# Genetic evidence for a tumor suppressor role of HIF-2α

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## Summary

**The hypoxia-inducible transcription factors HIF-1α and HIF-2α are activated in hypoxic tumor regions. However, their role in tumorigenesis remains controversial, as tumor growth promoter and suppressor activities have been ascribed to HIF-1α, while the role of HIF-2α remains largely unknown. Here, we show that overexpression of HIF-2α in rat glioma tumors enhances angiogenesis but reduces growth of these tumors, in part by increasing tumor cell apoptosis. Moreover, siRNA knockdown of HIF-2α reduced apoptosis in hypoxic human malignant glioblastoma cells. Furthermore, inhibition of HIF by overexpression of a dominant-negative HIF transgene in glioma cells or *HIF-2α* deficiency in teratomas reduced vascularization but accelerated growth of these tumor types. These findings urge careful consideration of using HIF inhibitors as cancer therapeutic strategies.**

## Introduction

Hypoxia is an important environmental determinant of tumor angiogenesis and growth. Hypoxia-inducible genes contain a hypoxia response element (HRE), to which the hypoxia-inducible transcription factors (HIF) HIF-1α and HIF-2α bind as a heterodimer with HIF-1β (Acker and Acker, 2004; Kaelin, 2004; Liu and Simon, 2004; Maxwell and Salnikow, 2004; Poellinger and Johnson, 2004; Schofield and Ratcliffe, 2004; Semenza, 2003). In addition, HIFs may also regulate gene transcription, independently of HRE, by counteracting the transcriptional activity of Myc (Koshiji et al., 2004, 2005). Despite structural similarities, HIF-1α and HIF-2α have a distinct spatiotemporal expression pattern and function in vivo. Indeed, *HIF-1α*-deficient embryos died of cardiovascular defects (Compernolle et al., 2003; Iyer et al., 1998; Ryan et al., 1998), while loss of *HIF-2α* impaired vascular remodeling and cardiac performance in embryos (Peng et al., 2000; Tian et al., 1998) and caused neonatal respiratory distress (Compernolle et al., 2002) and multiorgan pathology after birth (Scortegagna et al., 2003).

HIF-1α and HIF-2α are expressed in numerous tumors (Flamme et al., 1998; Jones et al., 2001; Krieg et al., 2000; Semenza, 2003; Talks et al., 2000; Wiesener et al., 1998; Xia et al., 2001). HIF-1α upregulates glycolytic enzymes and angiogenic factors and is a prognostic marker for a large number of tumors (Semenza, 2003). The importance of HIF-1α in regulating tumor angiogenesis was evidenced by the reduced vascularization of *HIF-1α*-deficient teratomas (Brown et al., 2001; Carmeliet et al., 1998; Ryan et al., 1998; Tsuzuki et al., 2000; Yu et al., 2001). However, the effect of *HIF-1α* deficiency on tumor growth differs widely. In some studies, loss of *HIF-1α* reduced the growth of teratomas and fibrosarcomas (Ryan et al., 1998, 2000), consistent with reports that HIF inhibitors attenuated tumorigenesis (Kung et al., 2000). However, in other

### SIGNIFICANCE

**The hypoxia-inducible transcription factors HIF-1α and HIF-2α have been proposed to promote tumor growth and angiogenesis. This has stimulated widespread interest in developing therapeutic strategies to block the HIF-dependent pathways. In the present study, we provide novel genetic evidence that HIF-2α acts as a tumor suppressor when overexpressed in glioma tumors, while gene transfer of a HIF inhibitor *accelerates*—not *delays*—growth of gliomas. Moreover, genetic inactivation of *HIF-2α* stimulates ES cell-derived tumor growth. Thus, based on our findings, we urge careful consideration of the use of HIF inhibitors as cancer therapeutic strategies.**

studies, *HIF-1α*-deficient teratomas grew faster, even despite a reduced tumor vascularization, due to a refractoriness of the mutant tumor cells to stress-induced apoptosis (Carmeliet et al., 1998). As a result, *HIF-1α*-deficient cells preferentially grew in hypoxic tumor regions (Brown et al., 2001; Yu et al., 2001). Thus, these genetic studies suggest that HIF-1α may act as either an oncogene or a tumor suppressor gene.

Even less is known about the role of HIF-2α in cancer, and the few published reports about its role are conflicting. A number of studies suggest a tumor-promoting activity of HIF-2α. Indeed, knockin replacement of *HIF-1α* by *HIF-2α* stimulated teratoma growth due to an increase in angiogenesis and cell proliferation (Covello et al., 2005), while a stable HIF-2α, but not HIF-1α, overrode the tumor-suppressing activity of the von Hippel-Lindau (VHL) protein in renal carcinoma cells (Kondo et al., 2002; Maranchie et al., 2002). Similarly, HIF-2α but not HIF-1α overexpression in *VHL*-defective renal carcinoma cells enhanced in vivo tumor growth (Raval et al., 2005). Furthermore, inhibition of HIF-2α suppressed tumor growth (Kondo et al., 2003; Zimmer et al., 2004). However, other studies suggest a tumor suppressor role for HIF-2α. Indeed, overexpression of HIF-2α has been reported to suppress tumor growth (Blancher et al., 2000), and loss of the *VHL* gene, resulting in increased activation of HIF-1α and HIF-2α, also impaired the growth of teratomas (Mack et al., 2003). Thus, the precise role of HIF-2α in tumor biology remains largely unknown. Nonetheless, a better understanding of how HIF-2α affects cancer is mandated, as the interest in developing anti-HIF strategies for cancer treatment is growing (Giaccia et al., 2003; Poellinger and Johnson, 2004; Semenza, 2003). In this study, we used gain- and loss-of-function methods to address this question in various tumor types.

## Results

### Generation of gliomas overexpressing HIF-2α

To evaluate the role of HIF-2α in tumorigenesis, we performed gain- and loss-of-function studies, using various tumor models of mouse, rat, and human origin. We first evaluated whether overexpression of HIF-2α affected tumor angiogenesis and growth. For such an experiment to be meaningful, the tumor cells would need to express endogenous HIF-2α, and the HIF-2α transgene would need to be overexpressed at physiological levels (i.e., comparable to the levels observed in hypoxic conditions), as supraphysiological transgene levels might affect tumor cell behavior nonspecifically. We therefore used a rat GS9L glioma tumor model, which expresses HIF-1α and HIF-2α (Figures 1A–1E; Figure S1 in the Supplemental Data available with this article online) and in which angiogenesis is dependent on hypoxic upregulation of VEGF.

We generated two stably transfected GS9L clones and a polyclonal pool of retrovirally infected GS9L cells that overexpress FLAG-tagged HIF-2α. Corresponding control cells were generated by mock transfection with pcDNA3 or infection with a LacZ virus. Expression of the *HIF-2α* transgene was analyzed by RT-PCR and FLAG immunoreactivity (data not shown), and by immunodetection of HIF-2α levels (Figures 1A–1E; Figure S2A; Table S1). These analyses revealed that HIF-2α levels in transgenic GS9L tumor cells in normoxia were comparable or slightly higher than those detected in mock-transfected cells in hypoxia (or exposed to hypoxia mimetics), while hypoxia upregulated HIF-2α expression levels to a similar extent (by ~30%) in control and HIF-2α-overexpressing cells. In addition, HIF-2α levels in the transfected GS9L glioma cells were comparable to those in various human glioblastoma lines (Figure S2B). Overexpression of HIF-2α upregulated mRNA transcript levels of VEGF in normoxia and hypoxia (Figure S2C; Table S2). For comparison, we also generated GS9L cells that overexpress HIF-1α at physiological levels (Figure 1E; Figure S1; Table S3). As this study focuses on HIF-2α, most data with HIF-1α were transferred to the Supplemental Data.

### HIF-2α stimulates tumor angiogenesis

HIF-2α-overexpressing and control GS9L cells were injected subcutaneously into nude mice. Histological analysis of established tumors revealed that necrosis was only rarely detected in control tumors (Figure 2A), while HIF-2α-overexpressing tumors had large necrotic areas, which were surrounded by microvessels and a rim of palisading cells (Figure 2B). The vascular tumor area was increased in HIF-2α-overexpressing tumors (Figures 2D and 2E; Table 1). Compared to control tumors, VEGF levels were increased in HIF-2α tumors, especially in the perinecrotic cells, as determined by Northern blotting (data not shown), in situ hybridization (Figures 2G and 2H), and immunohistochemistry (data not shown), thus indicating that regulation of tumor angiogenesis by HIF-2α is attributable, at least in part, to upregulation of VEGF expression. Similar findings were obtained in HIF-1α-overexpressing GS9L tumors (Figure S3).

### HIF-2α reduces tumor growth

Despite their increased vascularization, HIF-2α-overexpressing tumors grew more slowly. Control tumors grew to large sizes (>1000 $mm^3$) within 2.5 weeks, when the animals had to be sacrificed. In contrast, HIF-2α-overexpressing tumors grew only minimally during the first 2 weeks (<200 $mm^3$) and, even by 4 weeks, had still not obtained the size that control tumors obtained after 2 weeks ($p < 0.05$; $n = 10$; Figure 3A). Similar findings were obtained for another HIF-2α-overexpressing GS9L clone (Figure 3B) and a polyclonal pool of HIF-2α-overexpressing tumor cells (Figure S4), thus indicating that the reduced growth rate of HIF-2α-overexpressing tumors was not restricted to a single clone or a nonspecific clonal effect. The growth of HIF-1α-overexpressing tumors was similarly reduced (Figure S5A).

### Increased apoptosis in HIF-2α-expressing tumor cells

Compared to control tumors, apoptosis, quantified by determining the number of oligonucleosomes per 10 μg protein extract, was increased 5.1 ± 0.6 fold in HIF-2α tumors ($p < 0.005$; $n = 6$). The increased apoptosis of HIF-2α-overexpressing tumors was confirmed by measuring the tumor cell area, immunoreactive for activated caspase-3 (aCasp-3), an executer of apoptosis. In control tumors, aCasp-3-positive tumor cells were detected only occasionally, while numerous aCasp-3-positive cells were present in the perinecrotic area in tumors overexpressing HIF-2α (aCasp-$3^+$ area/optical field, 11.4% ± 1.9%; $n = 21$; Figures 3C and 3D). Similar findings were obtained for HIF-1α tumors (Figures S5B and S5C).

Protein levels of HIF-2α, both of the endogenous protein as well as of the transgene product, varied in individual cells, both in cultured tumor cells in vitro as well as in tumors in vivo—

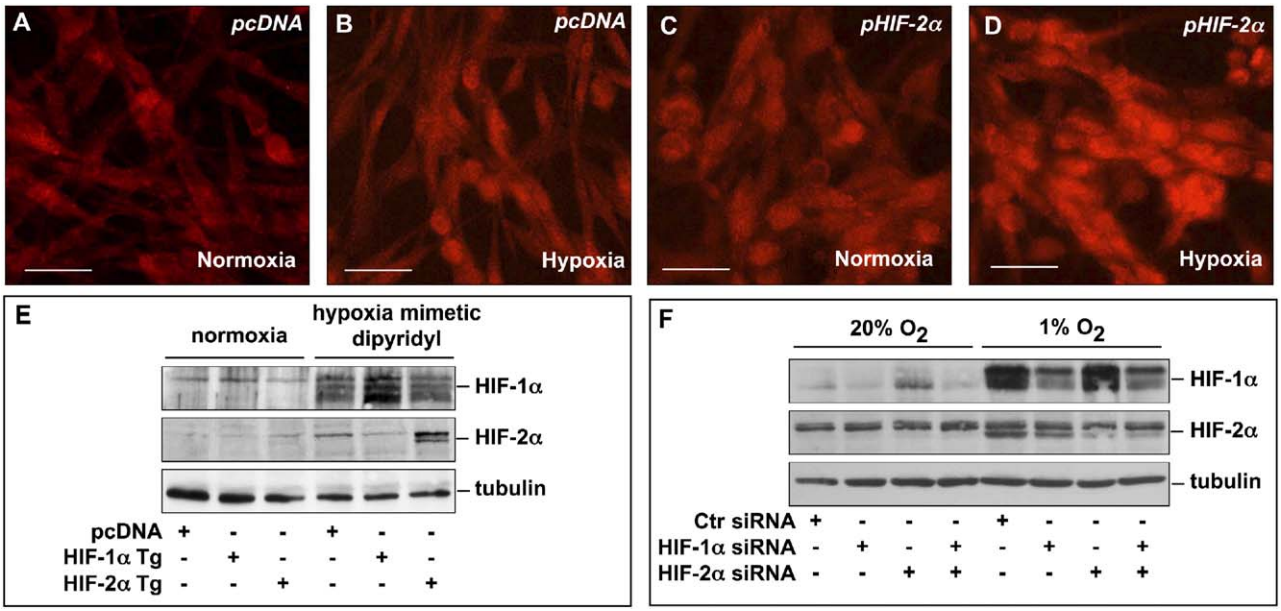



**Figure 1.** Generation of gliomas overexpressing HIF-2α

**A–D:** HIF-2α immunostaining of GS9L cells stably transfected with control vector (pcDNA3) or HIF-2α vector (*pHIF-2α*) in normoxia (**A and C**) or hypoxia (**B and D**). Scale bar, 20 μm in **A**–**D**.

**E:** Immunoblotting of HIF-1α and HIF-2α in GS9L cells stably transfected with control vector (pcDNA3), or with *HIF-1α* or *HIF-2α* transgenes (Tg) in normoxia and after treatment with dipyridyl.

**F:** Immunoblotting of HIF-1α and HIF-2α in human glioblastoma cells (G55), transiently transfected with control siRNA, *HIF-1α* siRNA, and/or *HIF-2α* siRNA.

presumably because of cellular differences in posttranslational stabilization. We took advantage of this heterogeneous expression pattern to examine whether those cells that expressed the highest HIF-2α levels were also labeled the strongest for aCasp3, as this might suggest that HIF-2α would switch on apoptosis in these cells. In both mock-transfected (data not shown) and HIF-2α-transfected tumor cells, the highest HIF-2α levels were detected in cultured cells that contained the highest aCasp-3 levels (Figures 4A–4C). When grown as tumors in vivo, aCasp-3 levels were maximal in a subset of glioma cells in the perinecrotic area that coexpressed abundant HIF-2α (Figures 4D–4F). These findings were not a peculiarity of rodent glioma tumors, as HIF-2α protein levels were also maximal in pseudopalisading cells in the perinecrotic area in human glioblastoma multiforme tumors (Figure 4G). Importantly, induction of apoptosis, assayed by TUNEL or aCasp-3 immunostaining, was also maximal in the pseudopalisading cells expressing maximal HIF-2α protein levels (Figures 4H and 4I).

### HIF-2α regulates tumor cell apoptosis

Even though the above findings show that elevated HIF-2α levels are correlated with increased tumor cell apoptosis, they do not necessarily establish a causal role of HIF-2α in tumor cell apoptosis. To address this question in more detail, we performed gain- or loss-of-HIF-2α function studies in cultured tumor cells. Overexpression of HIF-2α reduced the accumulation of cultured GS9L glioma cells. At 5 days after plating a comparable number of cells, HIF-2α-overexpressing cells accumulated at only 29% ± 2% of the number of mock-transfected cells ($p < 0.005$; $n = 6$). The reduced in vitro cell accumulation was, at least in part, attributable to an increase in apoptosis. Indeed, compared to control cells, apoptosis was increased in HIF-2α-overexpressing cells in normoxic and hypoxic conditions (Table 2). The increased apoptosis in HIF-2α-overexpressing cells was confirmed by staining for TUNEL (Figures 3F and 3G) and activated caspase-3 (Figures 3I and 3J). Apoptosis was also increased in the polyclonal pool of HIF-2α-overexpressing tumor cells (Supplemental Results). Similar findings were obtained after overexpression of HIF-1α in GS9L tumor cells (Figures S5D–S5G).

To further underscore a possible causal role of HIF-2α in apoptosis, we studied whether knockdown of HIF-2α, using siRNA, affected apoptosis of human glioblastoma tumor cells. Immunoblotting revealed that knockdown of *HIF-1α* or *HIF-2α* lowered (but did not completely eliminate) HIF-1α or HIF-2α protein levels (Figure 1F). Compared to normoxia, hypoxia increased apoptosis of cultured tumor cells by ~1.50-fold (Table 3). In both lines, knockdown of HIF-2α reduced tumor cell apoptosis in hypoxic conditions. A similar effect was observed when using siRNA to knock down the expression of HIF-1α, with the greatest effect resulting from a concomitant knockdown of HIF-1α and HIF-2α (Table 3). Thus, these over- and underexpression studies demonstrate that HIF-2α functions as a tumor suppressor gene, not only in rodent but also in human glioma tumors.

### HIF inhibition promotes tumor growth of gliomas despite reduced vascularization

Inhibition of HIF-1α has been proposed as a novel strategy to block tumor growth (Kung et al., 2000; Ratcliffe et al., 2000;

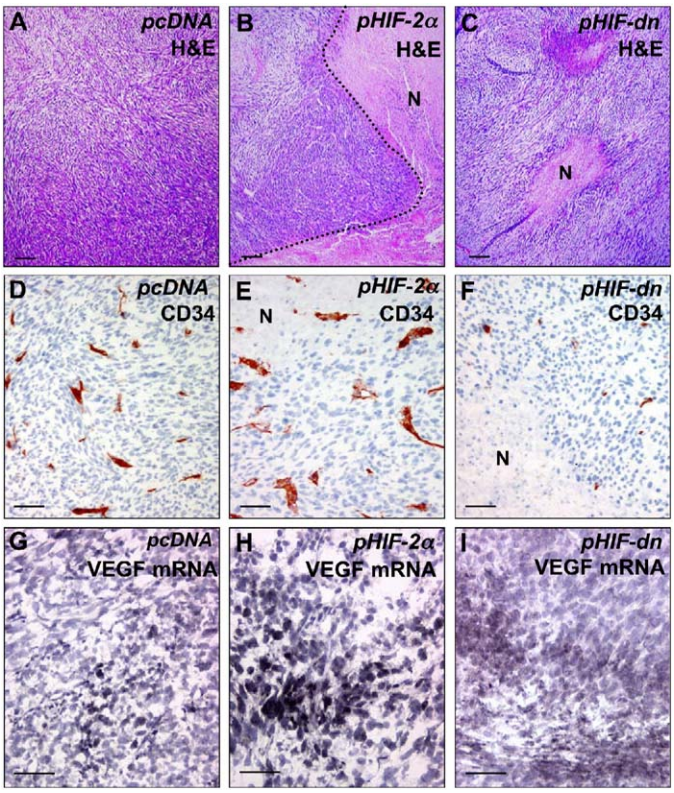



**Figure 2.** HIF-2α promotes tumor angiogenesis, whereas HIF inhibition reduces tumor angiogenesis

Microscopic analysis of GS9L tumors, stably transfected with control vector (pcDNA3) or constructs expressing HIF-2α (*pHIF-2α*) or dominant-negative HIF (pHIF-dn).

**A–C:** H&E staining showed the presence of large necrotic zones (N) in HIF-2α-overexpressing (**B**) tumors, but not or much less in control (**A**) tumors. HIF-dn tumors had micronecrotic regions (**C**).

**D–F:** CD34 staining (red) revealed more vascularization in HIF-2α-overexpressing (**E**) than control (**D**) tumors; in contrast, vascularization in HIF-dn tumors (**F**) was decreased.

**G–I:** In situ VEGF mRNA staining (black dots). In agreement with the vascularization patterns, HIF-2α-overexpressing tumors (**H**) showed high VEGF mRNA levels, whereas control tumors (**G**) showed moderate levels and HIF-dn tumors (**I**) showed low levels of VEGF.

Scale bar, 250 μm in **A**–**C**; 100 μm in **D**–**I**.

Zhong et al., 2000). Considering that overexpression of HIF-2α or HIF-1α retarded tumor growth, we evaluated whether inhibition of HIF-2α and HIF-1α would stimulate tumor angiogenesis and growth. Therefore, we generated both single clones (stable transfection) and a polyclonal pool (lentiviral infection) of GS9L cells overexpressing a FLAG-tagged dominant-negative-acting HIF (HIF-dn) that comprises the amino-terminal residues 24–325 of HIF-2α, lacks the DNA binding and transactivation domains, and blocks the activity of both HIF-1α and HIF-2α (Elvert et al., 2003). Compared to control cells, VEGF levels were significantly reduced in cells overexpressing the *HIF-dn* construct in normoxia and hypoxia (Figure S2C; Table S2).

Compared to controls, HIF-dn-expressing tumors, injected subcutaneously into nude mice, had multiple micronecrotic areas (Figure 2C). The vessel density and total vessel per tumor area were significantly decreased in HIF-dn tumors (Figure 2F; Table 1)—a likely cause why micronecrosis occurred in these tumors. VEGF mRNA (Figure 2I) and protein expression (data not shown) were also attenuated in HIF-dn tumors. Growth of monoclonal and polyclonal HIF-dn tumors was accelerated already at 1 week after inoculation ($p < 0.05$; $n = 10$; Figures 3A and 3B; Figure S4).

We also evaluated whether reduced apoptosis might explain the accelerated growth of HIF-dn tumors. Interestingly, despite the existence of multiple micronecrotic areas, only few aCasp$^+$ cells were detected in the perinecrotic tumor border. Unfortunately, however, the degree of apoptosis in the control tumor was too low to reliably detect any significant differences with the HIF-dn tumor (Figure 3E). We therefore examined whether we could detect any effect of HIF-dn on apoptosis of cultured monoclonal and polyclonal tumor cells in vitro. When counting the number of oligonucleosomes (Table 2), staining for 3′-hydroxylated DNA fragments (TUNEL) (Figure 3H) or activated caspase-3 (Figure 3K), or counting by flow cytometry the percentage of annexin V-positive propidium iodide-negative cells (Supplemental Results), no differences in apoptosis were detected between control and HIF-dn-transfected cells, cultured under normoxic conditions—presumably because the degree of apoptosis was minimal under normoxia. In hypoxic conditions, apoptosis was increased, but notably, apoptosis was lower in HIF-dn than in control cells (Table 2; Supplemental Results). Taken together, inhibition of HIF-2α (and HIF-1α) by a dominant-negative HIF-dn decreased apoptosis of tumor cells in vitro, thereby likely promoting tumor growth in vivo.

### Reduced vascularization of *HIF-2α*-deficient ES cell-derived tumors

Since the dominant-negative HIF construct inhibits both HIF-1α and HIF-2α, we also evaluated whether specific loss of *HIF-2α* alone would affect tumor angiogenesis and growth. We therefore used *HIF-2α*-deficient (*HIF-2α$^{-/-}$*) embryonic stem (ES) cells (Brusselmans et al., 2001) and evaluated the vascularization and growth of wild-type (wt) and *HIF-2α$^{-/-}$* ES cell-derived tumors, injected subcutaneously into nude mice. Macroscopic inspection after dissecting the tumors at 5 weeks after inoculation revealed that wt tumors appeared dark red and bled profusely, while *HIF-2α$^{-/-}$* tumors were pale and failed to bleed. When counting the total number of all vessels on tissue sections, we found no differences in vascular density in wt and *HIF-2α$^{-/-}$* tumors (CD34-positive vessels/mm$^2$, 190 ± 20 in *HIF-2α$^{-/-}$* tumors versus 160 ± 10 in wt tumors; $p = 0.13$; $n = 10$). Nonetheless, clear genotypic differences were detectable, when the vessels were categorized according to their size. Wt tumors contained large vessels and "blood lakes" in addition to medium-sized and small vessels (Figures 5A and 5C), while *HIF-2α$^{-/-}$* tumors only contained smaller vessels (Figures 5B and 5D). The density of small blood vessels (diameter <35 μm) was comparable in wt and *HIF-2α$^{-/-}$* tumors (vessels/mm$^2$, 180 ± 20 in *HIF-2α$^{-/-}$* tumors versus 140 ± 10 in wt tumors; $p = 0.09$; $n = 10$). In contrast, medium-sized (diameter 35–90 μm) and large blood vessels (diameter >90 μm) were significantly underrepresented in *HIF-2α$^{-/-}$* tumors (medium-sized and large vessels/mm$^2$, 8 ± 3 and 0.33 ± 0.21 in *HIF-2α$^{-/-}$* tumors versus 17 ± 1 and 2.6 ± 0.8 in wt tumors; $p < 0.05$; $n = 10$). As a result, the total vessel area per tumor area was reduced in *HIF-2α$^{-/-}$* tumors (CD34$^+$ vessel area per tumor area, 6.1% ± 0.4% in *HIF-2α$^{-/-}$* tumors versus 10.5% ± 1.4% in wt

**Table 1**. Effects of HIF-2α on vascularization of GS9L tumors

| | Transgene | All vessels | Small-sized vessels | Medium-sized vessels | Large-sized vessels |
|---|---|---|---|---|---|
| Vessel area (percentage) | *pcDNA3* | 7.2 ± 0.4 | 1.3 ± 0.2 | 2.2 ± 0.2 | 3.7 ± 0.4 |
| | *HIF-2α* | 8.8 ± 0.6* | 1.2 ± 0.1 | 1.9 ± 0.2 | 5.8 ± 0.8* |
| | *HIF-dn* | 3.6 ± 0.5* | 0.4 ± 0.03* | 0.8 ± 0.1* | 2.3 ± 0.4* |
| Vessels/mm$^2$ | *pcDNA3* | 190 ± 19 | 120 ± 15 | 50 ± 5 | 23 ± 1 |
| | *HIF-2α* | 190 ± 17 | 120 ± 15 | 43 ± 5 | 26 ± 2 |
| | *HIF-dn* | 70 ± 7* | 40 ± 4* | 19 ± 2* | 13 ± 3* |
| Mean vessel area (μm$^2$) | *pcDNA3* | 400 ± 40 | 100 ± 10 | 450 ± 10 | 1600 ± 100 |
| | *HIF-2α* | 500 ± 70 | 100 ± 10 | 440 ± 10 | 2200 ± 200* |
| | *HIF-dn* | 500 ± 50 | 100 ± 10 | 440 ± 10 | 1900 ± 130 |

GS9L glioma tumor lines were transfected with pcDNA3 (mock), *HIF-2α*, or HIF-dn. The vessel area was expressed as a percentage of the total tumor area analyzed. Vessel area and density and mean vessel area are represented for all vessels (all) or per category of vessels: small (0–250 μm$^2$), medium (250–750 μm$^2$), and large (>750 μm$^2$). The data represent the mean ± SEM (n = 7–8). *p < 0.05 versus pcDNA3.

tumors; p = 0.008; n = 10). Taken together, vascularization was significantly impaired in *HIF-2α$^{-/-}$* tumors.

To analyze whether the decreased vascularization in the *HIF-2α$^{-/-}$* tumors resulted in reduced blood supply, vascular volume and tumor oxygenation were analyzed by intravital microscopy. Therefore, wt or *HIF-2α$^{-/-}$* tumors were implanted in a dorsal skin chamber, and the tumor vasculature was visualized using fluorescence microscopy and epi-illumination of an intravenously injected fluorescent dye (FITC-dextran). No significant differences were observed in the total amount of vessels,

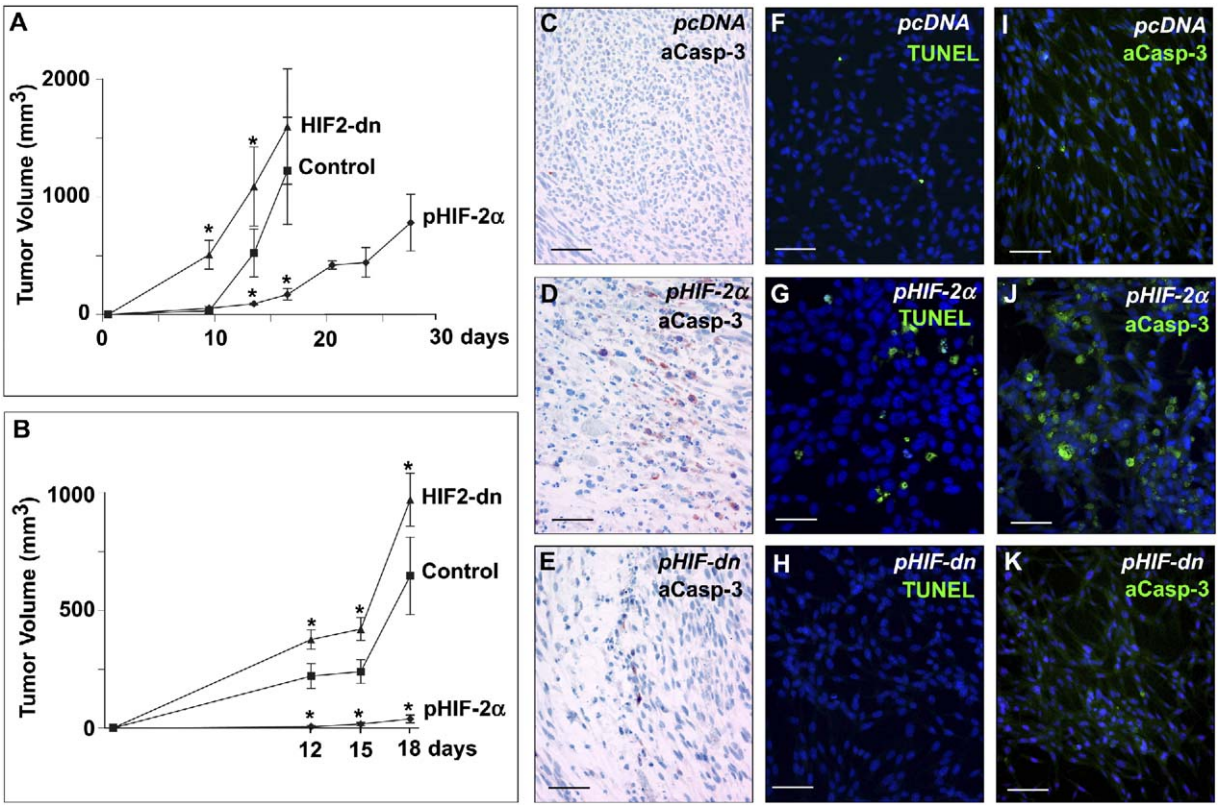



**Figure 3.** HIF-2α reduces tumor growth, whereas HIF inhibition promotes tumor growth

**A and B:** Growth rate of tumors derived from GS9L cells, stably transfected with control vector (pcDNA3) or with constructs expressing HIF-2α (*pHIF-2α*) or a dominant-negative HIF (pHIF-dn). **A** and **B** display two separate experiments, using the same control and *pHIF-dn* tumor clone but different individual HIF-2α-overexpressing tumor clones. Data represent mean ± SEM (n = 10). *p < 0.05 versus pcDNA3.

**C–K:** Apoptosis in GS9L cells and GS9L cell-derived tumors, stably transfected with control vector (pcDNA3) or with constructs expressing HIF-2α (*pHIF-2α*) or a dominant-negative HIF (*pHIF-dn*). **C–E:** In contrast to HIF-dn (**E**) and control tumors (**C**), HIF-2α-overexpressing tumors (**D**) showed excessive staining for activated caspase-3 (red staining), indicating that HIF-2α induced tumor cell apoptosis. TUNEL staining (**F–H**, green) and activated caspase-3 staining (**I–K**, green) of stably transfected GS9L cells cultured under hypoxia in vitro revealed that overexpression of HIF-2α (**G and J**) induced activation of caspase-3 and apoptosis in GS9L glioma cell. Scale bar, 100 μm in **C–E**; 50 μm in **F–K**.

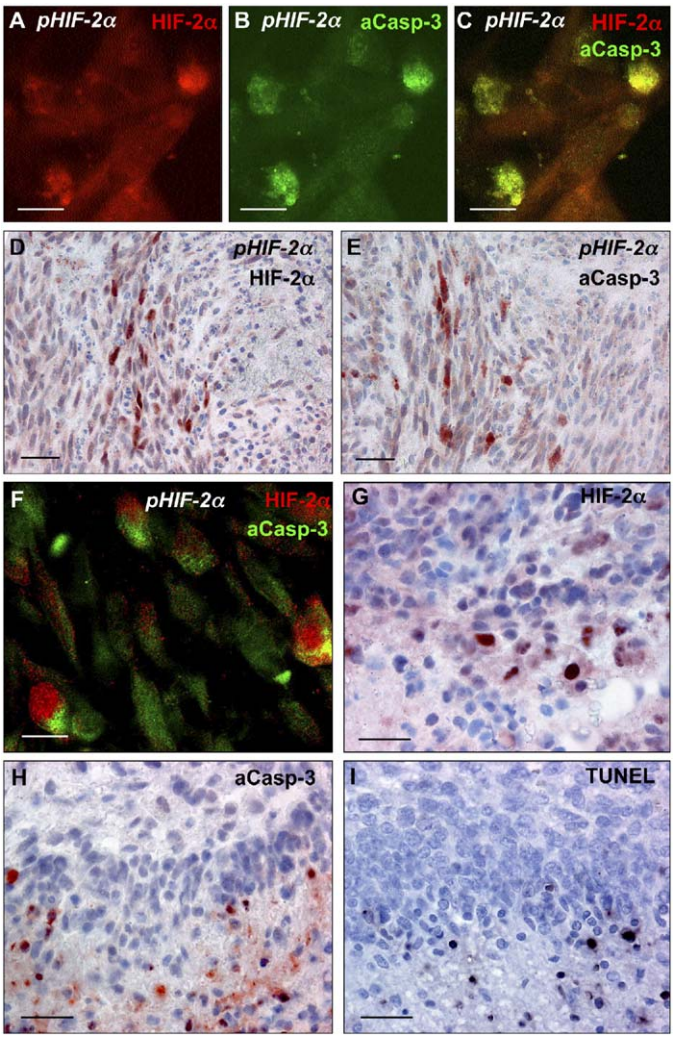



**Figure 4.** HIF-2α regulates tumor cell apoptosis

**A–C:** Coexpression of HIF-2α and activated caspase-3. Immunostainings for HIF-2α (**A**, red), activated caspase-3 (**B**, green), and merged (HIF-2α/aCasp-3) (**C**, yellow) stainings on GS9L cells in vitro, stably transfected with a construct expressing HIF-2α (*pHIF-2α*), showed that HIF-2α and activated caspase-3 were coexpressed in the same cells, indicating that HIF-2α induces apoptosis.

**D and E:** Tumors derived from GS9L cells, stably transfected with a construct expressing HIF-2α (*pHIF-2α*), showed excessive staining (red) of HIF-2α (**D**) and activated caspase-3 (**E**) in perinecrotic regions.

**F:** Double immunostaining of GS9L tumors, stably transfected with a construct expressing HIF-2α (*pHIF-2α*), for aCasp-3 (green) and HIF-2α (red) reveals coexpression in the same tumor cells (yellow color after merging).

**G–I:** HIF-2α (**G**), activated caspase-3 (**H**), and TUNEL (**I**) staining in human glioblastoma multiforme tumors. HIF-2α protein levels were highest in the pseudopalisading cells in the perinecrotic area (red staining), which showed maximal induction of apoptosis, as assayed by activated caspase-3 immunostaining (red) or TUNEL (brown/black staining), thereby confirming the colocalization of HIF-2α and apoptosis.

Scale bar, 10 μm in **A**–**C** and **F**; 75 μm in **D**, **E**, **G**, **H**, and **I**.

but the mean vessel diameter was significantly smaller in *HIF-2α*$^{-/-}$ than wt tumors (Figures 5E–5G). Also, the $pO_2$ was 30% lower in *HIF-2α*$^{-/-}$ than in wt tumors, likely as a result of the impaired vascularization (Figure 5G). Quantitative RT-PCR revealed that transcript levels of VEGF, but not of other angiogenic genes, were lower in *HIF-2α*$^{-/-}$ tumors (Table S4), suggesting that the reduced tumor vascularization was largely attributable to the reduced VEGF levels.

**Table 2.** Effects of HIF-2α and HIF-dn on apoptosis of GS9L clones

| Transgene | Normoxia | Hypoxia |
|---|---|---|
| Mock | 1.00 ± 0.02 | 2.06 ± 0.12+ |
| HIF-2α (clone 1) | 3.49 ± 0.33+ | 5.47 ± 0.14* |
| HIF-2α (clone 2) | 2.06 ± 0.09+ | 4.36 ± 0.6* |
| HIF-dn | 0.92 ± 0.03 | 1.27 ± 0.09* |

Apoptosis was quantified by measuring the amount of oligonucleosomes. The data (mean ± SEM; n = 4) are expressed as the fold induction (or reduction) obtained by normalization of the data relative to the data obtained in the mock-transfected cells in normoxia. +p < 0.05 versus control (normoxia).*p < 0.05 versus control (hypoxia).

### *HIF-2α*$^{-/-}$ tumors grow faster than wt tumors

We had anticipated that *HIF-2α*$^{-/-}$ ES cell tumors would grow more slowly because of their reduced vascularization. Surprisingly, however, *HIF-2α*$^{-/-}$ tumors grew faster than wt tumors. By 3 weeks, *HIF-2α*$^{-/-}$ tumors already tended to be larger than wt tumors (Figure 5H), but by 5 weeks, *HIF-2α*$^{-/-}$ tumors were twice as large as wt tumors (Figure 5H). The increased growth of *HIF-2α*$^{-/-}$ tumors might be attributable, at least in part, to reduced apoptosis, which was significantly lower in *HIF-2α*$^{-/-}$ than wt tumors at 5 weeks after inoculation (oligonucleosomes per mg tumor protein, 540 ± 70 for *HIF-2α*$^{-/-}$ tumors versus 1270 ± 190 for wt tumors; p = 0.016; n = 5). Similar observations were made using different wt and *HIF-2α*$^{-/-}$ ES cell clones (data not shown).

## Discussion

Little is known about the function of HIF-2α in cancer. Here, we report that overexpression of HIF-2α enhanced glioma tumor vascularization but impaired tumor growth, in part because of an increase in tumor cell apoptosis. Moreover, elevated expression of HIF-2α coincided with apoptosis in human malignant glioblastoma multiforme tumors, while inhibition of HIF-2α by siRNA attenuated hypoxic cell apoptosis. Furthermore, gene transfer of a dominant-negative HIF mutant, which inhibits the activity of HIF-2α (and HIF-1α), or genetic loss of HIF-2α accelerated tumor growth—even despite reduced tumor vascularization.

### Role of HIF-2α in tumor vascularization

Our genetic data that tumor vascularization was increased by overexpression of HIF-2α and reduced by loss or inhibition of HIF-2α indicate that HIF-2α regulates tumor vascularization. This effect was, at least in part, attributable to a reduction in VEGF production. Teratomas that lack *HIF-1α* or the HRE in the *VEGF* promoter are also less vascularized due to reduced VEGF levels (Carmeliet et al., 1998; Ryan et al., 1998; Tsuzuki et al., 2000). Blocking the interaction of HIF-1α with the transcriptional coactivator p300 or dysregulation of the HIF pathway by 2ME2 also reduced tumor vessel density (Kung et al., 2000; Mabjeesh et al., 2003). Thus, all these findings indicate that not only HIF-1α, but also HIF-2α, regulates tumor vascu-

**Table 3**. Effect of siRNA-mediated knockdown of HIF-1α and HIF-2α on hypoxia-induced apoptosis in human glioblastoma cell lines

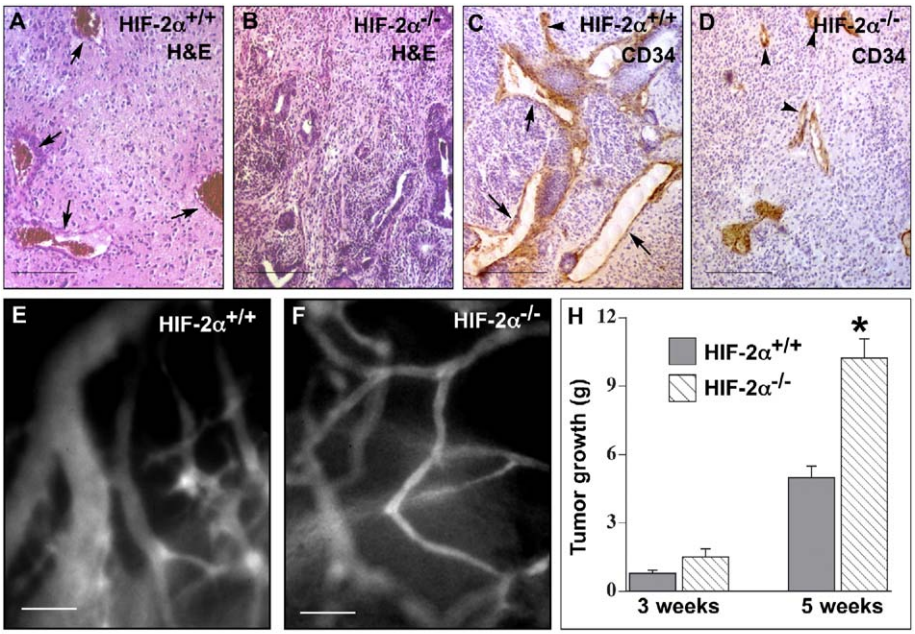
| Cell line | Normoxia control | Hypoxia control | Hypoxia/HIF-1α-kd | Hypoxia/HIF-2α-kd | Hypoxia/HIF-1α/HIF-2α-kd |
|---|---|---|---|---|---|
| G55 | 1.00 ± 0.009 | 1.54 ± 0.008+ | 0.71 ± 0.005* | 0.89 ± 0.007* | 0.66 ± 0.007* |
| G141 | 1.00 ± 0.008 | 1.43 ± 0.01+ | 1.33 ± 0.02* | 1.33 ± 0.016* | 1.13 ± 0.007* |

Human glioblastoma cell lines G55 and G141 were transiently transfected with control siRNA (mock) or with a siRNA directed to *HIF-1α*, *HIF-2α*, or a combination of both (kd, knockdown). Apoptosis was quantified by measuring the amount of oligonucleosomes. The data (mean ± SEM; n = 4) are expressed as the fold induction (or reduction) obtained by normalization of the data relative to the data obtained in the control mock under normoxia. *p < 0.05 versus control (normoxia).*p < 0.05 versus control (hypoxia).

larization via upregulation of VEGF. Obviously, we cannot exclude the possibility that HIF-1α and HIF-2α regulate the expression of additional angiogenic genes. An outstanding question is whether inhibition or deficiency of *HIF-2α* resulted in a partial normalization of the abnormal tumor vasculature, which would be expected to result in an increase in tumor perfusion and growth.

### Role of HIF-2α in tumor growth

We observed that loss of *HIF-2α* or suppression of HIF by a dominant-negative mutant stimulated the growth of teratomas and gliomas, even despite their reduced vascularization. Such a reduced vascular dependence was also observed for *HIF-1α*-deficient teratomas but, importantly, also for subpopulations of human melanoma cells growing in tumor regions devoid of a vascular supply (Yu et al., 2001). The increased growth of *HIF-2α* null or HIF-inhibited tumors was attributable, at least in part, to a reduction in tumor cell apoptosis. These in vivo data extend our previous in vitro data that HIF-2α regulates stress-induced ES cell apoptosis (Brusselmans et al., 2001). The *HIF-dn* construct blocks both HIF-1α and HIF-2α, and thus, the enhanced growth of HIF-dn tumors is likely attributable to the suppression of both HIFs. In line with these findings, siRNA knockdown of HIF-1α or HIF-2α, and especially a combined knockdown of HIF-1α and HIF-2α, attenuated hypoxia-induced apoptosis of human glioblastoma cells, suggesting that both factors cooperate. Further evidence for a tumor suppressor role of these HIFs is deduced from our present and previous findings that overexpression of HIF-1α or HIF-2α suppresses tumor growth, while loss or inhibition of these factors accelerates tumor growth (this study and Carmeliet et al., 1998).




| G | HIF-2α+/+ | HIF-2α-/- | p-value |
|---|---|---|---|
| vascular density (cm/cm$^2$) | 142.0 ± 13.0 | 154.0 ± 8.0 | 0.661 |
| vessel diameter (μm) | 19.6 ± 0.9 | 16.9 ± 1.1 | 0.023 |
| vascular volume (μm$^3$/μm$^2$) | 54.6 ± 4.7 | 45.4 ± 6.2 | 0.069 |
| $pO_2$ (mm Hg) | 17.5 ± 1.6 | 12.2 ± 0.9 | 0.014 |

**Figure 5.** Genetic inactivation of HIF-2α reduces angiogenesis but promotes growth of ES cell-derived tumors

**A–D:** H&E staining revealed the presence of large vascular structures (arrows) in 5-week-old wt (*HIF-2α$^{+/+}$*) tumors (**A**), whereas *HIF-2α$^{-/-}$* tumors appeared much less vascularized (**B**). Endothelial cell staining for CD34 showed the presence of both capillaries and small vessels (arrowheads) as well as larger vascular structures (arrows) in wt tumors (**C**), and only smaller vessels (arrowheads) in *HIF-2α$^{-/-}$* tumors (**D**).

**E–G:** By intravital microscopy, *HIF-2α$^{-/-}$* tumors (**F**) had a smaller vessel diameter and a reduced vascular volume than wt tumors (**E**). **G:** Vascularization parameters and oxygenation are shown in the table (mean ± SEM; n = 4). p values: Mann-Whitney U test.

**H:** Growth rate of wt and *HIF-2α$^{-/-}$* tumors: after 3 weeks, no significant difference was observed between wt and *HIF-2α$^{-/-}$* tumors; however, after 5 weeks *HIF-2α$^{-/-}$* tumors were larger than wt tumors. Mean ± SEM (n = 55–71). *p < 0.05 versus wt.

Scale bar, 250 μm in **A**–**D**; 25 μm in **E** and **F**.

### Hypoxia-inducible factors: A more complex role than anticipated?

Thus far, all studies, including the present, reported a positive role of HIF-1α, HIF-2α, and HIF-1β in tumor vascularization via upregulation of VEGF expression. They differ, however, widely in their reports on the role of these factors in tumor growth. Most studies documented that tumors express relatively high levels of HIF-1α, especially when growing more aggressively (Semenza, 2002), and that tumor growth (Ryan et al., 1998, 2000; Zimmer et al., 2004) or patient prognosis of various tumor types including breast (Dales et al., 2005), lung (Giatromanolaki et al., 2001), colorectal (Yoshimura et al., 2004), and cervical cancer (Bachtiary et al., 2003) correlated with the expression levels of HIF-1α or HIF-2α. Moreover, loss of *HIF-1α*, suppression of HIF by a dominant-negative HIF-1α, or pharmacological inhibition of HIF-1α by chemotin or topotecan impaired the growth of various cancers (Chen et al., 2003; Iyer et al., 1998; Kung et al., 2004; Maltepe et al., 1997; Rapisarda et al., 2004; Ryan et al., 1998; Stoeltzing et al., 2004).

In contrast, other studies did not report such an oncogenic activity of HIF-1α. Indeed, increased activation of the HIF pathway in *VHL*-deficient tumors or expression of a stable HIF-1α variant in renal carcinoma cells slowed down tumor growth (Mack et al., 2003; Maranchie et al., 2002; Raval et al., 2005). HIF-1α also upregulated the levels of the cell cycle inhibitors $p21^{CIP1}$ and $p27^{KIP1}$, explaining why growth of *VHL*-deficient fibrosarcomas was decreased (Mack et al., 2005). Furthermore, escape from stress-induced cellular death contributed to the uncontrolled growth of *HIF-1α* null teratomas despite their reduced vascularization (Carmeliet et al., 1998). In mixed tumors that contained both wt and *HIF-1α*-deficient ES cells, only *HIF-1α* null cells were able to survive in areas distant from tumor vessels (Brown et al., 2001; Yu et al., 2001). Also, patients with HIF-1α-positive nonsmall cell lung carcinomas showed increased tumor apoptosis and survived longer than patients with HIF-1α-negative carcinomas (Volm and Koomagi, 2000). Expression of HIF-1α in head-and-neck squamous cell carcinoma was associated with increased patient survival (Beasley et al., 2002). Another study reported that HIF-1α/HIF-2α levels were inversely correlated with the growth of human breast cancer cells and that HIF-2α overexpression impaired the growth of these tumors (Blancher et al., 2000).

How can we reconcile these apparently conflicting results? First, it is well known that HIF-1α and HIF-2α are required for the survival and differentiation of many nonmalignant cell types (Compernolle et al., 2002, 2003; Iyer et al., 1998; Peng et al., 2000; Ryan et al., 1998; Scortegagna et al., 2003; Tian et al., 1998), but it is also known that activation of the HIF pathway can induce growth arrest or death of primary cells, such as developing thymocytes and chondrocytes (Biju et al., 2004; Schipani et al., 2001). HIFs exert such proapoptotic activity by stabilizing p53, through a Bnip3/Nix-dependent mechanism, or by altering the expression of Bcl-2 and caspase family members (Greijer and van der Wall, 2004). While these dual activities of the HIFs may seem, at first sight, conflicting, they may, in fact, be necessary to allow the cell to respond optimally to hypoxia. Indeed, through the induction of angiogenesis and glycolysis, activation of HIF gives cells a growth advantage in hypoxia, but the induction of growth arrest is also to the cell's advantage to survive when the oxygen supply is limited (Koshiji and Huang, 2004). That these dual HIF-related activities are operational in tumors is evidenced by findings that, despite an increase in cell proliferation, *HIF-1α*-deficient hepatoma growth was impaired because of an even greater apoptosis rate (Leek et al., 2005). It has been proposed that tumor cells employ such a "stop-and-go" strategy to maintain growth and survival. However, how hypoxic cells strike a balance between these divergent HIF activities remains an outstanding question. Clearly, the overall effect on tumor growth is determined by the relative oncogenic or tumor-suppressive activity of the HIFs.

We speculate that the role of HIF-1α or HIF-2α in the growth/survival versus arrest/apoptosis decision during hypoxia may be influenced by a variety of genetic or environmental mechanisms. For example, oncogenes such as *Src* and *Ras* affect cell survival during hypoxia by upregulating HIF-1α (Chen et al., 2000). The pH and cell cycle are also involved, as hypoxia-induced apoptosis or accumulation of p53 requires acidosis (Pan et al., 2004; Schmaltz et al., 1998), while cells in S phase are more sensitive to hypoxia (Amellem and Pettersen, 1991). Interestingly, acidosis increases HIF-1α levels by nuclear sequestration of pVHL (Mekhail et al., 2004). Other questions are whether variations in cell density, degree of hypoxia, cell type, or perhaps even the relative HIF levels determine the oncogenic or tumor suppressor role of the HIF pathways. Alternatively, HIFs may determine clonal selection of tumor cell types, as observed in *HIF-1α* null tumors (Carmeliet et al., 1998). Since HIF-1α and HIF-2α differ in their spatiotemporal expression pattern, individual genetically unstable tumor cells that have lost HIF alone or in combination with another tumor suppressor gene may be prone to clonal selection and overgrowth. Such a mechanism is supported by evidence that p53-deficient tumor cells are clonally selected and overgrow wt cells in hypoxic tumor regions (Graeber et al., 1996). Finally, the tumor stroma and surrounding tissue influence the role of HIFs in cancer, as illustrated by the findings that *HIF-1α* null glial tumors grow slower at heterotopic implantation sites but faster at orthotopic sites (Blouw et al., 2003). Whatever the molecular mechanism, these data show that HIFs have dual—even opposite—activities in tumor growth.

### Medical implications

Our findings may have implications for the development of anticancer therapies. Inhibition of HIF has been proposed to be an attractive novel treatment to deprive the tumor cell of oxygen and nutrients by suppressing tumor angiogenesis, while at the same time disabling the mechanisms of the tumor to survive in a hostile microenvironment (Kung et al., 2000; Ratcliffe et al., 2000; Zhong et al., 2000). However, based on the present study, some caution is warranted to block HIF-2α in cancer, as it may bear a risk that individual tumor clones might become less dependent on vascular supply and develop resistance to stress-induced apoptosis, which would *promote*—instead of *suppress*—tumor growth.

#### Experimental procedures

##### Cell culture and tumor experiments

Generation of ES cell-derived tumors was performed as described (Carmeliet et al., 1998). GS9L cells were stably transfected with different HIF expression constructs (*HIF-1α*, *HIF-2α*, and *HIF-dn* in *pcDNA-3* [Elvert et al., 2003]) and clonally selected. Individual clones ($10^6$ cells) were used for tumor formation as described (Damert et al., 1997). To construct the recombinant lentiviral vectors, *HIF-2α* and *HIF-dn* cDNA were subcloned in the

Plenti6/UbC vector, and viral particles were produced using ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's directions. Cells were selected with 50 μg/ml of blasticidin and, after 5 days, with 10 μg/ml; the polyclonal pool of resistant cells was used for all experiments. To avoid any phenotypic change, polyclonal cells were used until passage 5 after infection. Gene expression was analyzed by RT-PCR (for primer sequence, see the Supplemental Experimental Procedures) or by Western blotting using antibodies specific for HIF-1α (Novus Biologicals, Littleton, CO), HIF-2α (Novus Biologicals), or FLAG (M2; Sigma-Aldrich, Saint-Louis, MO). Glioblastoma cell lines were kindly provided by M. Westphal (Hamburg). Transient siRNA transfection was performed with Oligofectamine using the control (SIMA), *HIF-1α*, and *HIF-2α* sequence as described (Berra et al., 2003; Sowter et al., 2003). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee for Medical Ethics and Clinical Research of the University of Leuven, the Regierungspräsidium Freiburg, or the University of Harvard and were performed according to the national guidelines for animal experiments.

**Intravital microscopy**

Intravital microscopy of ES cell-derived tumors in dorsal skin chambers was performed as described (Carmeliet et al., 1998). Mice with 17 day old tumors (8–10 mm in diameter) were injected intravenously with FITC-dextran (2 million MW), and vessel architecture was recorded using epi-illumination microscopy. Vessel diameter, vessel density (defined as the total length of vessels per unit area; cm/cm$^2$), and vascular volume (defined as the total volume of vessels per unit area; μm$^3$/μm$^2$) were analyzed offline. Local tissue $pO_2$ was quantified by measuring the $O_2$-dependent phosphorescence quenching of albumin-bound palladium meso-tetra-(4-carboxyphenyl)-porphyrin.

**Histology and apoptosis**

All methods for histology, immunostaining, TUNEL staining, and in situ hybridization have been described (Carmeliet et al., 1996, 1998). Vascular density of tumors was quantified after staining of endothelial cells, using antibodies against CD34 (BD Pharmingen, San Diego, CA) or CD31 (Pharmingen), as the percentage of area surrounded by endothelial cells (vascular lumen) per optical field (1.345 mm$^2$) using the Quantimet Q600 imaging system and the Soft Imaging Analysis system (Soft Imaging System GmbH, Muenster, Germany). Morphometric analysis was performed by counting the number of small vessels (diameter < 35 μm for teratoma; area < 250 μm$^2$ for GS9L tumors), medium-sized vessels (diameter between 35 and 90 μm for teratoma; area between 250 and 750 μm$^2$ for GS9L tumors), and large vessels (diameter > 90 μm for teratoma; area > 750 μm$^2$ for GS9L tumors) per optical field in at least 25 randomly chosen optical fields per tumor. To analyze apoptosis by immunohistochemistry, we used antibodies recognizing activated caspase-3 (Promega, Madison, WI). Apoptosis was measured by quantification of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) and confirmed by TUNEL staining (Carmeliet et al., 1998). Apoptosis of the polyclonal pool of infected cells, exposed to 30 hr of 2% $O_2$, was analyzed by flow cytometry after labeling the cells with propidium iodide and annexin V-FITC, as described (Chen et al., 2005). Addition of the apoptotic compound staurosporine (Sigma) was used as positive control (data not shown).

**Statistical analysis**

Results are represented as mean ± standard error of the mean (SEM). Comparisons of values were made using a nonparametric Mann-Whitney U test, Student's t test, or ANOVA, as indicated. Significance was assumed at $p < 0.05$.

**Supplemental data**

The Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/2/131/DC1/.

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**References**

Acker, T., and Acker, H. (2004). Cellular oxygen sensing need in CNS function: physiological and pathological implications. J. Exp. Biol. *207*, 3171–3188.

Amellem, O., and Pettersen, E.O. (1991). Cell inactivation and cell cycle inhibition as induced by extreme hypoxia: the possible role of cell cycle arrest as a protection against hypoxia-induced lethal damage. Cell Prolif. *24*, 127–141.

Bachtiary, B., Schindl, M., Potter, R., Dreier, B., Knocke, T.H., Hainfellner, J.A., Horvat, R., and Birner, P. (2003). Overexpression of hypoxia-inducible factor 1α indicates diminished response to radiotherapy and unfavorable prognosis in patients receiving radical radiotherapy for cervical cancer. Clin. Cancer Res. *9*, 2234–2240.

Beasley, N.J., Leek, R., Alam, M., Turley, H., Cox, G.J., Gatter, K., Millard, P., Fuggle, S., and Harris, A.L. (2002). Hypoxia-inducible factors HIF-1α and HIF-2α in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. Cancer Res. *62*, 2493–2497.

Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J. *22*, 4082–4090.

Biju, M.P., Neumann, A.K., Bensinger, S.J., Johnson, R.S., Turka, L.A., and Haase, V.H. (2004). Vhlh gene deletion induces Hif-1-mediated cell death in thymocytes. Mol. Cell. Biol. *24*, 9038–9047.

Blancher, C., Moore, J.W., Talks, K.L., Houlbrook, S., and Harris, A.L. (2000). Relationship of hypoxia-inducible factor (HIF)-1α and HIF-2α expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. Cancer Res. *60*, 7106–7113.

Blouw, B., Song, H., Tihan, T., Bosze, J., Ferrara, N., Gerber, H.P., Johnson, R.S., and Bergers, G. (2003). The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell *4*, 133–146.

Brown, E.B., Campbell, R.B., Tsuzuki, Y., Xu, L., Carmeliet, P., Fukumura, D., and Jain, R.K. (2001). In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. Nat. Med. *7*, 864–868.

Brusselmans, K., Bono, F., Maxwell, P., Dor, Y., Dewerchin, M., Collen, D., Herbert, J.M., and Carmeliet, P. (2001). Hypoxia-inducible factor-2α (HIF-2α) is involved in the apoptotic response to hypoglycemia but not to hypoxia. J. Biol. Chem. *276*, 39192–39196.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435–439.

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., et al. (1998).

Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature *394*, 485–490.

Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F., and Maity, A. (2000). Regulation of glut1 mRNA by HIF-1: Interaction between H-ras and hypoxia. J. Biol. Chem. *276*, 9519–9525.

Chen, J., Zhao, S., Nakada, K., Kuge, Y., Tamaki, N., Okada, F., Wang, J., Shindo, M., Higashino, F., Takeda, K., et al. (2003). Dominant-negative hypoxia-inducible factor-1α reduces tumorigenicity of pancreatic cancer cells through the suppression of glucose metabolism. Am. J. Pathol. *162*, 1283–1291.

Chen, G.G., Sin, F.L., Leung, B.C., Ng, H.K., and Poon, W.S. (2005). Glioblastoma cells deficient in DNA-dependent protein kinase are resistant to cell death. J. Cell. Physiol. *203*, 127–132.

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., et al. (2002). Loss of HIF-2α and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat. Med. *8*, 702–710.

Compernolle, V., Brusselmans, K., Franco, D., Moorman, A., Dewerchin, M., Collen, D., and Carmeliet, P. (2003). Cardia bifida, defective heart development and abnormal neural crest migration in embryos lacking hypoxia-inducible factor-1α. Cardiovasc. Res. *60*, 569–579.

Covello, K.L., Simon, M.C., and Keith, B. (2005). Targeted replacement of hypoxia-inducible factor-1α by a hypoxia-inducible factor-2α knock-in allele promotes tumor growth. Cancer Res. *65*, 2277–2286.

Dales, J.P., Garcia, S., Meunier-Carpentier, S., Andrac-Meyer, L., Haddad, O., Lavaut, M.N., Allasia, C., Bonnier, P., and Charpin, C. (2005). Overexpression of hypoxia-inducible factor HIF-1α predicts early relapse in breast cancer: Retrospective study in a series of 745 patients. Int. J. Cancer *116*, 734–739.

Damert, A., Ikeda, E., and Risau, W. (1997). Activator-protein-1 binding potentiates the hypoxia-inducible factor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells. Biochem. J. *327*, 419–423.

Elvert, G., Kappel, A., Heidenreich, R., Englmeier, U., Lanz, S., Acker, T., Rauter, M., Plate, K., Sieweke, M., Breier, G., and Flamme, I. (2003). Cooperative interaction of hypoxia-inducible factor-2α (HIF-2α) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). J. Biol. Chem. *278*, 7520–7530.

Flamme, I., Krieg, M., and Plate, K.H. (1998). Up-regulation of vascular endothelial growth factor in stromal cells of hemangioblastomas is correlated with up-regulation of the transcription factor HRF/HIF-2α. Am. J. Pathol. *153*, 25–29.

Giaccia, A., Siim, B.G., and Johnson, R.S. (2003). HIF-1 as a target for drug development. Nat. Rev. Drug Discov. *2*, 803–811.

Giatromanolaki, A., Koukourakis, M.I., Sivridis, E., Turley, H., Talks, K., Pezzella, F., Gatter, K.C., and Harris, A.L. (2001). Relation of hypoxia inducible factor 1α and 2α in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. Br. J. Cancer *85*, 881–890.

Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., and Giaccia, A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature *379*, 88–91.

Greijer, A.E., and van der Wall, E. (2004). The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. J. Clin. Pathol. *57*, 1009–1014.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., and Semenza, G.L. (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1α. Genes Dev. *12*, 149–162.

Jones, A., Fujiyama, C., Blanche, C., Moore, J.W., Fuggle, S., Cranston, D., Bicknell, R., and Harris, A.L. (2001). Relation of vascular endothelial growth factor production to expression and regulation of hypoxia-inducible factor-1α and hypoxia-inducible factor-2α in human bladder tumors and cell lines. Clin. Cancer Res. *7*, 1263–1272.

Kaelin, W.G., Jr. (2004). Proline hydroxylation and gene expression. Annu. Rev. Biochem. Annu. Rev. Biochem. *74*, 115–128.

Kondo, K., Klco, J., Nakamura, E., Lechpammer, M., and Kaelin, W.G., Jr. (2002). Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell *1*, 237–246.

Kondo, K., Kim, W.Y., Lechpammer, M., and Kaelin, W.G., Jr. (2003). Inhibition of HIF2α is sufficient to suppress pVHL-defective tumor growth. PLoS Biol. *1*, e83. 10.1371/journal.pbio.0000083.

Koshiji, M., and Huang, L.E. (2004). Dynamic balancing of the dual nature of HIF-1α for cell survival. Cell Cycle *3*, 853–854.

Koshiji, M., Kageyama, Y., Pete, E.A., Horikawa, I., Barrett, J.C., and Huang, L.E. (2004). HIF-1α induces cell cycle arrest by functionally counteracting Myc. EMBO J. *23*, 1949–1956.

Koshiji, M., To, K.K., Hammer, S., Kumamoto, K., Harris, A.L., Modrich, P., and Huang, L.E. (2005). HIF-1α induces genetic instability by transcriptionally downregulating MutSα expression. Mol. Cell *17*, 793–803.

Krieg, M., Haas, R., Brauch, H., Acker, T., Flamme, I., and Plate, K.H. (2000). Up-regulation of hypoxia-inducible factors HIF-1α and HIF-2α under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene *19*, 5435–5443.

Kung, A.L., Wang, S., Klco, J.M., Kaelin, W.G., and Livingston, D.M. (2000). Suppression of tumor growth through disruption of hypoxia-inducible transcription. Nat. Med. *6*, 1335–1340.

Kung, A.L., Zabludoff, S.D., France, D.S., Freedman, S.J., Tanner, E.A., Vieira, A., Cornell-Kennon, S., Lee, J., Wang, B., Wang, J., et al. (2004). Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway. Cancer Cell *6*, 33–43.

Leek, R.D., Stratford, I., and Harris, A.L. (2005). The role of hypoxia-inducible factor-1 in three-dimensional tumor growth, apoptosis, and regulation by the insulin-signaling pathway. Cancer Res. *65*, 4147–4152.

Liu, L., and Simon, M.C. (2004). Regulation of transcription and translation by hypoxia. Cancer Biol. Ther. *3*, 492–497.

Mabjeesh, N.J., Escuin, D., LaVallee, T.M., Pribluda, V.S., Swartz, G.M., Johnson, M.S., Willard, M.T., Zhong, H., Simons, J.W., and Giannakakou, P. (2003). 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell *3*, 363–375.

Mack, F.A., Rathmell, W.K., Arsham, A.M., Gnarra, J., Keith, B., and Simon, M.C. (2003). Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. Cancer Cell *3*, 75–88.

Mack, F.A., Patel, J.H., Biju, M.P., Haase, V.H., and Simon, M.C. (2005). Decreased growth of Vhl–/– fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. Mol. Cell. Biol. *25*, 4565–4578.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*, 403–407.

Maranchie, J.K., Vasselli, J.R., Riss, J., Bonifacino, J.S., Linehan, W.M., and Klausner, R.D. (2002). The contribution of VHL substrate binding and HIF1-α to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell *1*, 247–255.

Maxwell, P., and Salnikow, K. (2004). HIF-1: An oxygen and metal responsive transcription factor. Cancer Biol. Ther. *3*, 29–35.

Mekhail, K., Gunaratnam, L., Bonicalzi, M.E., and Lee, S. (2004). HIF activation by pH-dependent nucleolar sequestration of VHL. Nat. Cell Biol. *6*, 642–647.

Pan, Y., Oprysko, P.R., Asham, A.M., Koch, C.J., and Simon, M.C. (2004). p53 cannot be induced by hypoxia alone but responds to the hypoxic microenvironment. Oncogene *23*, 4975–4983.

Peng, J., Zhang, L., Drysdale, L., and Fong, G.H. (2000). The transcription factor EPAS-1/hypoxia-inducible factor 2α plays an important role in vascular remodeling. Proc. Natl. Acad. Sci. USA *97*, 8386–8391.

Poellinger, L., and Johnson, R.S. (2004). HIF-1 and hypoxic response: the plot thickens. Curr. Opin. Genet. Dev. *14*, 81–85.

Rapisarda, A., Zalek, J., Hollingshead, M., Braunschweig, T., Uranchimeg, B., Bonomi, C.A., Borgel, S.D., Carter, J.P., Hewitt, S.M., Shoemaker, R.H., and Melillo, G. (2004). Schedule-dependent inhibition of hypoxia-inducible factor-1α protein accumulation, angiogenesis, and tumor growth by topotecan in U251-HRE glioblastoma xenografts. Cancer Res. *64*, 6845–6848.

Ratcliffe, P.J., Pugh, C.W., and Maxwell, P.H. (2000). Targeting tumors through the HIF system. Nat. Med. *6*, 1315–1316.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L., and Ratcliffe, P.J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol. Cell. Biol. *25*, 5675–5686.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J. *17*, 3005–3015.

Ryan, H.E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J.M., and Johnson, R.S. (2000). Hypoxia-inducible factor-1α is a positive factor in solid tumor growth. Cancer Res. *60*, 4010–4015.

Schipani, E., Ryan, H.E., Didrickson, S., Kobayashi, T., Knight, M., and Johnson, R.S. (2001). Hypoxia in cartilage: HIF-1α is essential for chondrocyte growth arrest and survival. Genes Dev. *15*, 2865–2876.

Schmaltz, C., Hardenbergh, P.H., Wells, A., and Fisher, D.E. (1998). Regulation of proliferation-survival decisions during tumor cell hypoxia. Mol. Cell. Biol. *18*, 2845–2854.

Schofield, C.J., and Ratcliffe, P.J. (2004). Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell Biol. *5*, 343–354.

Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L.J., Marck, B.T., Matsumoto, A.M., Shelton, J.M., Richardson, J.A., et al. (2003). Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1–/– mice. Nat. Genet. *35*, 331–340.

Semenza, G.L. (2002). HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol. Med. *8*, S62–S67.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer *3*, 721–732.

Sowter, H.M., Raval, R.R., Moore, J.W., Ratcliffe, P.J., and Harris, A.L. (2003). Predominant role of hypoxia-inducible transcription factor (Hif)-1α versus Hif-2α in regulation of the transcriptional response to hypoxia. Cancer Res. *63*, 6130–6134.

Stoeltzing, O., McCarty, M.F., Wey, J.S., Fan, F., Liu, W., Belcheva, A., Bucana, C.D., Semenza, G.L., and Ellis, L.M. (2004). Role of hypoxia-inducible factor 1α in gastric cancer cell growth, angiogenesis, and vessel maturation. J. Natl. Cancer Inst. *96*, 946–956.

Talks, K.L., Turley, H., Gatter, K.C., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., and Harris, A.L. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1α and HIF-2α in normal human tissues, cancers, and tumor-associated macrophages. Am. J. Pathol. *157*, 411–421.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W., and McKnight, S.L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev. *12*, 3320–3324.

Tsuzuki, Y., Fukumura, D., Oosthuyse, B., Koike, C., Carmeliet, P., and Jain, R.K. (2000). Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1α→ hypoxia response element→ VEGF cascade differentially regulates vascular response and growth rate in tumors. Cancer Res. *60*, 6248–6252.

Volm, M., and Koomagi, R. (2000). Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Res. *20*, 1527–1533.

Wiesener, M.S., Turley, H., Allen, W.E., Willam, C., Eckardt, K.U., Talks, K.L., Wood, S.M., Gatter, K.C., Harris, A.L., Pugh, C.W., et al. (1998). Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1α. Blood *92*, 2260–2268.

Xia, G., Kageyama, Y., Hayashi, T., Kawakami, S., Yoshida, M., and Kihara, K. (2001). Regulation of vascular endothelial growth factor transcription by endothelial PAS domain protein 1 (EPAS1) and possible involvement of EPAS1 in the angiogenesis of renal cell carcinoma. Cancer *91*, 1429–1436.

Yoshimura, H., Dhar, D.K., Kohno, H., Kubota, H., Fujii, T., Ueda, S., Kinugasa, S., Tachibana, M., and Nagasue, N. (2004). Prognostic impact of hypoxia-inducible factors 1α and 2α in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. Clin. Cancer Res. *10*, 8554–8560.

Yu, J.L., Rak, J.W., Carmeliet, P., Nagy, A., Kerbel, R.S., and Coomber, B.L. (2001). Heterogeneous vascular dependence of tumor cell populations. Am. J. Pathol. *158*, 1325–1334.

Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M.M., Simons, J.W., and Semenza, G.L. (2000). Modulation of hypoxia-inducible factor 1α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res. *60*, 1541–1545.

Zimmer, M., Doucette, D., Siddiqui, N., and Iliopoulos, O. (2004). Inhibition of hypoxia-inducible factor is sufficient for growth suppression of VHL–/– tumors. Mol. Cancer Res. *2*, 89–95.