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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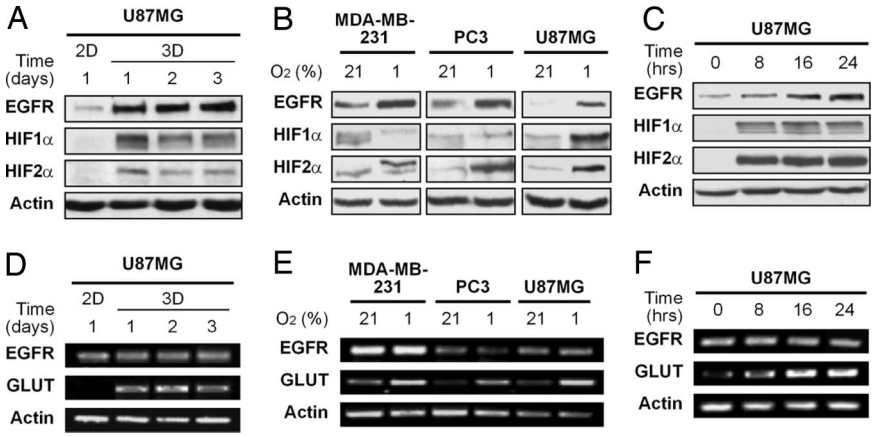

Fig. 1. The hypoxic tumor microenvironment triggers EGFR expression. (*A*) Western blot analysis of total EGFR and HIFα levels in U87MG glioma cells grown as 3D spheroids for 1–3 days. Control cells were grown in 2D culture. (*B*) Western blot analysis of EGFR and HIFα levels in U87MG, MDA-MB-231 breast, 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

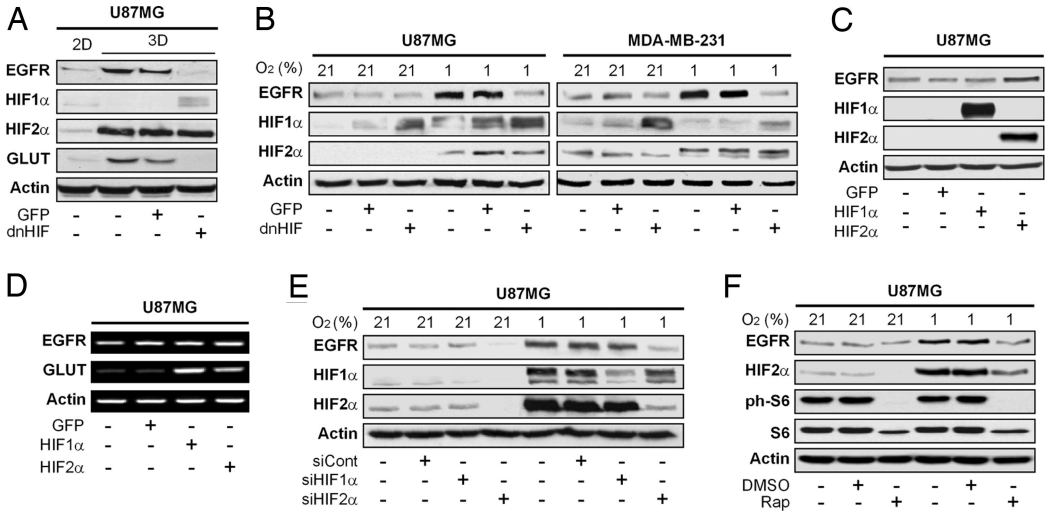

Fig. 2. HIF2α activation results in induction of EGFR protein levels. (*A*) Western blot analysis of EGFR, HIFα, and GLUT levels in U87MG cells infected with adenovirus expressing flag-tagged GFP or a dominant-negative form of HIF (dnHIF). Cells were grown as 3D spheroids for 3 days. Control cells were 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

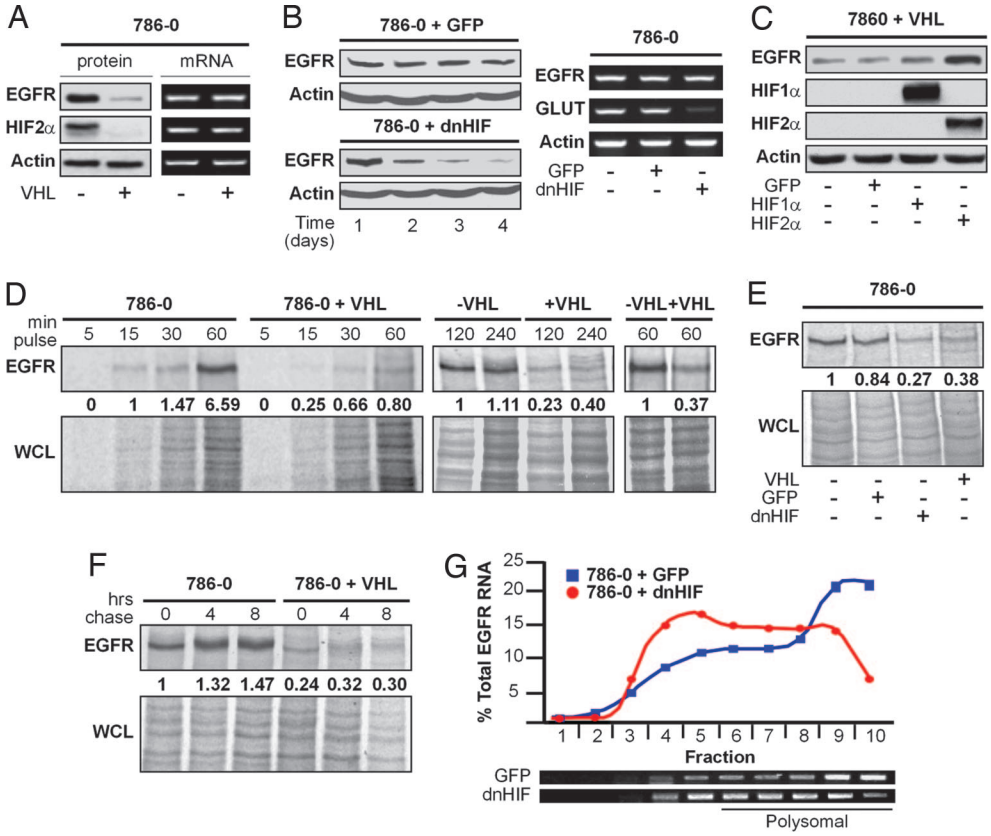

Fig. 3. HIF2α activation results in increased EGFR mRNA translation. (*A*) Western blot and RT-PCR analysis of EGFR protein and mRNA levels in the VHL$^{-/-}$ RCC, 786-0, cell line (786-0), and 786-0 cells stably expressing wild-type VHL (786-0 + VHL). (*B*) Western blot (*Left*) and RT-PCR (*Right*) 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

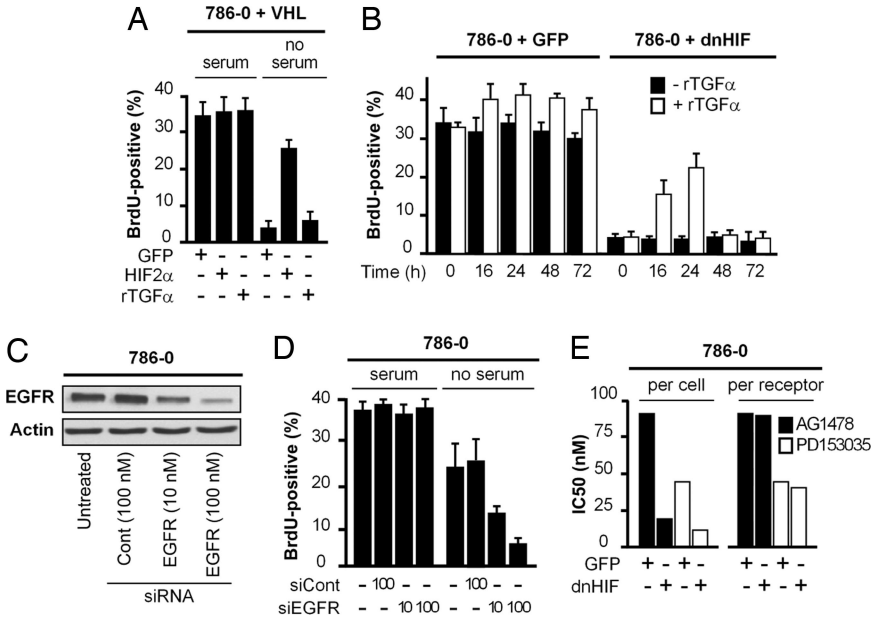

Fig. 4. Overexpression of EGFR results in cancer cell growth autonomy but does not affect sensitivity to anti-EGFR drugs. (*A*) VHL-competent RCC cells (786-0 + VHL) were infected with adenovirus expressing GFP or HIF2α or treated with 20 ng/ml recombinant TGFα (rTGFα) and then cultured in the absence 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 $\times$ *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 $\times$ *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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1. Salomon DS, Brandt R, Ciardiello F, Normanno N (1995) *Crit Rev Oncol Hematol* 19:183–232.
2. Yarden Y, Sliwkowski MX (2001) *Nat Rev* 2:127–137.
3. Hanahan D, Weinberg RA (2000) *Cell* 100:57–70.
4. Arteaga CL (2002) *Oncologist* 7:31–39.
5. Mendelsohn J, Baselga J (2000) *Oncogene* 19:6550–6565.
6. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS (2006) *Gene* 366:2–16.
7. Bache KG, Slagsvold T, Stenmark H (2004) *EMBO J* 23:2707–2712.
8. Halatsch, M-E, Schmidt U, Behnke-Mursch J, Unterberg A, Wirtz CR (2006) *Cancer Treat Rev* 32:74–89.
9. Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, Harris PL, Driscoll DR, Fidias P, Lynch TJ, *et al.* (2005) *Proc Natl Acad Sci USA* 102:7665–7670.
10. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, *et al.* (2004) *Science* 304:1497–1500.
11. Spindler KL, Olsen DA, Nielsen JN, Brandslund I, Poulsen HS, Villingshoj M, Jakobsen A (2006) *Anticancer Res* 26:4889–4893.
12. Blehm KN, Spiess PE, Bondaruk JE, Dujka ME, Villares GJ, Zhao Y-J, Bogler O, Aldape KD, Grossman HB, Adam L, *et al.* (2006) *Clin Cancer Res* 12:4671–4677.
13. Sharma SV, Bell DW, Settleman J, Haber DA (2007) *Nat Rev Cancer* 7:169–181.
14. Brown JM, Wilson WR (2004) *Nat Rev Cancer* 4:437–447.
15. Gatenby RA, Gillies RJ (2004) *Nat Rev Cancer* 4:891–899.
16. Semenza GL (2001) *Cell* 107:1–3.
17. Harris AL (2002) *Nat Rev Cancer* 2:38–47.
18. Vaupel P (2004) *Semin Radiat Oncol* 14:198–206.
19. Swinson DE, O'Byrne KJ (2006) *Clin Lung Cancer* 7:250–256.
20. Lieubeau-Teillet B, Rak J, Jothy S, Iliopoulos O, Kaelin W, Kerbel RS (1998) *Cancer Res* 58:4957–4962.
21. Gordan JD, Simon MC (2007) *Curr Opin Genet Dev* 17:71–77.
22. Holmquist-Mengelbier L, Fredlund E, Lofstedt T, Noguera R, Navarro S, Nilsson H, Pietras A, Vallon-Christersson J, Borg A, Gradin K, *et al.* (2006) *Cancer Cell* 10:413–423.
23. Maemura K, Hsieh CM, Jain MK, Fukumoto S, Layne MD, Liu Y, Kourembanas S, Yet SF, Perrella MA, Lee ME (1999) *J Biol Chem* 274:31565–31570.
24. Gunaratnam L, Morley M, Franovic A, de Paulsen N, Mekhail K, Parolin DAE, Nakamura E, Lorimer IAJ, Lee S (2003) *J Biol Chem* 278:44966–44974.
25. Smith K, Gunaratnam L, Morley M, Franovic A, Mekhail K, Lee S (2005) *Cancer Res* 65:5221–5230.
26. Kondo K, Klco J, Nakamura E, Lechpammer M, Kaelin WG, Jr (2002) *Cancer Cell* 1:237–246.
27. Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, Giaccia AJ, Abraham RT (2002) *Mol Cell Biol* 22:7004–7014.
28. Kaelin WG, Iliopoulos O, Lonergan KM, Ohh M (1998) *J Intern Med* 243:535–539.
29. Linehan WM, Lerman MI, Zbar B (1995) *J Am Med Assoc* 273:564–570.
30. Gemmill RM, Zhou M, Costa L, Korch C, Bukowski RM, Drabkin HA (2005) *Brit J Cancer* 92:2266–2277.
31. Gamou S, Shimizu N (1987) *J Biol Chem* 262:6708–6713.
32. Decker SJ (1984) *Mol Cell Biol* 4:571–575.
33. de Paulsen N, Brychzy A, Fournier, M-C, Klausner RD, Gnarra JR, Pause A, Lee S (2001) *Proc Natl Acad Sci USA* 98:1387–1392.
34. Franovic A, Robert I, Smith K, Kurban G, Pause A, Gunaratnam L, Lee S (2006) *Cancer Res* 66:8083–8090.
35. Dikic I, Giordano S (2003) *Curr Opin Cell Biol* 15:128–135.
36. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H (2005) *PLoS Med* 2:e73.
37. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B (2005) *N Engl J Med* 352:786–792.
38. Dancey JE (2004) *J Clin Oncol* 22:2975–2977.
39. Wang MY, Lu KV, Zhu S, Dia EQ, Vivanco I, Shackleford GM, Cavenee WK, Mellinghoff IK, Cloughesy TF, Sawyers CL, *et al.* (2006) *Cancer Res* 66:7864–7869.