at 4 °C. Relative levels of phosphorylated receptor tyrosine kinases were determined using a Human Phospho-RTK Array kit (R&D Systems). Briefly, blocked arrays were incubated with lysates (1 mg) overnight at 4 °C, washed three times with the provided buffer, and incubated with anti-phospho-tyrosine-HRP detection antibody for 2 h at room temperature. Positive signals were detected by enhanced chemiluminescence (Pierce).

EGFR Inhibition Assays. Cells were treated with increasing concentrations (0.001–1 μM) of the EGFR tyrosine kinase inhibitor, ZD1839 (Iressa; AstraZeneca), for 3 h and then stimulated with 20 ng/mL human recombinant TGFα (Chemicon) for 15 min. Cells were lysed in 4% SDS in PBS before immunoblotting. Relative receptor phosphorylation was determined by measurement of band intensities, less the background readings for equivalent areas, using Adobe Photoshop 7.0 software.

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6.2. Appendix B – Additional Contributions

Silencing of Epidermal Growth Factor Receptor Suppresses Hypoxia-Inducible Factor-2–Driven *VHL*$^{-/-}$ Renal Cancer

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Abstract

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

Introduction

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

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

Whereas the role of HIF in promoting the highly vascularized phenotype of VHL loss RCC tumors is well characterized, the mechanisms by which this transcription factor can promote tumorigenesis remain debatable (19, 20). There are two active forms of HIFα called HIF-1α and HIF-2α, which activate common as well as distinct targets (21). Recent reports have led to the suggestion that HIF-2α is the oncogenic form of HIFα, at least in human RCC (22–24). This would imply that HIF-2α differs from HIF-1α in its ability to activate a specific target(s) that is involved in oncogenic growth of RCC cells. We recently reported that HIF activates the transforming growth factor-α/epidermal growth factor (TGF-α/EGFR) pathway in VHL$^{-/-}$ RCC cells (25). We suggested that HIF-mediated constitutive EGFR activation provides permanent self-sufficiency in growth signaling that drives the growth autonomy of VHL-defective RCC cells, a hallmark of cancer (26). In this report, we put forward an explanation for the differential oncogenic potential of HIF-2α in RCC cells by

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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identifying TGF-α as a HIF-2α-specific target. We show that HIF-2α, but not HIF-1α, is able to promote *in vitro* and *in vivo* tumorigenesis of $VHL^{-/-}$ RCC cells by constitutively activating the TGF-α/EGFR oncogenic pathway. Transient and stable silencing of EGFR is sufficient to prevent HIF-2α-dependent tumorigenesis in multiple $VHL^{-/-}$ RCC cell lines. These data reaffirm EGFR as a crucial therapeutic target for VHL-defective renal cancer treatment.

Materials and Methods

Cell culture and reagents. Normoxic cells were incubated at 37°C under a 5% CO_2 environment. Hypoxia was achieved by incubation in a hypoxic chamber at 37°C under 1% O_2, 5% CO_2, and N_2-balanced atmosphere. VHL-deficient, 786-0 and A498 RCC cells, and HCT116 colon carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD). HOP62, MCF7, SKMEL, and PC3 cells were a kind gift from John Bell (Ottawa Regional Cancer Center, Ottawa, Ontario, Canada) and U87MG cells a kind gift from Ian Lorimer (Ottawa Regional Cancer Center, Ottawa, Ontario, Canada). 786-0 and A498 cells stably transfected with HA-VHL (786-0 + VHL and A498 + VHL, respectively) were a kind from Dr. W.G. Kaelin (Harvard University, Boston, MA). KTCL140 cell line was a kind gift from Dr. Peter Ratcliffe (University of Oxford, Oxford, United Kingdom). Primary cultures of human renal cortical epithelial cells were purchased from Clonetics (San Diego, CA). Serum containing medium consisted of DMEM or McCoys 5A (HCT116) medium with 10% fetal bovine serum (FBS). Serum-free medium consisted of DMEM supplemented with 1% insulin-transferrin-selenium (Invitrogen, Burlington, Ontario, Canada). PD153035 (Calbiochem, San Diego, CA) was used as indicated. Treatment of VHL-deficient cells with antisense targeting TGF-α was carried out as previously described (27).

Adenovirus construction. Adenoviruses encoding the HIF-α mutants, VHL, GFP, and DN-HIF were generated through Cre-lox recombination as described elsewhere (25, 28). Vectors encoding HIF-α subunits mutated at proline hydroxylation sites encoding HA-HIF-2α (P405A and P531α) and HA-HIF1α (P402A and P564A) were a kind gift from Dr. W.G. Kaelin. cDNA encoding the constitutively active variants of HIFα were released by *Bam*HI/*Not*I restriction digest from their original pcDNA 3.0 vector and subcloned, in-frame, downstream of a Flag-GFP moiety previously cloned into the *Hin*dIII and *Bam*HI sites of a pAdlox adenoviral vector. DNA sequencing analysis confirmed the alanine substitutions of the targeted proline residues. Experiments used approximately equal multiplicity of infection.

RNA isolation and reverse transcription-PCR analysis. One microgram of total RNA collected using TRIPURE isolation reagent (Roche, Indianapolis, IN) was used. All primers and cycle details for reverse transcription-PCR (RT-PCR) for TGF-α, Glut-1, and actin are described elsewhere (25). Semiquantitative RT-PCR was done by taking samples after 30 amplification cycles. Products were analyzed with gel electrophoresis and ethidium bromide staining, and visualized using a Kodak Digital Science IC440 system.

For real-time RT-PCR, triplicate (25 μL) multiplex RT-PCR reactions were done on 50 ng of total RNA using Taqman One-Step RT-PCR master mix reagents (Applied Biosystems, Streetville, Ontario, Canada) and 0.25 μmol/L 5′ -VIC TGF-α and 5′ -6FAM actin modified probes. Cycling conditions were 30 minutes at 48°C, 10 minutes at 95°C, then 40 cycles of alternating 15 seconds at 95°C and 1 minutes at 60°C. Amplification and analysis of real-time data was done using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The quantity of TGF-α detected in each reaction tube was normalized to the level of coamplified actin endogenous control. Each RNA sample was analyzed in triplicate to obtain an average normalized quantity of TGF-α transcript (arbitrary units). TGF-α forward GCACGTCCCCGCTGAGT and TGF-α reverse CAGGTTCCATGGAAGCAGAA; TGF-α probe TTTTAATGACTGCCCAGATTCCCACACTCA; β-actin probe CCGCCGCCCGTCCACACCCGCC. The sequences all other primers used are described elsewhere (25).

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

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

Western blot. Cells were washed with PBS and harvested in 4% SDS in PBS. Samples (20-100 μg of each) were separated on denaturing polyacrylamide gels containing SDS and transferred to methanol-activated polyvinylidene difluoride membrane (NEN, Boston, MA). Membranes were blocked in skimmed milk before incubation with Flag (Sigma) or EGFR (Ab-12; LabVision, Fremont, CA) monoclonal antibodies. Polyclonal antibodies were used to detect py-EGFR (sc-12351; Santa Cruz Biotechnology, Santa Cruz, CA), HIF-2α (Novus, Littleton, CO), Glut-1 (Alpha Diagnostic International, San Antonio, TX), and actin (Sigma). After washing with 0.2% Tween-PBS solution, membranes were blotted with secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) and detected by enhanced chemiluminescence (Pierce).

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

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

***In vitro* tumor spheroid.** Multicellular spheroids were prepared by the liquid overlay technique (31–33) and as described by our group elsewhere (34). Briefly, 24-well plates were coated with 250 μL of preheated 1% Seaplaque agarose (Cambrex, Rockland, ME) in serum-free medium; 10^5 of indicated cells were plated per 1 mL of DMEM per well. To promote cell-cell adhesion, the plates were gently swirled (32 times) 30 minutes after plating. Spheroids were grown for 6 days at 37°C under 5% CO_2 in serum-containing medium. Spheroids were harvested in lysis buffer (4% SDS in PBS) before

immunoblotting. Alternatively, spheroids were fixed in 10% formaldehyde, embedded in paraffin, sectioned, mounted on slides, and stained with H&E.

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

Results

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

Transforming growth factor-α is a hypoxia-inducible factor-2α–specific target. Next, we wanted to uncover the

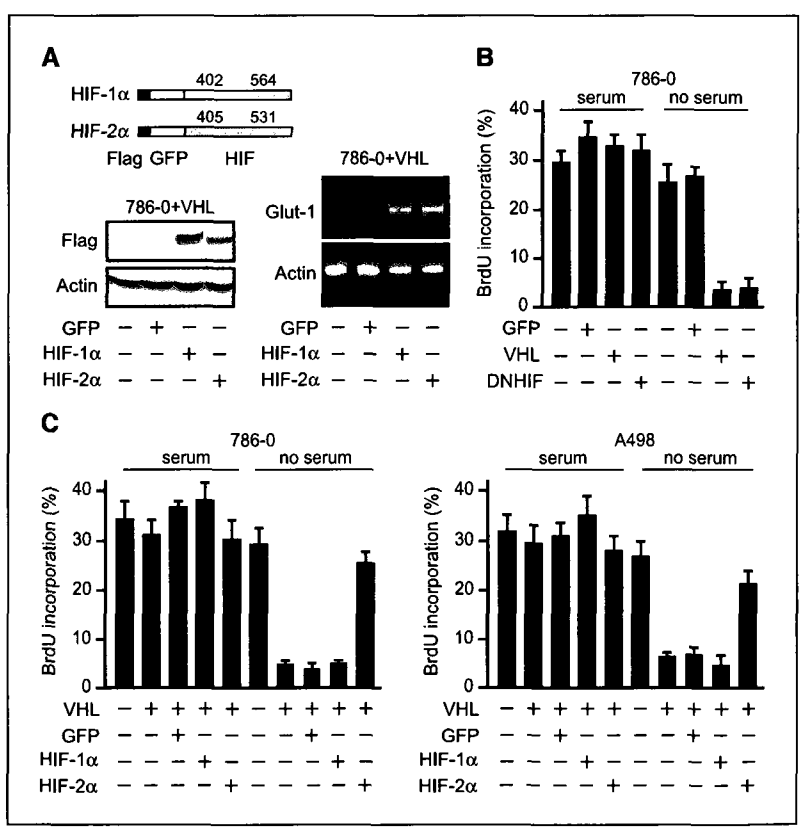
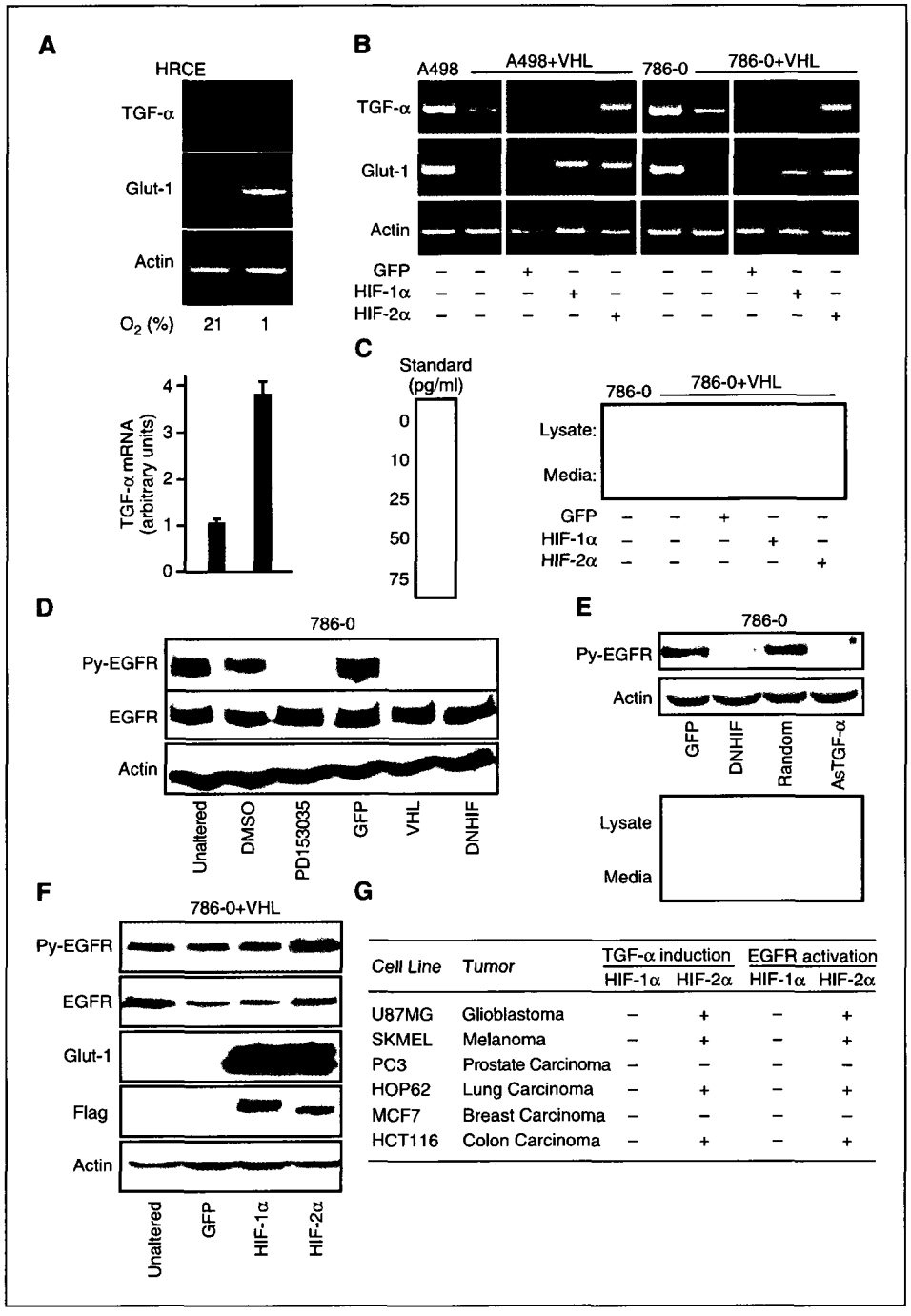


Figure 1. HIF-2α promotes autonomous growth of RCC cells. *A*, HIF-1α and HIF-2α variants carrying P-to-A mutations at prolyl hydroxylation sites (schematic) are both able to activate HIF target genes. VHL-deficient 786-0 cells were stably transfected to express HA-VHL and infected with adenoviruses expressing flag-tagged HIF-1α, HIF-2α, or GFP. After 48 hours post-infection immunoblotting or RT-PCR was done to detect flag protein or Glut-1 mRNA, respectively. *B*, HIF activation is required for serum independent growth of VHL-deficient RCCs. 786-0 cells infected to express GFP, VHL, or dominant-negative HIF were incubated for 72 hours in the absence or presence of serum and assessed for BrdUrd incorporation. *Columns*, average mean of at least three independent experiments in triplicates; *bars*, SE. *C*, HIF-2α but not HIF-1α is able to promote serum-independent growth in VHL-competent cells. Growth was measured in 786-0 or A498 cells deficient or competent for VHL expression (786-0 + VHL or A498 + VHL, respectively) following infection with an adenovirus encoding GFP, HIF-1α, or HIF-2α. *Columns*, average mean of at least three independent experiments in triplicates; *bars*, SE.



Cell Line	Tumor	TGF-α induction		EGFR activation	
		HIF-1α	HIF-2α	HIF-1α	HIF-2α
U87MG	Glioblastoma	–	+	–	+
SKMEL	Melanoma	–	+	–	+
PC3	Prostate Carcinoma	–	–	–	–
HOP62	Lung Carcinoma	–	+	–	+
MCF7	Breast Carcinoma	–	–	–	–
HCT116	Colon Carcinoma	–	+	–	+

Figure 2. Specific induction of TGF-α by HIF-2α activates the EGFR. *A*, hypoxic induction of TGF-α mRNA as measured by RT-PCR in primary human renal cortical epithelial cells. Real-time PCR results (*bottom inset*). *Columns*, average mean of three independent experiments in triplicates; *bars*, SE. *B*, HIF-2α but not HIF-1α induces TGF-α mRNA synthesis in VHL-competent cells. RT-PCR was used to detect TGF-α and Glut-1 in VHL-deficient A498 and 786-0 cells and in A498 and 786-0 cells that stably express reintroduced VHL (A498 + VHL and 786-0 + VHL). Cells were infected to express indicated proteins. *C*, HIF-2α but not HIF-1α induces TGF-α protein in VHL-competent cells. ELISA was done to detect TGF-α protein in cellular lysates and media. VHL-deficient 786-0 cells and VHL-competent 786-0 + VHL cells were infected to express the indicated proteins. *D*, immunoblotting detecting EGFR and phosphorylated, activated EGFR in 786-0 cells treated with PD153035, or infected to express GFP, VHL or DNHIF. Cells were incubated for 48 hours in serum-free conditions before collection of lysates. *E*, blockade of HIF activation or silencing of its target TGF-α reduces EGFR activation. 786-0 cells were infected to express GFP or DNHIF, or were treated with TGF-α antisense or random oligomers. Total cell lysates were submitted to anti-Py-EGFR immunoblotting. In parallel, ELISA was used to detect TGF-α. Cells were incubated for 48 hours in serum-free conditions before collection of lysates. *F*, HIF-2α but not HIF-1α induces EGFR activation in VHL-competent 786-0 cells. Cells were infected to express the indicated proteins and lysates were submitted to immunoblotting to detect the indicated proteins. Cells were incubated for 48 hours in serum-free conditions before collection of lysates. *G*, HIF-2α induces TGF-α mRNA/protein production and EGF-R activation in cancer cells from various tissue origin. Cells were infected to express HIF-1α or HIF-2α and submitted to RT-PCR analysis to detect TGF-α mRNA and to immunoblotting to detect increased phosphorylation of EGFR as described in (*B*), (*C*), and (*F*). Data is presented as a table for the sake of simplicity.

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

We next asked if HIF-2α was able to activate EGFR through production of TGF-α ligand. VHL-deficient 786-0 RCC cells

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

Transient silencing of epidermal growth factor receptor by small interfering RNA prevents formation of dense renal cell carcinoma tumors *in vitro*. We next asked whether EGFR activation plays a central role in the ability of HIF-2α to drive VHL loss RCC tumorigenesis. To do so, a panel of VHL-defective

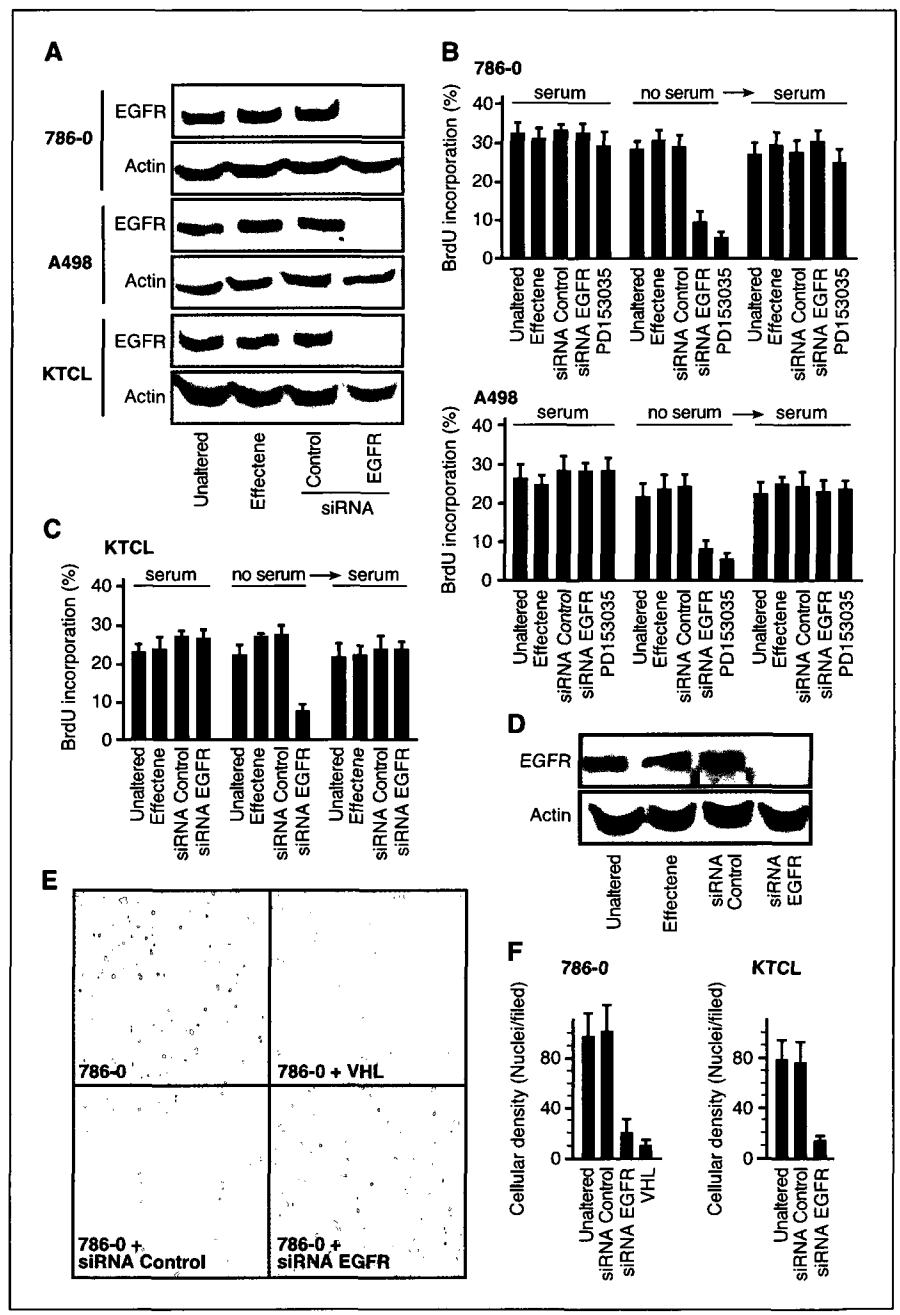


Figure 3. Transient silencing of EGFR with siRNA prevents serum-free growth and formation of *dense tumor spheroids. A, transient incubation* with siRNA directed against EGFR mRNA suppresses EGFR protein production in multiple VHL-defective cells. Immunoblots were done to detect EGFR in untreated cells, cells treated with effectene, control siRNA, or siRNA against EGFR. *B-C,* siRNA directed against EGFR prevents serum-independent growth of multiple $VHL^{-/-}$ RCC cells. Cells were incubated in the presence of 10% serum or in the absence of serum for 72 hours followed by addition of serum for 48 hours. BrdUrd labeling was for 3 hours in all conditions. Transient incubation with siRNA was as described in Materials and Methods. PD153035 was added for the duration of the experiments without noticeable toxic effects. *Columns,* average mean of at least three independent experiments in triplicates; *bars,* SE. *D-E,* siRNA directed against EGFR prevents dense spheroid formation. *In vitro* tumor spheroids were produced as described in Materials and Methods for 6 days in the presence or absence of control siRNA or siRNA directed against EGFR. Histology from spheroids is visualized at a magnification of 400×. Immunoblot detection of EGFR shown in (*D*) was from lysates of spheroids incubated for 6 days to show inhibition of EGFR protein production by siRNA. *F,* density was measured at 400× magnification in four independent spheroids in at least three sections per spheroid and reported as nuclei/field. *Columns,* average mean of at least three independent experiments in triplicates; *bars,* SE.

RCC cell lines was incubated in the presence of a siRNA directed against EGFR mRNA. The cell lines included the widely characterized VHL-defective RCC 786-0 and A498 cell lines. In addition, we used the KTCL140 line reported to harbor VHL inactivating mutations and overexpress HIF-2α and its targets (8). KTCL140 overproduce TGF-α and display strong EGFR phosphorylation that can be repressed by reintroduction of VHL or expression of DNHIF (data not shown; Fig. 5). The siRNA efficiently decreased levels of EGFR protein in $VHL^{-/-}$ RCC cells (Fig. 3*A*) and, importantly, prevented HIF-2α-mediated autonomous growth as efficiently as the EGFR tyrosine kinase inhibitor PD153035 for 786-0 and A498 (Fig. 3*B-C*). The effect of silencing EGFR on growth was restricted to serum-free conditions because addition of fresh serum abolished the growth inhibitory effect of the siRNA, as expected. These data show that the ability of HIF-2α to drive growth autonomy of $VHL^{-/-}$ RCC cells requires activation of EGFR.

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

Stable silencing of epidermal growth factor receptor suppresses tumorigenesis of von Hippel-Lindau–defective renal cell carcinoma. Based on the data obtained in Fig. 3, we decided to engineer $VHL^{-/-}$ RCC cells with stably inactivated EGFR by expression of shRNA. For these experiments, we used the $VHL^{-/-}$ RCC cells 786-0, which has been extensively characterized by several other groups (4, 33, 40, 41). The 786-0 cells form tumors

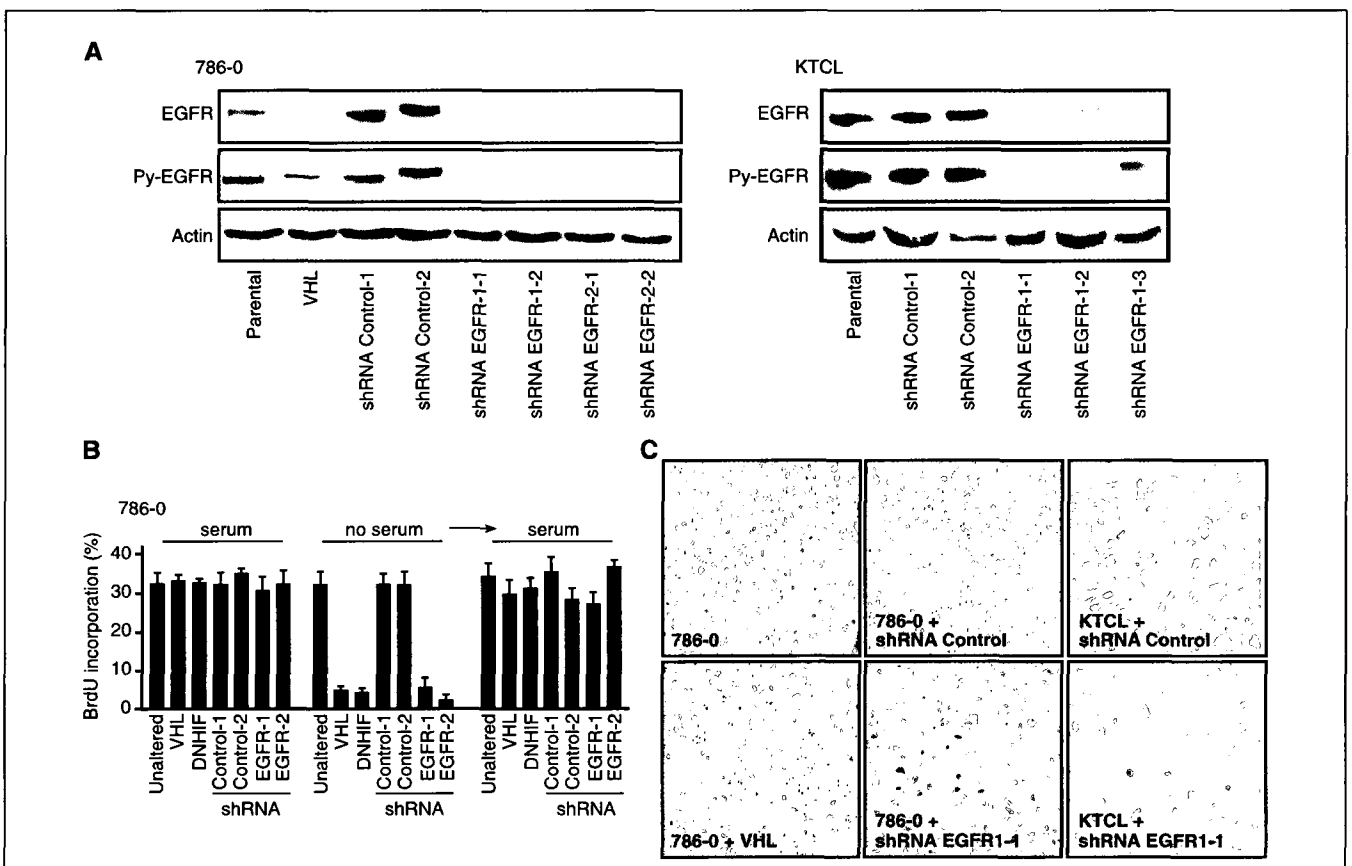


Figure 4. Stable inhibition of EGFR protein and activity by shRNA in $VHL^{-/-}$ RCC cells. *A*, shRNA directed against EGFR mRNA suppresses EGFR protein levels. $VHL^{-/-}$ RCC 786-0 and KTCL140 were stably transfected with pSilencer expressing control shRNA (called shRNA control) or expressing shRNA1 or shRNA2 directed against EGFR mRNA (called shRNA EGFR-1 or shRNA EGFR-2, respectively). Cell lines of 786-0 cells stably expressing reintroduced VHL are denoted VHL. Immunoblots were done to detect EGFR or phosphorylated EGFR. *B*, stable silencing of EGFR by shRNA abolishes growth autonomy of $VHL^{-/-}$ RCC cells as efficiently as expression of VHL or DNHIF. $VHL^{-/-}$ RCC 786-0 cells infected to express GFP, VHL, or DNHIF as well as stably expressing control shRNAs or shRNA EGFR-1 or EGFR-2 were incubated in 10% serum, transferred to serum-free conditions for 72 hours followed by addition of serum for 48 hours. BrdUrd incubation was for 3 hours in each case. Similar data were obtained for KTCL140 cell line but omitted for the sake of clarity. *Columns*, average mean of at least three independent experiments in triplicates; *bars*, SE. *C*, stable silencing of EGFR by shRNA prevents dense tumor formation. *In vitro* tumor spheroids were produced as described in Materials and Methods for 6 days. Histology from spheroids is visualized at a magnification of 400×.

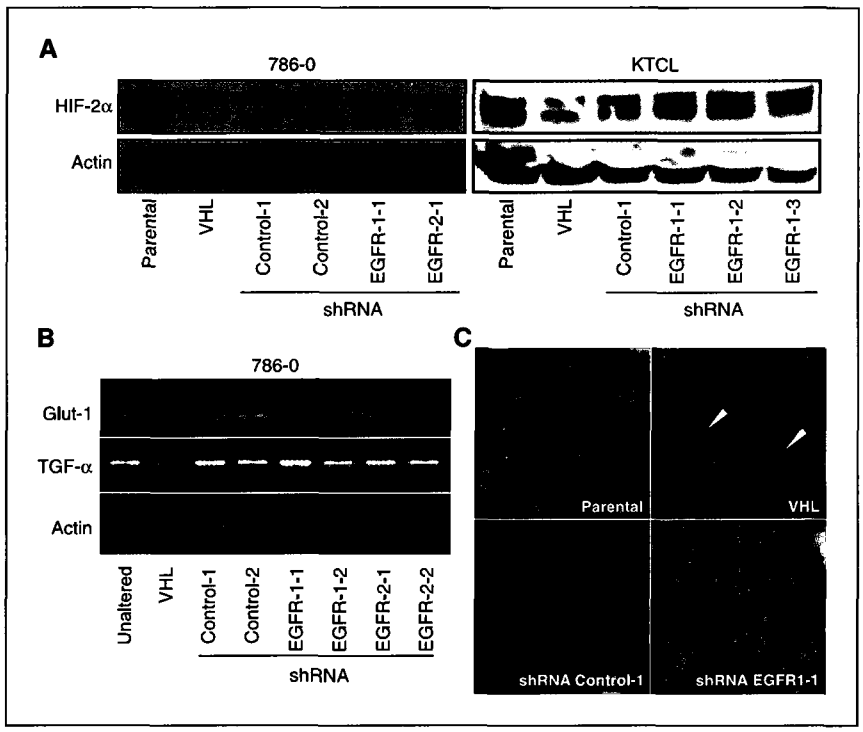


Figure 5. Inhibition of EGFR does not correct other defects associated with VHL loss. *A*, stable inhibition of EGFR does not alter HIF-2α protein levels. *Immunoblots were done to monitor HIF-2α protein* levels in 786-0 or KTCL140 cells stably expressing shRNA directed against EGFR. *B*, stable silencing of EGFR by shRNA does not prevent activation of HIF-2α-regulated genes. RT-PCR was done from RNA isolated from the indicted clones to detect TGF-α and Glut-1 mRNA as well as actin mRNA for loading comparison. *C*, inhibition of EGFR does not correct *defects of VHL$^{-/-}$ RCC cells in fibronectin matrix* deposition. Immunofluorescence to detect fibronectin was as described in Material and Methods. Arrows point to fibronectin matrix only found in VHL-expressing cells.

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

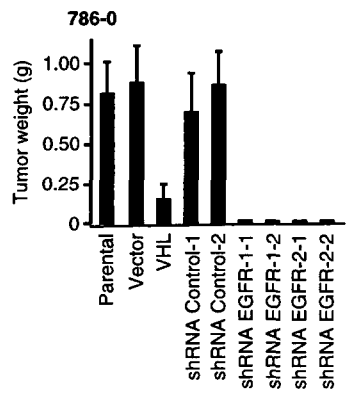
We next wanted to examine the effect of EGFR knockdown on defects associated with VHL loss. shRNA-mediated silencing of EGFR did not affect HIF-2α protein levels and its ability to activate downstream targets, such as Glut-1 and TGF-α (Fig. 5*A-B*). In addition, EGFR knockdown failed to restore the ability of VHL$^{-/-}$ RCC 786-0 cells to form a fibronectin extracellular matrix (Fig. 5*C*; ref. 17). Similar data were obtained with the KTCL140 cell lines stably expressing shRNA1 against EGFR (Fig. 5*A*; data not shown). These data indicate that RCC cells expressing shRNA against EGFR display all of the cancer-like biochemical characteristics associated with VHL loss including constitutive activation of HIF-2α-regulated genes and loss of fibronectin deposition.

Finally, we examined the effect of EGFR knockdown on the tumorigeneic potential of VHL-deficient RCC cells by injecting clones into nude mice and monitoring tumor formation for a period of 9 weeks. Parental 786-0 cells as well as 786-0 cells expressing vector alone or control shRNA cells produced large tumors detectable after 4 to 5 weeks. VHL$^{-/-}$ RCC cells expressing reintroduced VHL did not form tumors after 9 weeks of incubation (Fig. 6*A-B*). RCC 786-0 cells expressing either shRNA1 or shRNA2 directed against EGFR mRNA failed to form tumors after 9 weeks post-injection (Fig. 6*A-C*). It should be noted that prolong periods of incubation resulted in measurable tumor formation by VHL-competent RCC cells. In contrast, we were unable to detect small tumors in shRNA-expressing cells even after 15 weeks of incubation suggesting that EGFR knockdown abolishes latent tumor formation observed in VHL-competent RCC cells (data not shown). Similar data were obtained with VHL$^{-/-}$ RCC KTCL140 cells expressing shRNA1, although tumor size observed with KTCL140 cells

expressing control shRNA was smaller than that observed with the 786-0 cells (Fig. 6*C*). These results show that shRNA-mediated silencing of EGFR phenocopies the effect of reintroduction of VHL, or silencing of HIF-2α, suggesting that EGFR is a central downstream target of HIF-dependent tumorigenesis.

Discussion

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



C

Cell Lines	Tumor	Injection	Tumor	Tumor size (mm)
786-0	Parental	4	4	9.3 +/- 1.1
	Vector	4	4	10.2 +/- 1.3
	VHL	8	0	0
	Control shRNAs	16	14	9.6 +/- 0.9
	EGFR shRNAs	16	0	0
KTCL	Control shRNAs	8	4	3.2 +/- 0.5
	EGFR shRNAs	8	0	0

Figure 6. Silencing of EGFR suppresses $VHL^{-/-}$ RCC tumor formation. *A*, representative nude mice injected with $VHL^{-/-}$ *RCC 786-0 cells expressing vector alone*, reintroduced VHL (*VHL*), control shRNA and shRNA-1 directed against EGFR. *B*, average of tumor mass 9 weeks post-injection of at least four injections per cell line. In all cases, we failed to detect a tumor even after 15 weeks following injection of shRNA to EGFR-expressing $VHL^{-/-}$ RCC cells; thus, no statistical analysis is shown. Small tumors were detected after ~12 weeks in the cases of 786-0 cells expressing reintroduced VHL. Notice that the tumor mass is lower in the KTCL140 cells compared with 786-0. *Columns*, average mean of tumor weight; *bars*, SE. *C*, cell line used in this study, number of injections, number of tumors observed after 9 weeks of incubation, and average size of tumors (±SE) when detectable. Experiments were blinded.

Hanahan and Weinberg proposed that the malignant phenotype is the manifestation of six essential alterations in cell physiology called hallmarks of cancer (26). Oncogenic dysregulation or environmental activation of HIF-2α may play a central role in at least two of these hallmarks. First, HIF-2α activation provides an environment of continued production of endogenous growth factor (TGF-α) and permanent EGFR signaling reducing the dependence of cancer cells on exogenous growth factors to maintain proliferation. Therefore, HIF-2α-expressing cells acquire the ability to engage in permanent EGFR signaling, which provides the necessary cellular milieu for growth autonomy. Whereas these data were obtained from renal cancer cells, we suggest that HIF-2α activation and subsequent EGFR signaling may have a broad implication in human malignancies. HIF-2α is induced in the vast majority of human cancer as a consequence of altered microenvironment conditions (e.g., hypoxia and acidosis) found in the core of tumors (34, 42). TGF-α overexpression and oncogenic dysregulation of EGFR activity are common features of human malignancies and are thought to provide permanent self-sufficiency in growth signaling that drives growth autonomy of cancer cells. Whereas still unproven, TGF-α/EGFR activation found in many tumors may be rationalized by HIF-2α activation and may explain why the hypoxic core of tumors is associated with increased probability of metastasis and poor prognosis. This hypothesis is supported by data shown in this report, which suggest that HIF-2α activation drives TGF-α production and EGFR activation in human cancer cell lines from different tissue origins. Second, although the data presented here focuses on the oncogenic potential of HIF-2α, its role in tumor angiogenesis has been previously well documented. HIF-2α, in cooperation with HIF-1α, activates genes such as *VEGF* and *TGF-β* to sustain proper tumor vascularization required for growth and metastasis, another hallmark of tumorigenesis.

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

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