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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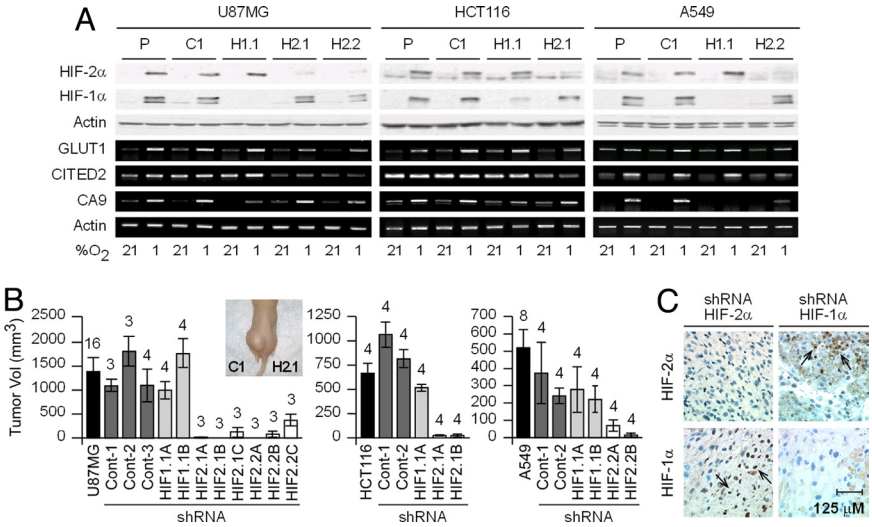

Fig. 1. Inhibition of HIF-2α prevents the tumorigenesis of genetically diverse human cancers. (*A*) Western blot (*Upper*) and RT-PCR (*Lower*) analysis of HIF-α subunit and target gene expression in serum-starved U87MG glioblastoma, HCT116 colorectal and A549 lung carcinoma cells stably expressing scramble 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

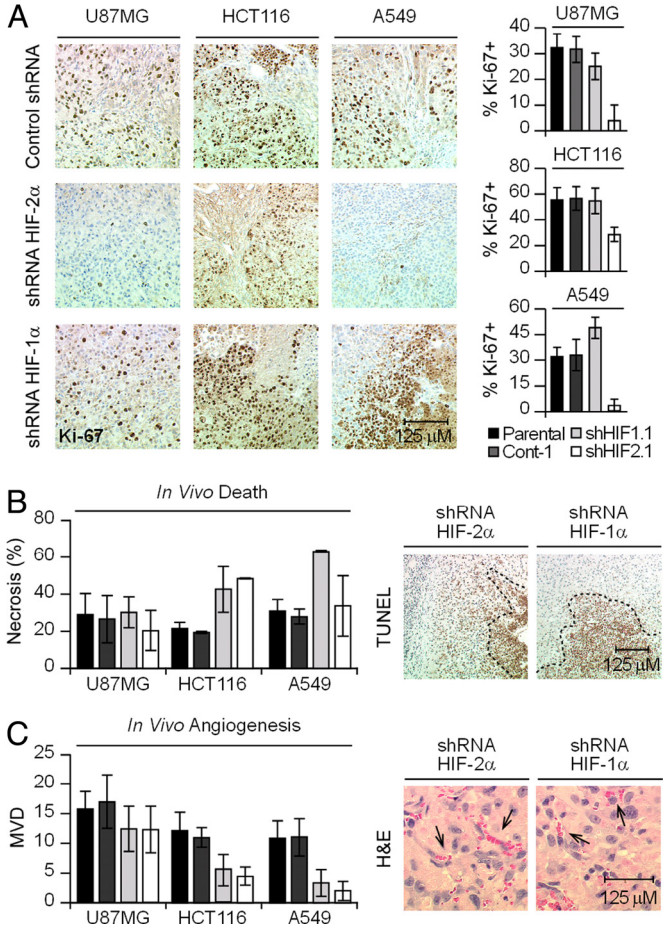

Fig. 2. Inhibition of HIF-2α prevents the proliferation of human cancer cells in vivo. (*A*) Representative images of Ki-67-stained initial mass tumor sections, derived from human cancer cell lines stably expressing scramble control, HIF-1α or HIF-2α shRNA, excised 1 week post-injection and visualized 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.

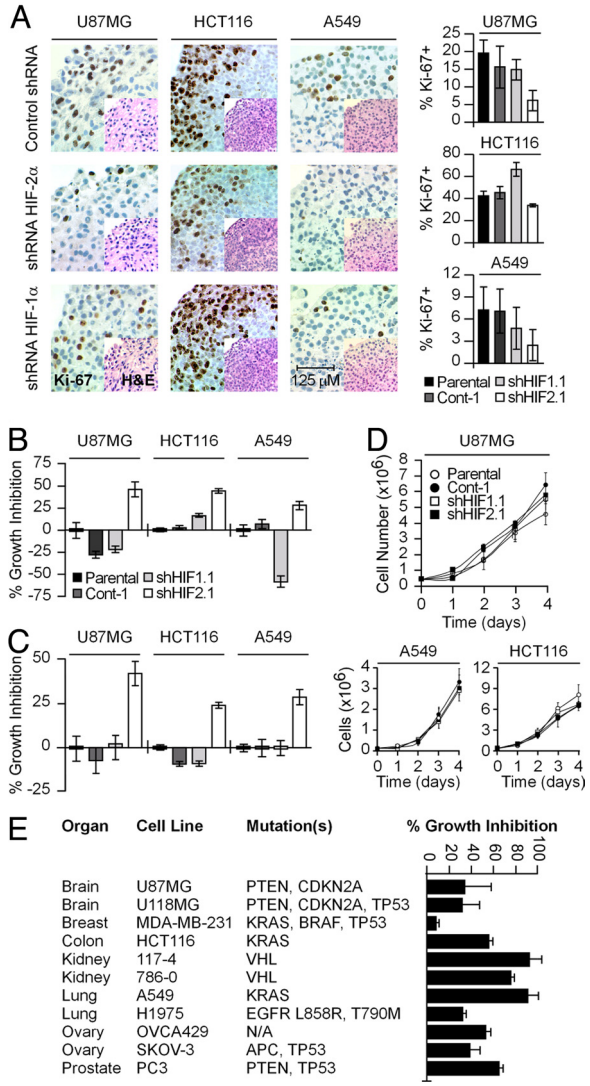

Fig. 3. Silencing HIF-2α prevents the autonomous growth of human cancer cells in vitro. (*A*) The ability of human cancer cell lines stably expressing scramble control, HIF-1α or HIF-2α shRNA to form multicellular spheroids and proliferate in an avascular setting was assessed by Ki-67-staining. Spheroids 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

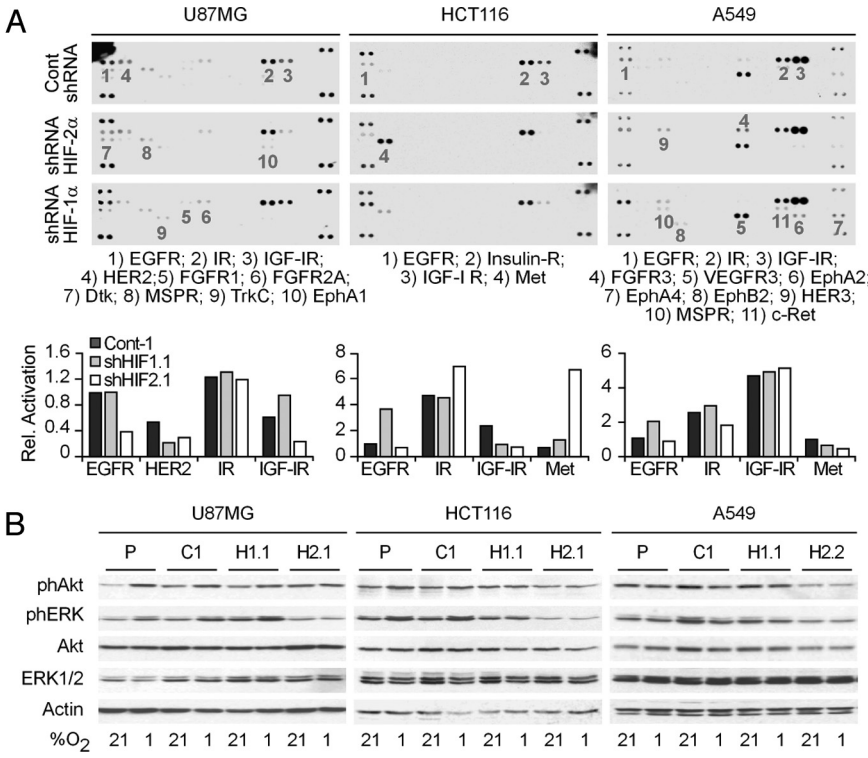

Fig. 4. HIF-2α activates the EGFR and IGF1R tyrosine kinases and their downstream signaling molecules. (*A*) Relative levels of RTK phosphorylation in serum-starved human cancer cell lines stably expressing scramble control, HIF-1α or HIF-2α shRNA after incubation in hypoxia for 24 h, as assessed using 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

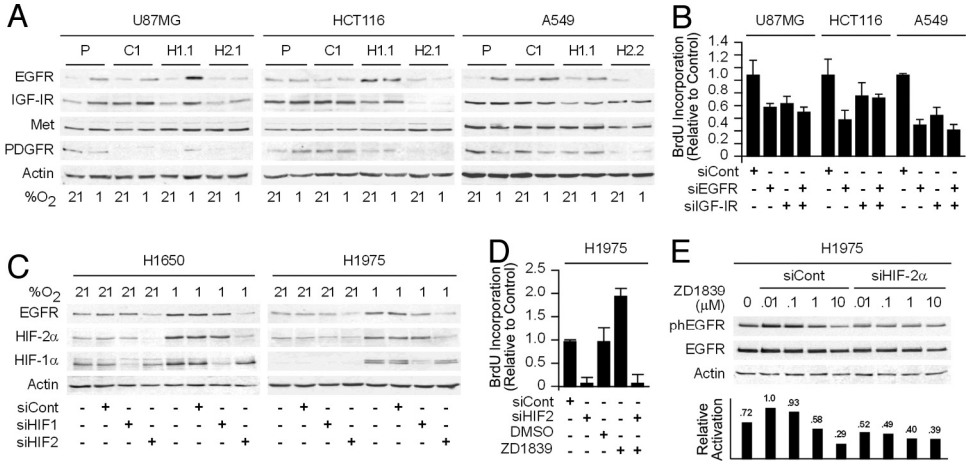

Fig. 5. HIF-2α regulates the expression of wild-type and mutant RTKs required for cell growth autonomy. (*A*) Western blot analysis of total cellular levels of various RTKs in human cancer cell lines stably expressing scramble control, HIF-1α, or HIF-2α shRNA after incubation in normoxia or hypoxia for 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 $\pm$ 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.

be mTOR-dependent as activation of this pathway was unaffected by silencing HIF-2α (Fig. S5). 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 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 *Bam*HI/*Hin*dIII 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.), phospho-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$\times$ 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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