Tumor growth and hypoxia

It's all about location, location and location

Barbara Blouw

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Tumor growth and hypoxia:
it's all about location, location and location

Tumor groei en hypoxie: van belang is de lokatie, lokatie en lokatie (met een samenvatting in het Nederlands)

Proefschrift

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Voor mijn ouders

Voor Daniel

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CHAPTER 1

Introduction: Hypoxia and Cancer

HYPOXIA AND CANCER

Hypoxia, a reduction in oxygen tension, occurs during normal biological processes such as wound healing and exercise, but also during pathological situations such as ischemia (cerebral and pulmonary) and cancer (1). Tumors become hypoxic because the high proliferation rate of the cancer cells causes the tumor to outgrow its normal blood supply, and the newly formed blood vessels that result from angiogenesis, have a poor blood flow (2). The physiological response of cells when exposed to reduced oxygen levels is activation of the hypoxic pathway, which leads to significant changes in gene expression that are mediated by an increase in expression of the Hypoxia Inducible transcription Factors (HIFs) (3). The processes that are initiated within the tumor upon activation of the hypoxic response, involve the formation of new blood vessels (angiogenesis) (4) and the switch from oxidative phosphorylation to glycolysis as a source of ATP (known as the the Pasteur's effect) (5). Cancer cells however, preferentially use glycolysis to generate energy regardless of a reduction the oxygen levels, a phenomenon known as the Warburg effect (6). Another important consequence of hypoxia with regard to tumor malignancy, is the activation of genes that allow cancer cells to invade the surrounding tissue and metastasize to distant organs (the 'invasive switch') (7). This adaptation to lower oxygen tension is crucial for the survival of tumor cells. Moreover, hypoxic tumors are more aggressive and have shown to be resistant to radiation and chemotherapy. (8).

Cells undergo a variety of biological adaptations in response to hypoxia, but how do cells sense hypoxia and what signaling pathways mediate these cellular responses?

REGULATION OF THE HYPOXIC RESPONSE: THE HIF-1α PATHWAY

The hypoxic response of mammalian cells is controlled by members of the HIF-family including, HIF-1α, HIF-2α and HIF-3α. HIF-1α was the first protein that was identified as a Hypoxia Inducible Factor, by Semenza and Wang in 1992 (9). Later, they determined that in cells growing under hypoxic conditions, HIF-1α is expressed in the nucleus and interacts directly with a hypoxia response element (HRE) located in the 3-flanking region of the human erythropoietin gene (EPO) (9-11). In 1997 Ema and colleagues identified a homologue; HIF-2α, which was shown to be regulated in a similar manner as HIF-1α, and also was able to activate transcription via HRE sequences (12). HIF-2α has been implicated to play a role in lung maturation, angiogenesis of the retina, heamatopoiesis and the development of the central nervous system (13-15). In addition, it has been shown that HIF-2α is involved in the Von Hippel Lindau disease and specifically in the oncogenesis of clear renal cell carcinoma, as is described below. In 1998, Gu *et al*. identified the third hypoxia indubible factor, HIF-3α, which in contrast to HIF-1α and HIF-2α, gives rise to multiple splice variants (16). The mouse HIF-3α

variant known as the inhibitory PAS domain protein (IPAS), lacks the oxygen-dependent degradation and transactivation domain of full length HIF-1α. Furthermore, it was demonstrated that HIF-3α contains extra DNA binding elements and protein-protein interaction motifs not found in HIF-1α or HIF-2α. HIF-3α functions as a dominant negative HIF subunit, and was shown to attenuate HIF-1α transcription under hypoxic conditions (16-18).

HIF-1 is the best characterized member of the hypoxia inducible factors, and is known as the master regulator of the oxygen homeostasis. HIF-1 is a heterodimer consisting of a constitutively active subunit HIF-1β (also named ARNT- aryl hydrocarbon receptor nuclear translocator) and the oxygen sensitive subunit HIF-1α. Both HIF-1α and HIF-1β are members of the basic helix-loop-helix PER/ARNT/SIM (bHLH-PAS) family of transcription factors (19). The regulation of HIF-1α is complex and occurs through both oxygen dependent and independent mechanisms, as is described in detail below.

Oxygen dependent regulation of HIF-1α

HIF-1α consists of a bHLH and PAS domain, but also of a C-terminal and a N-terminal transactivation domain, the latter is also known as the oxygen-dependent degradation domain, or ODD (depicted in figure 1a). At normal oxygen levels and in the presence of iron and 2-oxoglutarate, HIF-1α is hydroxylated on two different prolyl sites, P402 and P564, the latter being located in the ODD domain of HIF-1α (figure 1a). In mammalian cells this is carried out by three different prolyl hydroxylases (PHDs), PHD1 through 3 (20). These proteins were originally designated EGLN-1-3 on the basis of sequence homology to EGL9, the HIF-1 prolyl hydroxylase in *Caenorhabditis elegans* (21). The hydroxylation of the proline sites in HIF-1α is required for recognition by the Von Hippel Lindau tumor suppressor protein (pVHL) (22-25). Acetylation of HIF-1α at lysine 532 by the ARD1 acetyltransferase enhances the interaction of pVHL with HIF-1α (27).

pVHL is part of a multiprotein complex containing Elongin B, Elongin C, Cul-2 and Rbx1 (Figure 1a) (28-33). Elongin C and Cul2 were noted to be similar to the SCF (Skp1, Cdc53, F-box protein) multiprotein complexes in yeast (Skp1 and Cdc53 respectively). SCF complexes target specific proteins for proteosomal degradation. In SCF complexes, the F-box protein serves as the substrate binding molecule and thereby confers specificity to the SCF complexes. Structure studies of pVHL bound to Elongin B and C confirmed that Elongin C resembled Skp1 and that pVHL resembles an F-box.

This observation led to the hypothesis that pVHL was a ubiquitin ligase. In keeping with this, two groups demonstrated that pVHL complexes contained ubiquitin ligase activity (34, 35). In 1999 Maxwell *et al.* showed that cells lacking pVHL fail to degrade HIF-1α in the presence of oxygen and that pVHL interacts directly with HIF-1α (26). Later it was established that the pVHL complex was indeed a ubiquitin ligase and that it directed the polyubiqutylation and hence the destruction of HIF-1α subunits in the presence of oxygen (36-38).

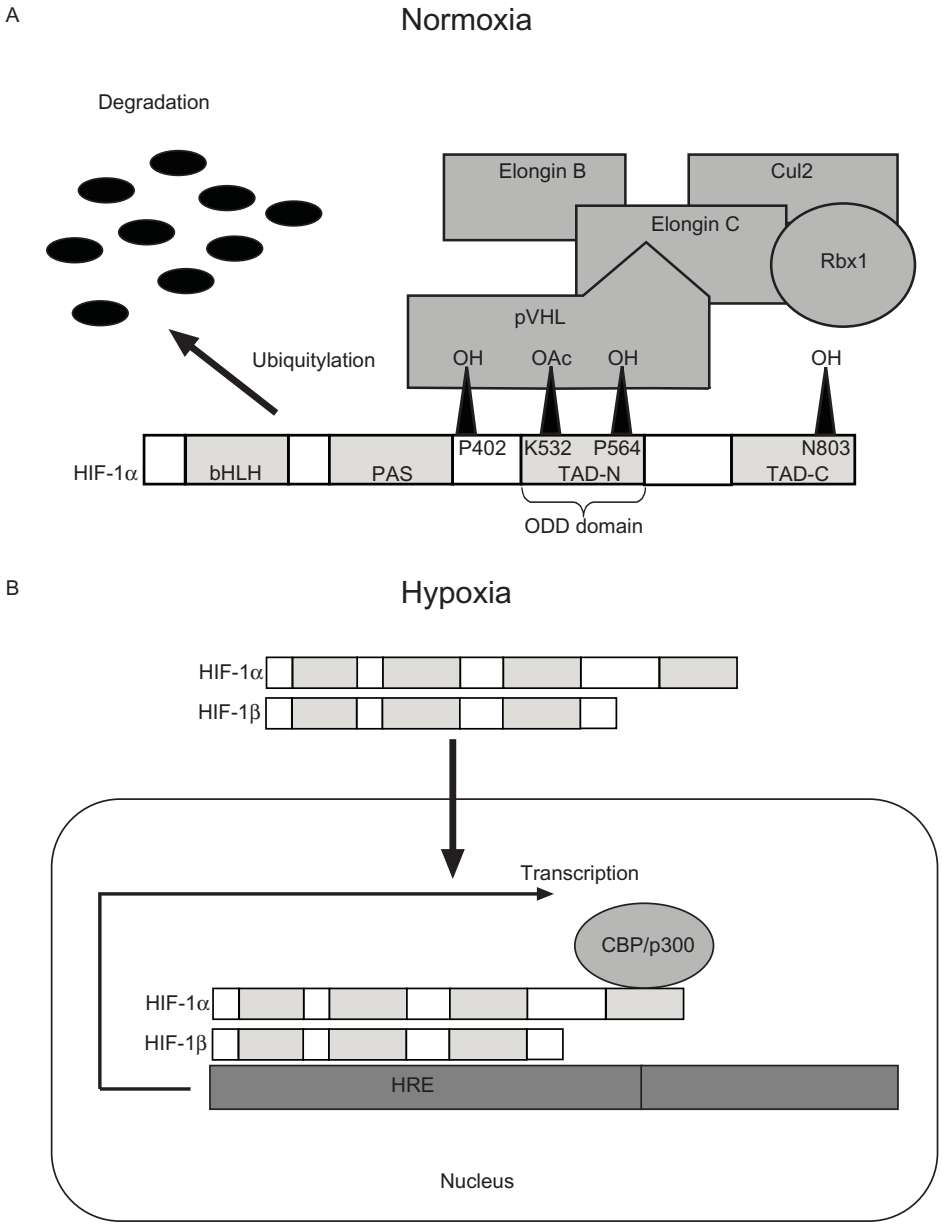

Figure 1. Model of the oxygen-dependent regulation of HIF-1α. A. Under normoxic conditions, O_2-dependent hydroxylation of the proline residues (P) 402 and 564 in HIF-1α is required for binding to the Von Hippel Lindau (pVHL) tumor suppressor gene, which is the recognition component of an E3 ubiquitin ligase and targets HIF-1α for degradation. Hydroxylation of an asparigine (N) residue at 803 by the enzyme Factor Inhibiting HIF (FIH) prevents the binding of the co-activators CBP/p300. B. Under hypoxic conditions, the hydroxylation does not take place and pVHL cannot bind, resulting in the stabilization of HIF-1α which then forms a heterodimer with HIF-1β (constitutively expressed). The complex translocates to the nucleus and binds to hypoxia response elements (HREs) in the DNA. Binding of HIF-1α to the CBP/p300 results in the transcriptional activation of the HIF-1α target genes. Adapted from Semenza, 2003 (39).

The transcriptional activity of HIF-1α is regulated by oxygen-dependent hydroxylation as well (40, 41). This particular hydroxylation is mediated by an enzyme known as Factor Inhibiting HIF (FIH), that recognizes an asparigine located (N803) at the C-terminus of HIF-1α and prevents interaction with the p300/CBP co-activator (42-44). FIH does not influence HIF-1α stability but allows for modulation of HIF-1α transactivation (42).

Taken together, under normal oxygen conditions, HIF-1α is constantly being degraded as a result of the hydroxylation. In the absence of oxygen however, the hydroxylation from either the PHDs or FIH cannot take place, causing HIF-1α to be stabilized which allows it to bind to HIF-1β and the heterodimer translocates to the nucleus (10). In the nucleus, HIF-1α/HIF-1β binds to HRE sequences in the DNA, and interaction of the C-terminal domain of HIF-1α (now released from inhibition by FIH) with the co-activator CBP/p300 activates the transcription of the hypoxia inducible genes, see figure 1b (45, 46).

The relative contribution of each of the prolyl hydroxylases to the regulation of HIF-1α is incompletely understood. By using small interfering RNA sequences (siRNA) directed against the 3 different prolyl hydroxylases, it was shown that PHD2 is the key limiting enzyme targeting HIF-1α for degradation (47). More recently it was demonstrated that the expression levels of the different PHDs is cell type specific and that PHD3 contributes more to the regulation of HIF-2α versus HIF-1α (48). Furthermore, it was shown that the prolyl hydroxylases can be up regulated by HIF-1α under hypoxia, thus providing an auto-regulatory mechanism for oxygen-driven HIF-1α regulation (49, 50).

Why are both the PHDs and FIH required for the regulation of HIF-1α? Dayan and colleagues suggested that this type of dual regulation allows cells to fine-tune HIF-1α target gene transcription, depending on the exposure to varying degrees of hypoxia. By downregulating PHD2 and FIH by siRNA, they were able to identify two different sets of HIF-1α target genes in human cancer cells. One group of genes such as the Glucose transporter (Glut-1), Nip3-like protein X (Nix) and p21 were induced under moderate hypoxic conditions at which PHD2 is active, but their expression strongly increased at drastic hypoxic conditions at which FIH is also inactivated. Another group of genes, including Phosphoglycerate kinase 1 (PGK-1), Bcl2/adenovirus EIB Interacting Protein 3 (BNIP3) and Enolase (ENO) were induced at moderate hypoxia, but their expression levels did not change upon severe hypoxic conditions and thus was independent of FIH activity (51).

Oxygen independent regulation of HIF-1α expression: the PI(3)K pathway

Signaling via growth factor receptors induces HIF-1α expression by increasing its synthesis rate through the activation of the phophatidylinositol 3-kinase (PI(3)K) and the mitogen-activated protein kinase (MAPK) pathway (39). Upon binding of a ligand to a tyrosine kinase receptor, Ras (which activates the MAPK pathway) and PI(3)K are activated (52). It has been shown that growth factors such as the insulin growth factor (IGF) (53), the platelet derived

growth factor (PDGF), epidermal growth factor (EGF) (54), fibroblast growth factor (FGF) and transforming growth factor-α and β (TGF-α and TGF-β) can increase the expression of HIF-1α by activation of the PI(3) pathway. Upon its activation, PI(3)K activates Akt by phosphorylation. This reaction can be inhibited by the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) which acts as a phosphatase. By means of different phosphorylation steps, eventually the inhibition of a protein called the mammalian target of Rapamycin (mTOR) is alleviated, leading to its activation and ultimately to the increase of translation of different genes including HIF-1α. Activation of the MAPK can also increase translation because it can activate the downstream targets of mTOR. The effect of the activation of both the PI(3)K and MAPK pathway is an increase in the rate of HIF-1α mRNA synthesis. The increase in HIF-1α titrates out the inhibitory hydroxylases and ultimately causes an increased expression of HIF-1α target genes (reviewed by Shaw and Cantley, 2006 (52).

Many human tumors harbor activating mutations in the regulators of the PI(3)K pathway, such as K-Ras, H-Ras, N-Ras and tyrosine kinase receptors such as PDGFR and HER2 (55). In addition, inactivating mutations in the negative regulators of this pathway such as PTEN and neurofibromin (NF1) which deactivates Ras, are also frequently detected in human tumors (52). Loss of PTEN is correlated with an increased expression of HIF-1α and tumor vascularization in gliomas (56). Conversely, it has been shown that overexpression of recombinant PTEN in glioma cells leads to a marked reduction in HIF-1α expression (57).

HIF-1α AND ITS PATHWAYS IN CANCER

HIF-1α and tumor progression

Immunohistochemical analysis has revealed that HIF-1α and its target genes are overexpressed in many human cancers (58). Table 1 summarizes the findings from studies examining the prognostic significance of HIF-1α expression in different tumors. The general trend is that presence of HIF-1α in the tumor correlates with a diminished prognosis, and this is improved if HIF-1α is not expressed. In rare cases a favorable prognosis correlates with strong HIF-1α staining. These studies often used different scoring methods and HIF-1α detection systems. In addition, the number of patients and follow-up times was variable. Besides these study-oriented factors, differences in the expression of the specific HIF-1α target genes (e.g. pro-apoptotic genes or pro-angiogenic genes) that are up regulated in these tumors, may explain contrasting results between studies.

For cervical cancer, Birner *et al.* (2000) (59) reported a diminished disease free survival (DSF) which is defined as lack of a relapse or occurrence of a distant metastasis with strong HIF-1α expression, whereas Haugland *et al.* (2002) (60) reported no such correlation in the same cancer type. Possibly, differences in scoring methods may explain these contrasting

Table 1: HIF-1α expression and prognosis in different tumor sites

Cancer type	Disease Free Survival	Overall Survival	Ref
Cervical	Diminished	Diminished	(59)
Cervical	N/A	Diminished	(66)
Cervical	Trend to diminished[a]	Diminished	(67)
Cervical	No correlation	N/A	(60)
Cervical - small[b] Cervical - large	Diminished Improved	N/A N/A	(61)
Transitional Cell Carc. Of the Upper Urinary Tract	Diminished	Diminished	(68)
Head and Neck	Improved	Improved	(62)
Squamous Cell Head and Neck	Diminished	No correlation	(63)
Nasopharyngeal	Diminished	Trend to diminished[c]	(69)
Oropharyngeal[d]	Diminished	Diminished	(70)
Esophageal Squamous Cell	N/A	Diminished	(71)
Esophageal Squamous Cell	Diminished	Trend to diminished[e]	(72)
Breast	Diminished	Diminished	(73, 74)
Invasive Breast	Diminished[f]/ Improved[g]	N/A	(75)
Ovarian	N/A	Diminished	(76)
Ovarian	No correlation	No correlation	(77)[h]
Gastric	N/A	No correlation	(78)
Gastric	N/A	Diminished	(79)
Bladder	Diminished	Diminished	(80)
Colorectal	N/A	Trend to diminished[ij]	(81, 82)
Non Small Cell Lung	Diminished (83)	Diminished (84)	(83, 84)
Oligodendroglioma	N/A	Diminished	(85)
Astrocytoma	N/A	Diminished	(86)
Squamous Cell Carc. of the oral floor	Improved[g]	Improved[g]	(87)
Malignant Pleural Mesothelioma	N/A	No correlation	(88)
Superficial Urothelial Bladder	Diminished	N/A	(89)

[a]p= 0.15; [b]Tumor size measured by NMR imaging or by taking the average diameter of 4 different planes, < 4 cm is small, ≥ 4 cm is large; [c]p= 0.06; [d]Biopsies from radiation treated patients; [e]p= 0.109; [f]Perinecrotic HIF-1α expression; [g]Diffuse HIF-1α expression; [h]Tissues analyzed by RT PCR; [i]p= 0.07 (81); [j]p> 0.05 (82); N/A Not Available.

observations. The study of Birner *et al.* (2000) used two independent observers to score staining intensity and the percentage of positive cells in a semiquantitative manner. In contrast, Haugland *et al.* (2002), who used image software to calculate the number of positive cells, with the threshold set to include the weakest staining. This may have resulted in the two groups observing different subsets of HIF-1α expressing tumors.

Tumor size can also determine the prognostic value of HIF-1α expression, as was shown by Hutchison *et al.* (2004) (61). They reported in cervical cancer that high levels of HIF-1α expression in smaller tumors (< 4 cm in diameter) was correlated with a diminished DFS. In larger tumors (≥ 4 cm in diameter) HIF-1α expression correlated with an improved DFS. One

possible explanation could be that HIF-1α induces different genes depending on the hypoxic demands of the tumor as it grows. In the smaller tumors HIF-1α may stimulate angiogenesis, conferring a growth advantage to the tumor cells and leading to a poor prognosis. On the other hand, as the tumor grows HIF-1α may activate the expression of pro-apoptotic genes and that could explain the correlation with an improved prognosis.

In head and neck cancer, contrasting correlations between DFS and HIF-1α expression have been reported. Beasley *et al.* (2002) (62) reported an improved DFS, whereas Koukourakis *et al.* (2002) (63) showed the DFS to be diminished. After removal of the tumor, the patients in the two different studies received different post-operative therapies. In the Beasley study, the majority did not receive additional treatment. In contrast, in the Koukourakis study patients were treated with a combination of carboplatin and radiotherapy. It is still unclear how radiation or chemotherapy affect HIF-1α gene expression and how the expression of HIF-1α influences the response of the tumor to these treatments. Upon irradiation, HIF-1α can induce apoptosis through stabilization of p53. but it can also cause the up regulation of VEGF and bFGF which promote angiogenesis, the latter would reduce the response to treatment (64). In this regard, it was recently shown that downregulation of HIF-1α after irratidation of a subcutaneously growing human xenograft, improved the response (65). It is possible that in the Koukourakis study the combination of both radiation and carboplatin may have favored the expression of pro-angiogenic genes. In the Beasley study, lack of additional radiation may have increased the expression of pro-apoptotic genes. Indeed, they observed a positive correlation between HIF-1α expression and necrosis (62).

The staining pattern of HIF-1α in the tumor can also help to predict the prognosis. Two studies suggest that non-hypoxia induced, diffuse HIF-1α staining correlates with an improved DFS and that this is diminished when HIF-1α staining follows a hypoxic and perinecrotic pattern. These data were reported by Vleugel *et al.* (2005) (75) and Fillies *et al.* (2005) (87), in studies of invasive breast cancer and of early stage squamous cell carcinoma of the oral floor, respectively. Both groups showed that non-hypoxia induced HIF-1α expression did not correlate with the up regulation of Glut-1 and Carbonic anhydrase 9 (CA IX), proteins which are major factors in the shift to glycolysis as a result of hypoxia. These studies imply that hypoxia independent HIF-1α expression may not lead to the switch to glycolysis, which results in a less efficient tumor growth and this may explain the improved prognosis with a high expression of HIF-1α.

Collectively, these observations strongly suggest that depending on the cancer type and type of stimulus which induces HIF-1α, different target genes can be activated during tumor growth. The correlation between the expression of HIF-1α and DFS or overall survival, depends on the balance between the transcription of the pro-angiogenic genes and pro-apoptotic genes. These data suggest that detection of HIF-1α in combination with different target genes may be a better indicator of prognosis. In this regard, it was recently demonstrated that a micro-array comprising a 'hypoxic signature' (gene expression profiles derived

from epithelial cells cultured under hypoxia) was a strong predictor of clinical outcomes in breast and ovarian cancers. Moreover, the prognostic information in the hypoxia signature was more predictive than currently used clinical parameters such as the lymph node status or tumor grade (90).

Activation of HIF-1α pathways in cancer

During tumor growth, hypoxia-inducible genes are implicated in the regulation of glycolysis, proliferation, apoptosis, angiogenesis, invasion and metastasis. Expression profiling studies have highlighted HIF-1α in regulating the expression of many of the genes involved in these processes. The criteria used to determine whether a gene is a target of HIF-1α include the induction of gene expression in response to hypoxia, the presence of a HIF-1α binding site in the gene, and an effect of the HIF-1α gain-of-function or loss-of-function mutation on the expression of the gene (1).

Glycolysis

Under hypoxic conditions, cells shift their methods of glucose metabolism from the oxidative phosphorylation in the mitochondria, to the glycolysis in the cytoplasm; the oxygen-independent metabolic pathway the Pasteur effect (5). The oxidative phosporylation provides 38 molecules of ATP per glucose molecule, whereas the glycolysis provides only 2 molecules of ATP. In order to compensate for this reduction in net ATP yield, cells must increase the rates of glycolysis and glucose uptake. Indeed, HIF-1α has been shown to regulate expression of many enzymes in the glycolytic pathway such as phosphofructo kinase (PFKL), aldolase A (ALDA), phosphoglycerate kinase 1 (PGK1), enolase (ENO), pyruvate kinase (PKM) and lactate dehydrogenase (LDH A) (91). The expression of the glucose transporters glut-1 and glut-3 which mediate cellular glucose uptake are also regulated by HIF-1α (92). Moreover, cells lacking HIF-1α fail to undergo the switch to glycolysis and as a result generate less ATP, and these cells show a reduced proliferation rate under hypoxic conditions (93).

Cell Proliferation and Apoptosis

Hypoxia induces expression of various growth factors that have been shown to promote cell proliferation. This proliferation is normally involved in initiating cell migration and tissue regeneration after acute or chronic hypoxia damage. HIF-1α induces the production of growth factors such as transforming growth factor–β and platelet derived growth factor (PDGF) (8). In addition to promoting proliferation, HIF-1α also has been shown to induce the expression of genes that can cause a cell cycle arrest or apoptosis. In this regard it was reported that in primary B lymphocytes exposed to hypoxia, the expression of p21 and p27 was increased. Upon conditional deletion of HIF-1α, the expression of p21 and p27 was decreased and these cells showed an increase entry in the S-phase compared to wild type controls (94) . Further-

more, HIF-1α has been shown to activate the pro-apoptotic genes Nip3-like protein X (NIX) and Bcl2/adenovirus EIB Interacting Protein 3 (BNIP3) in human tumors (95). In an animal model of ischemia, HIF-1α was demonstrated to induce BH3-only Bcl-2 family protein Noxa, which mediates hypoxic cell death (96).

Angiogenesis

At an early stage of tumor growth, when the tumor consists only of a small number of cells, it is able to obtain nutrients and oxygen from existing blood vessels in the micro-environment. When the mass grows beyond a diameter of approximately 1 mm however (97), and outgrows the available blood supply, the tumor cells become hypoxic which results in the induction of angiogenesis or 'the angiogenic switch'. HIF-1α has been shown to orchestrate the process of angiogenesis, by regulating the transcription of genes that promote endothelial cell proliferation and survival, such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) and by the transcription of genes that break down the extracellular matrix (ECM), such as matrix metalloproteinase 7 (MMP7), plasminogen activator inhibitor 1(PAI1), urokinase plasminogen activator receptor (uPAR) (7). Several lines of evidence support a critical role of HIF-1α in angiogenesis. First, in many different human tumors the expression of HIF-1α is correlated with an increase in vessel density and HIF-1α co-localizes with VEGF in necrotic regions of tumors such as glioblastoma (98). Second, mice lacking HIF-1α die in utero between embryonic day (E) 8.5 and 9.5 with defects in vascularization of the yolk sac and embryo (99).

On the transcriptional level, HIF-1α regulates VEGF directly by both increasing its transcription and its post-transcriptional stabilization (100). The increased levels of VEGF promote the proliferation of endothelial cells and cause an increased vessel dilation and permeability. This allows the detached endothelial cells to extravasate the blood vessels. The genes activated by HIF-1α that promote the degradation of the extracellular matrix, permit the endothelial cells to migrate through the stroma and form a new vessel network.

The ability of tumors to induce their own blood vessel supply allows them to survive, however the newly formed vasculature is very different from the normal vasculature. Tumor blood vessels display a broad range of structural and functional abnormalities including dilations, incomplete or absent endothelial linings and basement membranes, leakiness, irregular and tortuous architecture and blind ends. The abnormal structure of the tumor vasculature leads to areas of hypoxia within the tumors, resulting in another cycle of angiogenesis mediated by HIF-1α (101).

Migration and invasion

In many different solid tumors HIF-1α can predict or metastasis free survival (see table 1). This suggests that besides the 'angiogenic switch' tumor cells may also go through an 'invasive switch', which confers a survival advantage to the tumor cells by allowing the cells to escape a hostile site, and eventually home in a more oxygenated environment.

The first step of tumor metastasis involves degradation of the ECM. Many of the same genes that are up regulated by HIF-1α in ECM degradation during angiogenesis, are also involved in tumor cell migration and metastasis, and therefore include the same HIF-1α target genes namely, the members of the plasmin and metalloproteinase pathway as mentioned earlier, the urokinase plasminogen receptor, - activator and, - activator inhibitor (uPAR, uPA, and uPAI) and the metalloproteinase 7 (MMP7) (7). Following the ECM degradation, the tumor cells migrate through the ECM, penetrate lymphatic and vascular channels and extravasate from the circulation into a foreign tissue where they have to implant, proliferate and establish new vasculature.

Recently, evidence has emerged that cytokines and chemokines which are normally involved in the immune response to pathogens, are critical in directing tumor cell migration. Many of these factors have shown to be targets of HIF-1α. The receptor for the cytokine scatter factor/hepatocyte growth factor (SF/HGF) known as *met*, was shown to contain an HRE site in the 5′ region of the gene. Hypoxia increased the HGF-induced cell motility and this effect was abrogated by inhibition of *met* expression using RNA interference (102). The chemokine receptor CXCR4 was demonstrated to be a HIF-1α target gene (103) and was later implicated with organ specific metastasis of tumor cells. In this regard it was shown that breast cancer cells that overexpress CXCR4 preferentially metastasize to sites that produce large amounts of its ligand, the stromal derived factor –1α (SDF-1α) such as the lung or bone (104). More recently it was reported that lysyl oxidase (LOX) is a HIF-1α target and upon its downregulation using siRNA, tumor growth and metastasis was prevented in an orthotopic mouse breast cancer model (105).

Von Hippel Lindau disease

Germline and somatic mutations in the von Hippel-Lindau tumor suppressor gene give rise to highly vascular tumors in a variety of organs including the central nervous system, the kidney, the adrenal gland and pancreas Most aspects of the tumor pathology can be explained by up regulation of the HIF target genes (reviewed by Kaelin, 2002 (16)). It has been shown that in VHL disease, additional mutations are required to initiate tumor growth in VHL disease consistent with the Knudsen 'two-hit' hypothesis. First, neither forced activation of HIF target genes, nor conditional loss of pVHL, has led to tumor formation (107-109). Furthermore, loss of pVHL in primary mouse ES cells was surprisingly correlated with a reduction in tumor growth (110, 111). In addition, fibrosarcomas lacking pVHL also showed a decrease in tumor growth and an increase in p27 and p21 expression (112).

Gene expression profiling studies suggest that pVHL has HIF-1α -independent targets (113, 114). These HIF-1α independent targets of pVHL have shown to include atypical protein kinase C (115-117), Rpb1 (a subunit of RNA polymerase II) (118), the plant homeodomain protein Jade-1 (119). More recently, it has been established that pVHL stabilizes p53 and that

loss of pVHL attenuates p53 activation upon DNA damage, thereby promoting tumor growth under genotoxic conditions (120).

It has been suggested that pVHL plays a role in ECM degradation and fibronectin matrix assembly (121). The highly invasive phenotype of the cells derived from clear renal cell carcinoma (CRCC cells) was associated with an increased secretion of metalloproteinases 1 and -9 (MMP2 and MMP9), together with a decreased expression of a matrix metalloproteinase antagonist called TIMP-2 (tissue inhibitor of metalloproteinase 2) (122). In addition, it was shown that CRCC cells lacking pVHL secrete higher levels of the plasminogen activator inhibitor-1 (PAI-1) (123) which was shown to be essential for cancer cell invasion (124). Certain cell types lacking pVHL harbor defects in fibronectin matrix assembly (112, 125, 126) that can be restored upon re-introduction of wild type pVHL (121, 127). The fibronectin assembly defects in cells deficient in pVHL were demonstrated to be independent of HIF-1α (125).

Furthermore, it has been shown that pVHL interacts directly with the cytoskeleton, by binding to the microtubules and inhibiting their depolymerization (128).

pVHL has also been implicated in the regulation of cell cycle control. CRCC cells fail to exit the cell cycle upon serum withdrawal (129) and gene expression profiling identified pVHL as a negative regulator of Cyclin D1 (130, 131) and TGF-α (132). How dysregulation of these proteins contributes to tumor growth associated with VHL disease, is incompletely understood. The work carried out using the CRCC cell lines has revealed that re-introduction of wild type VHL is sufficient to prevent subcutaneous tumor growth and that the binding to HIF-2α is required for the tumor suppression (133-137). In addition, it was established that in kidneys from VHL patients, both HIF-2α and HIF-1α are overexpressed in early stage lesions and overt CRCC (138). How loss of pVHL causes hemangioblastoma, pheochromocytoma or pancreatic cysts is still unclear however, and more research is warranted.

MODULATING HIF TO ELUCIDATE ITS ROLE IN TUMOR PROGRESSION

Since the realization that hypoxia is an important physiological feature of solid tumor growth and that HIF-1α is a prognostic factor for different types of cancer, much work has been done trying to determine its role in tumorigenesis. Table 2 depicts the approaches that have been used to study the role of HIF-1α in tumor formation by modulating its expression and activation.

The first mouse models used to study the role of HIF-1α in tumor formation, were created by injecting mouse embryonic stem (ES) cells lacking HIF-1α subcutaneously into mice. ES cells are non-transformed cells that can grow as xenografts in immunocompromised mice. Two independently derived sets of HIF-1α null ES cell tumors showed a markedly reduced tumor angiogenesis (99, 139). In one study however, these tumors showed an increased tumor growth, which was correlated with a reduced apoptosis (139). In the other study, tumor growth

Table 2: Effects HIF-1α reduction or overexpression on *in vivo* tumor growth (overview 1998-2003)

Cell type	Method	Tumor growth	Angiogenesis	Ref
ES cells (mouse)	HIF-1α knockout	Reduced	Reduced	(99)
ES cells (mouse)	HIF-1α knockout	Increased	Reduced	(139)
T-Ag/H-Ras transformed ES cells (mouse)	Cre/LoxP knockout	Reduced	Reduced	(140)
T-Ag/H-Ras transformed mEFs	Cre/LoxP knockout	Reduced	Reduced	(140)
T-Ag/H-Ras transformed mEFs[a]	Cre/LoxP knockout	Reduced	ND	(143)
Colon (HCT 116)	Overexpression	Increased	Increased	(141)
Breast (MDA-231) and Colon (HCT116)	GAL4-TAD-C	Reduced	ND	(144)
Pancreas (PC-10)	Overexpression	Increased	ND	(142)
Pancreas (PCI-43)	Dominant Negative	Reduced	No Effect	(145)
Thymic Lymphoma (EL-4)	Antisense	Reduced	Reduced	(146)
5 Human Cancer Cell lines[b]	YC-1	Reduced	Reduced	(147)

[a]Treated with Carboplatin and Etoposide;[b]Hep3B hepatoma, NCI-H87 stomach, Caki-1 renal, SiHa cervical, SK-N-MC neuroblastoma ; ND - not determined; NE - no effect. Adapted from Semenza, 2003 (39).

was reported to be decreased (99). These data imply that differences in the genetic background of the ES cells or in the xenograft recipients in the two studies had a key role in determining the effect of HIF-1α deficiency on xenograft growth. To determine the effect of HIF-1α deficiency on tumor growth of differentiated cells, fibroblasts were isolated from HIF-1α embryos, immortalized by SV 40 Large T antigen and transformed by H-Ras. In these cells, loss of HIF-1α was associated with reduced tumor mass, although angiogenesis was not affected (140).

HIF-1α activity has also been manipulated in human cancer cell lines. Expression of VEGF, xenograft growth and angiogenesis were markedly increased in HCT116 colon cancer cells that were transfected with an expression vector that encoded HIF-1α (141). Subcutaneously grown pancreatic cells (PCI-10 cells) that overexpressed HIF-1α showed an increase in tumor growth, but the vessel density was not affected (142).

Two strategies have been used to inhibit HIF-1α activity in human cancer cells. The first approach was based on the demonstration that deletion of the DNA-binding and transactivation domain results in a dominant-negative form of HIF-1α that can bind to HIF-1β resulting in formation of an inactive heterodimer (148). Overexpression of this dominant negative form of HIF-1α in PI-43 pancreatic cancer cells, which constitutively express high levels of HIF-1α, led to an increase in the number of cells undergoing apoptosis under conditions of glucose and oxygen deprivation and a decreased ability to form tumors. Tumor vascularization was not affected, however (145).

The second approach was based on the demonstration that HIF-1α contains two transactivation domains– TAD-N and TAD-C (figure 1). The interaction between TAD-C and its coactivators CBP and p300, is regulated by FIH (figure 1). A fusion protein that consists of Gal4 fused to TAD-C inhibits the ability of HIF-1α to interact with these coactivators and therefore

blocks the transcription. Human breast (MDA435) and colon (HCT116) cancer cells infected with a retrovirus that encodes this fusion protein showed reduced growth when injected into nude mice (144). However, the fusion protein also disrupts the ability of many other transcription factors to interact with CBP/p300 and therefore limits the conclusions that can be drawn from this work.

In conclusion, most studies have shown that HIF-1α is a contributing factor for tumor growth. The role of HIF-1α expression in angiogenesis was less consistent however, and dependent on the cell type. Furthermore, the studies mentioned above, involve injection of cells into the subcutaneous space which does not recapitulate the interaction between tumor cells and surrounding tissue in which the tumors normally develop (149).

OUTLINE OF THIS THESIS

Glioblastomas, like other solid tumors have extensive areas of hypoxia and necrosis. In gliomagenesis, HIF-1α is believed to be most important in activating angiogenesis and invasion, leading to a more malignant tumor growth. Moreover, HIF-1α expression is detected at the invasive front of glioblastomas and correlates with grade and vessel density (150). Generating a mouse model of glioblastoma, in which HIF-1α was conditionally deleted in the transformed astrocytes, provided the basis for the experiments to determine the role of HIF-1α in brain tumor formation (chapter 2). We found that HIF-1α has different roles in the tumor growth, dependent on the micro-environment in which cells are grown. When grown as subcutaneous tumors, loss of HIF-1α in transformed astrocytes reduced tumor formation and vascularization. In contrast, when grown in the brain the same cells did not reduce tumor growth and instead, increased the invasiveness of the cells which was accompanied with a reduced survival of the mice. These date emphasize that the physiological effects of an activated hypoxic pathway during tumor growth, such as angiogenesis and apoptosis, depend on the micro-environment. Further evidence for this observation came from work showing that transformed astrocytes in which VEGF is conditionally deleted in a pVHL null background, tumor growth was reduced when these cells were grown as subcutaneous tumors. When the same cells were grown in the brain, it did not affect tumor growth or survival time of the mice (chapter 3). Finally, in chapter 4 it is described how an increased expression of HIF-1α due to loss of pVHL sensitizes tumor cells to cell death mediated by inhibition of the glycolytic pathway by 2-deoxyglucose.

REFERENCES

1. Hirota, K. and Semenza, G. L. Regulation of hypoxia-inducible factor 1 by prolyl and asparaginyl hydroxylases. Biochem Biophys Res Commun, *338:* 610-616, 2005.
2. Brown, J. M. and Wilson, W. R. Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer, *4:* 437-447, 2004.
3. Gu, Y. Z., Moran, S. M., Hogenesch, J. B., Wartman, L., and Bradfield, C. A. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expr, *7:* 205-213, 1998.
4. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57-70, 2000.
5. Racker, E. History of the Pasteur effect and its pathobiology. Mol Cell Biochem, *5:* 17-23, 1974.
6. Gatenby, R. A. and Gillies, R. J. Why do cancers have high aerobic glycolysis? Nat Rev Cancer, *4:* 891-899, 2004.
7. Le, Q. T., Denko, N. C., and Giaccia, A. J. Hypoxic gene expression and metastasis. Cancer Metastasis Rev, *23:* 293-310, 2004.
8. Harris, A. L. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer, *2:* 38-47, 2002.
9. Semenza, G. L. and Wang, G. L. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol, *12:* 5447-5454, 1992.
10. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A, *92:* 5510-5514, 1995.
11. Wang, G. L. and Semenza, G. L. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem, *270:* 1230-1237, 1995.
12. Wiesener, M. S., Jurgensen, J. S., Rosenberger, C., Scholze, C. K., Horstrup, J. H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U. A., Pugh, C. W., Ratcliffe, P. J., Bachmann, S., Maxwell, P. H., and Eckardt, K. U. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. Faseb J, *17:* 271-273, 2003.
13. Scortegagna, M., Morris, M. A., Oktay, Y., Bennett, M., and Garcia, J. A. The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. Blood, *102:* 1634-1640, 2003.
14. 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., Bennett, M. J., and Garcia, J. A. Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nat Genet, *35:* 331-340, 2003.
15. Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., Nemery, B., Dewerchin, M., Van Veldhoven, P., Plate, K., Moons, L., Collen, D., and Carmeliet, P. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med, *8:* 702-710, 2002.
16. Makino, Y., Kanopka, A., Wilson, W. J., Tanaka, H., and Poellinger, L. Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. J Biol Chem, *277:* 32405-32408, 2002.
17. Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A., and Poellinger, L. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature, *414:* 550-554, 2001.
18. Maynard, M. A., Evans, A. J., Hosomi, T., Hara, S., Jewett, M. A., and Ohh, M. Human HIF-3alpha4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. Faseb J, *19:* 1396-1406, 2005.
19. Semenza, G. L. Hypoxia-inducible factor 1 and the molecular physiology of oxygen homeostasis. J Lab Clin Med, *131:* 207-214, 1998.

20. Bruick, R. K. and McKnight, S. L. A conserved family of prolyl-4-hydroxylases that modify HIF. Science, *294:* 1337-1340, 2001.
21. Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell, *107:* 43-54, 2001.
22. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science, *292:* 464-468, 2001.
23. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science, *292:* 468-472, 2001.
24. Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. Embo J, *20:* 5197-5206, 2001.
25. Yu, F., White, S. B., Zhao, Q., and Lee, F. S. HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc Natl Acad Sci U S A, *98:* 9630-9635, 2001.
26. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature, *399:* 271-275, 1999.
27. Jeong, J. W., Bae, M. K., Ahn, M. Y., Kim, S. H., Sohn, T. K., Bae, M. H., Yoo, M. A., Song, E. J., Lee, K. J., and Kim, K. W. Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell, *111:* 709-720, 2002.
28. Kishida, T., Stackhouse, T. M., Chen, F., Lerman, M. I., and Zbar, B. Cellular proteins that bind the von Hippel-Lindau disease gene product: mapping of binding domains and the effect of missense mutations. Cancer Res, *55:* 4544-4548, 1995.
29. Kibel, A., Iliopoulos, O., DeCaprio, J. A., and Kaelin, W. G., Jr. Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. Science, *269:* 1444-1446, 1995.
30. Duan, D. R., Pause, A., Burgess, W. H., Aso, T., Chen, D. Y., Garrett, K. P., Conaway, R. C., Conaway, J. W., Linehan, W. M., and Klausner, R. D. Inhibition of transcription elongation by the VHL tumor suppressor protein. Science, *269:* 1402-1406, 1995.
31. Pause, A., Lee, S., Worrell, R. A., Chen, D. Y., Burgess, W. H., Linehan, W. M., and Klausner, R. D. The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc Natl Acad Sci U S A, *94:* 2156-2161, 1997.
32. Lonergan, K. M., Iliopoulos, O., Ohh, M., Kamura, T., Conaway, R. C., Conaway, J. W., and Kaelin, W. G., Jr. Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. Mol Cell Biol, *18:* 732-741, 1998.
33. Kamura, T., Conrad, M. N., Yan, Q., Conaway, R. C., and Conaway, J. W. The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. Genes Dev, *13:* 2928-2933, 1999.
34. Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R. C., Conaway, J. W., Klausner, R. D., and Pause, A. Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. Proc Natl Acad Sci U S A, *96:* 12436-12441, 1999.
35. Lisztwan, J., Imbert, G., Wirbelauer, C., Gstaiger, M., and Krek, W. The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. Genes Dev, *13:* 1822-1833, 1999.

36. Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R. C., and Conaway, J. W. Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. Proc Natl Acad Sci U S A, *97:* 10430-10435, 2000.
37. Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol, *2:* 423-427, 2000.
38. Tanimoto, K., Makino, Y., Pereira, T., and Poellinger, L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. Embo J, *19:* 4298-4309, 2000.
39. Semenza, G. L. Targeting HIF-1 for cancer therapy. Nat Rev Cancer, *3:* 721-732, 2003.
40. Jiang, B. H., Zheng, J. Z., Leung, S. W., Roe, R., and Semenza, G. L. Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. J Biol Chem, *272:* 19253-19260, 1997.
41. Pugh, C. W., O'Rourke, J. F., Nagao, M., Gleadle, J. M., and Ratcliffe, P. J. Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. J Biol Chem, *272:* 11205-11214, 1997.
42. Mahon, P. C., Hirota, K., and Semenza, G. L. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev, *15:* 2675-2686, 2001.
43. Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y. M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M., Ratcliffe, P. J., Pugh, C. W., and Schofield, C. J. Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem, *277:* 26351-26355, 2002.
44. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science, *295:* 858-861, 2002.
45. Dames, S. A., Martinez-Yamout, M., De Guzman, R. N., Dyson, H. J., and Wright, P. E. Structural basis for Hif-1 alpha /CBP recognition in the cellular hypoxic response. Proc Natl Acad Sci U S A, *99:* 5271-5276, 2002.
46. Freedman, S. J., Sun, Z. Y., Poy, F., Kung, A. L., Livingston, D. M., Wagner, G., and Eck, M. J. Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha. Proc Natl Acad Sci U S A, *99:* 5367-5372, 2002.
47. Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. Embo J, *22:* 4082-4090, 2003.
48. Appelhoff, R. J., Tian, Y. M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem, *279:* 38458-38465, 2004.
49. D'Angelo, G., Duplan, E., Boyer, N., Vigne, P., and Frelin, C. Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. J Biol Chem, *278:* 38183-38187, 2003.
50. Berra, E., Richard, D. E., Gothie, E., and Pouyssegur, J. HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1alpha degradation. FEBS Lett, *491:* 85-90, 2001.
51. Dayan, F., Roux, D., Brahimi-Horn, M. C., Pouyssegur, J., and Mazure, N. M. The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. Cancer Res, *66:* 3688-3698, 2006.
52. Shaw, R. J. and Cantley, L. C. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature, *441:* 424-430, 2006.
53. Fukuda, R., Hirota, K., Fan, F., Jung, Y. D., Ellis, L. M., and Semenza, G. L. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression,

which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem, *277:* 38205-38211, 2002.

54. Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M. M., Simons, J. W., and Semenza, G. L. Modulation of hypoxia-inducible factor 1alpha 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, 2000.
55. Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C., and Semenza, G. L. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol, *21:* 3995-4004, 2001.
56. Sansal, I. and Sellers, W. R. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol, *22:* 2954-2963, 2004.
57. Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A. R., Ryan, H. E., Johnson, R. S., Jefferson, A. B., Stokoe, D., and Giaccia, A. J. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev, *14:* 391-396, 2000.
58. Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L., and Simons, J. W. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res, *59:* 5830-5835, 1999.
59. Birner, P., Schindl, M., Obermair, A., Plank, C., Breitenecker, G., and Oberhuber, G. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res, *60:* 4693-4696, 2000.
60. Haugland, H. K., Vukovic, V., Pintilie, M., Fyles, A. W., Milosevic, M., Hill, R. P., and Hedley, D. W. Expression of hypoxia-inducible factor-1alpha in cervical carcinomas: correlation with tumor oxygenation. Int J Radiat Oncol Biol Phys, *53:* 854-861, 2002.
61. Hutchison, G. J., Valentine, H. R., Loncaster, J. A., Davidson, S. E., Hunter, R. D., Roberts, S. A., Harris, A. L., Stratford, I. J., Price, P. M., and West, C. M. Hypoxia-inducible factor 1alpha expression as an intrinsic marker of hypoxia: correlation with tumor oxygen, pimonidazole measurements, and outcome in locally advanced carcinoma of the cervix. Clin Cancer Res, *10:* 8405-8412, 2004.
62. Beasley, N. J., Leek, R., Alam, M., Turley, H., Cox, G. J., Gatter, K., Millard, P., Fuggle, S., and Harris, A. L. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. Cancer Res, *62:* 2493-2497, 2002.
63. Koukourakis, M. I., Giatromanolaki, A., Sivridis, E., Simopoulos, C., Turley, H., Talks, K., Gatter, K. C., and Harris, A. L. Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. Int J Radiat Oncol Biol Phys, *53:* 1192-1202, 2002.
64. Moeller, B. J. and Dewhirst, M. W. HIF-1 and tumour radiosensitivity. Br J Cancer, *95:* 1-5, 2006.
65. Moeller, B. J., Dreher, M. R., Rabbani, Z. N., Schroeder, T., Cao, Y., Li, C. Y., and Dewhirst, M. W. Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. Cancer Cell, *8:* 99-110, 2005.
66. Bachtiary, B., Schindl, M., Potter, R., Dreier, B., Knocke, T. H., Hainfellner, J. A., Horvat, R., and Birner, P. Overexpression of hypoxia-inducible factor 1alpha indicates diminished response to radiotherapy and unfavorable prognosis in patients receiving radical radiotherapy for cervical cancer. Clin Cancer Res, *9:* 2234-2240, 2003.
67. Burri, P., Djonov, V., Aebersold, D. M., Lindel, K., Studer, U., Altermatt, H. J., Mazzucchelli, L., Greiner, R. H., and Gruber, G. Significant correlation of hypoxia-inducible factor-1alpha with treatment outcome in cervical cancer treated with radical radiotherapy. Int J Radiat Oncol Biol Phys, *56:* 494-501, 2003.
68. Nakanishi, K., Hiroi, S., Tominaga, S., Aida, S., Kasamatsu, H., Matsuyama, S., Matsuyama, T., and Kawai, T. Expression of hypoxia-inducible factor-1alpha protein predicts survival in patients with transitional cell carcinoma of the upper urinary tract. Clin Cancer Res, *11:* 2583-2590, 2005.

69. Hui, E. P., Chan, A. T., Pezzella, F., Turley, H., To, K. F., Poon, T. C., Zee, B., Mo, F., Teo, P. M., Huang, D. P., Gatter, K. C., Johnson, P. J., and Harris, A. L. Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. Clin Cancer Res, *8:* 2595-2604, 2002.
70. Aebersold, D. M., Burri, P., Beer, K. T., Laissue, J., Djonov, V., Greiner, R. H., and Semenza, G. L. Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res, *61:* 2911-2916, 2001.
71. Kurokawa, T., Miyamoto, M., Kato, K., Cho, Y., Kawarada, Y., Hida, Y., Shinohara, T., Itoh, T., Okushiba, S., Kondo, S., and Katoh, H. Overexpression of hypoxia-inducible-factor 1alpha(HIF-1alpha) in oesophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Br J Cancer, *89:* 1042-1047, 2003.
72. Matsuyama, T., Nakanishi, K., Hayashi, T., Yoshizumi, Y., Aiko, S., Sugiura, Y., Tanimoto, T., Uenoyama, M., Ozeki, Y., and Maehara, T. Expression of hypoxia-inducible factor-1alpha in esophageal squamous cell carcinoma. Cancer Sci, *96:* 176-182, 2005.
73. Dales, J. P., Garcia, S., Meunier-Carpentier, S., Andrac-Meyer, L., Haddad, O., Lavaut, M. N., Allasia, C., Bonnier, P., and Charpin, C. Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients. Int J Cancer, *116:* 734-739, 2005.
74. Schindl, M., Schoppmann, S. F., Samonigg, H., Hausmaninger, H., Kwasny, W., Gnant, M., Jakesz, R., Kubista, E., Birner, P., and Oberhuber, G. Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. Clin Cancer Res, *8:* 1831-1837, 2002.
75. Vleugel, M. M., Greijer, A. E., Shvarts, A., van der Groep, P., van Berkel, M., Aarbodem, Y., van Tinteren, H., Harris, A. L., van Diest, P. J., and van der Wall, E. Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. J Clin Pathol, *58:* 172-177, 2005.
76. Birner, P., Schindl, M., Obermair, A., Breitenecker, G., and Oberhuber, G. Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. Clin Cancer Res, *7:* 1661-1668, 2001.
77. Nakayama, K., Kanzaki, A., Hata, K., Katabuchi, H., Okamura, H., Miyazaki, K., Fukumoto, M., and Takebayashi, Y. Hypoxia-inducible factor 1 alpha (HIF-1 alpha) gene expression in human ovarian carcinoma. Cancer Lett, *176:* 215-223, 2002.
78. Urano, N., Fujiwara, Y., Doki, Y., Tsujie, M., Yamamoto, H., Miyata, H., Takiguchi, S., Yasuda, T., Yano, M., and Monden, M. Overexpression of hypoxia-inducible factor-1 alpha in gastric adenocarcinoma. Gastric Cancer, *9:* 44-49, 2006.
79. Mizokami, K., Kakeji, Y., Oda, S., Irie, K., Yonemura, T., Konishi, F., and Maehara, Y. Clinicopathologic significance of hypoxia-inducible factor 1alpha overexpression in gastric carcinomas. J Surg Oncol, *94:* 149-154, 2006.
80. Theodoropoulos, V. E., Lazaris, A., Sofras, F., Gerzelis, I., Tsoukala, V., Ghikonti, I., Manikas, K., and Kastriotis, I. Hypoxia-inducible factor 1 alpha expression correlates with angiogenesis and unfavorable prognosis in bladder cancer. Eur Urol, *46:* 200-208, 2004.
81. Kuwai, T., Kitadai, Y., Tanaka, S., Onogawa, S., Matsutani, N., Kaio, E., Ito, M., and Chayama, K. Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma. Int J Cancer, *105:* 176-181, 2003.
82. Yoshimura, H., Dhar, D. K., Kohno, H., Kubota, H., Fujii, T., Ueda, S., Kinugasa, S., Tachibana, M., and Nagasue, N. Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. Clin Cancer Res, *10:* 8554-8560, 2004.

83. Kim, S. J., Rabbani, Z. N., Dewhirst, M. W., Vujaskovic, Z., Vollmer, R. T., Schreiber, E. G., Oosterwijk, E., and Kelley, M. J. Expression of HIF-1alpha, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. Lung Cancer, *49:* 325-335, 2005.
84. Swinson, D. E., Jones, J. L., Cox, G., Richardson, D., Harris, A. L., and O'Byrne, K. J. Hypoxia-inducible factor-1 alpha in non small cell lung cancer: relation to growth factor, protease and apoptosis pathways. Int J Cancer, *111:* 43-50, 2004.
85. Birner, P., Gatterbauer, B., Oberhuber, G., Schindl, M., Rossler, K., Prodinger, A., Budka, H., and Hainfellner, J. A. Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis. Cancer, *92:* 165-171, 2001.
86. Korkolopoulou, P., Patsouris, E., Konstantinidou, A. E., Pavlopoulos, P. M., Kavantzas, N., Boviatsis, E., Thymara, I., Perdiki, M., Thomas-Tsagli, E., Angelidakis, D., Rologis, D., and Sakkas, D. Hypoxia-inducible factor 1alpha/vascular endothelial growth factor axis in astrocytomas. Associations with microvessel morphometry, proliferation and prognosis. Neuropathol Appl Neurobiol, *30:* 267-278, 2004.
87. Fillies, T., Werkmeister, R., van Diest, P. J., Brandt, B., Joos, U., and Buerger, H. HIF1-alpha overexpression indicates a good prognosis in early stage squamous cell carcinomas of the oral floor. BMC Cancer, *5:* 84, 2005.
88. Klabatsa, A., Sheaff, M. T., Steele, J. P., Evans, M. T., Rudd, R. M., and Fennell, D. A. Expression and prognostic significance of hypoxia-inducible factor 1alpha (HIF-1alpha) in malignant pleural mesothelioma (MPM). Lung Cancer, *51:* 53-59, 2006.
89. Theodoropoulos, V. E., Lazaris, A. C., Kastriotis, I., Spiliadi, C., Theodoropoulos, G. E., Tsoukala, V., Patsouris, E., and Sofras, F. Evaluation of hypoxia-inducible factor 1alpha overexpression as a predictor of tumour recurrence and progression in superficial urothelial bladder carcinoma. BJU Int, *95:* 425-431, 2005.
90. Chi, J. T., Wang, Z., Nuyten, D. S., Rodriguez, E. H., Schaner, M. E., Salim, A., Wang, Y., Kristensen, G. B., Helland, A., Borresen-Dale, A. L., Giaccia, A., Longaker, M. T., Hastie, T., Yang, G. P., Vijver, M. J., and Brown, P. O. Gene Expression Programs in Response to Hypoxia: Cell Type Specificity and Prognostic Significance in Human Cancers. PLoS Med, *3:* e47, 2006.
91. Semenza, G. L., Roth, P. H., Fang, H. M., and Wang, G. L. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem, *269:* 23757-23763, 1994.
92. Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F., and Maity, A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem, *276:* 9519-9525, 2001.
93. Seagroves, T. N., Ryan, H. E., Lu, H., Wouters, B. G., Knapp, M., Thibault, P., Laderoute, K., and Johnson, R. S. Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol Cell Biol, *21:* 3436-3444, 2001.
94. Goda, N., Ryan, H. E., Khadivi, B., McNulty, W., Rickert, R. C., and Johnson, R. S. Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. Mol Cell Biol, *23:* 359-369, 2003.
95. Sowter, H. M., Ratcliffe, P. J., Watson, P., Greenberg, A. H., and Harris, A. L. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Res, *61:* 6669-6673, 2001.
96. Kim, J. Y., Ahn, H. J., Ryu, J. H., Suk, K., and Park, J. H. BH3-only protein Noxa is a mediator of hypoxic cell death induced by hypoxia-inducible factor 1alpha. J Exp Med, *199:* 113-124, 2004.
97. Vaupel, P. The role of hypoxia-induced factors in tumor progression. Oncologist, *9 Suppl 5:* 10-17, 2004.
98. Zagzag, D., Zhong, H., Scalzitti, J. M., Laughner, E., Simons, J. W., and Semenza, G. L. Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. Cancer, *88:* 2606-2618, 2000.
99. Ryan, H. E., Lo, J., and Johnson, R. S. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. Embo J, *17:* 3005-3015, 1998.

100. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature, *359:* 843-845, 1992.
101. Bergers, G. and Benjamin, L. E. Tumorigenesis and the angiogenic switch. Nat Rev Cancer, *3:* 401-410, 2003.
102. Pennacchietti, S., Michieli, P., Galluzzo, M., Mazzone, M., Giordano, S., and Comoglio, P. M. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell, *3:* 347-361, 2003.
103. Staller, P., Sulitkova, J., Lisztwan, J., Moch, H., Oakeley, E. J., and Krek, W. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature, *425:* 307-311, 2003.
104. Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordon-Cardo, C., Guise, T. A., and Massague, J. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell, *3:* 537-549, 2003.
105. Erler, J. T., Bennewith, K. L., Nicolau, M., Dornhofer, N., Kong, C., Le, Q. T., Chi, J. T., Jeffrey, S. S., and Giaccia, A. J. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature, *440:* 1222-1226, 2006.
106. Kaelin, W. G., Jr. Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer, *2:* 673-682, 2002.
107. Haase, V. H., Glickman, J. N., Socolovsky, M., and Jaenisch, R. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci U S A, *98:* 1583-1588, 2001.
108. Rankin, E. B., Higgins, D. F., Walisser, J. A., Johnson, R. S., Bradfield, C. A., and Haase, V. H. Inactivation of the arylhydrocarbon receptor nuclear translocator (Arnt) suppresses von Hippel-Lindau disease-associated vascular tumors in mice. Mol Cell Biol, *25:* 3163-3172, 2005.
109. Rankin, E. B., Tomaszewski, J. E., and Haase, V. H. Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. Cancer Res, *66:* 2576-2583, 2006.
110. Mack, F. A., Rathmell, W. K., Arsham, A. M., Gnarra, J., Keith, B., and Simon, M. C. Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. Cancer Cell, *3:* 75-88, 2003.
111. Rathmell, W. K., Hickey, M. M., Bezman, N. A., Chmielecki, C. A., Carraway, N. C., and Simon, M. C. In vitro and in vivo models analyzing von Hippel-Lindau disease-specific mutations. Cancer Res, *64:* 8595-8603, 2004.
112. Mack, F. A., Patel, J. H., Biju, M. P., Haase, V. H., and Simon, M. C. Decreased growth of Vhl-/- fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. Mol Cell Biol, *25:* 4565-4578, 2005.
113. Jiang, Y., Zhang, W., Kondo, K., Klco, J. M., St Martin, T. B., Dufault, M. R., Madden, S. L., Kaelin, W. G., Jr., and Nacht, M. Gene expression profiling in a renal cell carcinoma cell line: dissecting VHL and hypoxia-dependent pathways. Mol Cancer Res, *1:* 453-462, 2003.
114. Wykoff, C. C., Pugh, C. W., Maxwell, P. H., Harris, A. L., and Ratcliffe, P. J. Identification of novel hypoxia dependent and independent target genes of the von Hippel-Lindau (VHL) tumour suppressor by mRNA differential expression profiling. Oncogene, *19:* 6297-6305, 2000.
115. Okuda, H., Hirai, S., Takaki, Y., Kamada, M., Baba, M., Sakai, N., Kishida, T., Kaneko, S., Yao, M., Ohno, S., and Shuin, T. Direct interaction of the beta-domain of VHL tumor suppressor protein with the regulatory domain of atypical PKC isotypes. Biochem Biophys Res Commun, *263:* 491-497, 1999.
116. Pai, J. T., Guryev, O., Brown, M. S., and Goldstein, J. L. Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. J Biol Chem, *273:* 26138-26148, 1998.
117. Okuda, H., Saitoh, K., Hirai, S., Iwai, K., Takaki, Y., Baba, M., Minato, N., Ohno, S., and Shuin, T. The von Hippel-Lindau tumor suppressor protein mediates ubiquitination of activated atypical protein kinase C. J Biol Chem, *276:* 43611-43617, 2001.

118. Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, J. W., Conaway, R. C., and Czyzyk-Krzeska, M. F. von Hippel-Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination. Proc Natl Acad Sci U S A, *100:* 2706-2711, 2003.
119. Zhou, M. I., Wang, H., Ross, J. J., Kuzmin, I., Xu, C., and Cohen, H. T. The von Hippel-Lindau tumor suppressor stabilizes novel plant homeodomain protein Jade-1. J Biol Chem, *277:* 39887-39898, 2002.
120. Roe, J. S., Kim, H., Lee, S. M., Kim, S. T., Cho, E. J., and Youn, H. D. p53 stabilization and transactivation by a von Hippel-Lindau protein. Mol Cell, *22:* 395-405, 2006.
121. Ohh, M., Yauch, R. L., Lonergan, K. M., Whaley, J. M., Stemmer-Rachamimov, A. O., Louis, D. N., Gavin, B. J., Kley, N., Kaelin, W. G., Jr., and Iliopoulos, O. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. Mol Cell, *1:* 959-968, 1998.
122. Koochekpour, S., Jeffers, M., Wang, P. H., Gong, C., Taylor, G. A., Roessler, L. M., Stearman, R., Vasselli, J. R., Stetler-Stevenson, W. G., Kaelin, W. G., Jr., Linehan, W. M., Klausner, R. D., Gnarra, J. R., and Vande Woude, G. F. The von Hippel-Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells. Mol Cell Biol, *19:* 5902-5912, 1999.
123. Los, M., Zeamari, S., Foekens, J. A., Gebbink, M. F., and Voest, E. E. Regulation of the urokinase-type plasminogen activator system by the von Hippel-Lindau tumor suppressor gene. Cancer Res, *59:* 4440-4445, 1999.
124. Bajou, K., Noel, A., Gerard, R. D., Masson, V., Brunner, N., Holst-Hansen, C., Skobe, M., Fusenig, N. E., Carmeliet, P., Collen, D., and Foidart, J. M. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat Med, *4:* 923-928, 1998.
125. Tang, N., Mack, F., Haase, V. H., Simon, M. C., and Johnson, R. S. pVHL function is essential for endothelial extracellular matrix deposition. Mol Cell Biol, *26:* 2519-2530, 2006.
126. Kurban, G., Hudon, V., Duplan, E., Ohh, M., and Pause, A. Characterization of a von Hippel Lindau pathway involved in extracellular matrix remodeling, cell invasion, and angiogenesis. Cancer Res, *66:* 1313-1319, 2006.
127. Lieubeau-Teillet, B., Rak, J., Jothy, S., Iliopoulos, O., Kaelin, W., and Kerbel, R. S. von Hippel-Lindau gene-mediated growth suppression and induction of differentiation in renal cell carcinoma cells grown as multicellular tumor spheroids. Cancer Res, *58:* 4957-4962, 1998.
128. Hergovich, A., Lisztwan, J., Barry, R., Ballschmieter, P., and Krek, W. Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pVHL. Nat Cell Biol, *5:* 64-70, 2003.
129. Pause, A., Lee, S., Lonergan, K. M., and Klausner, R. D. The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. Proc Natl Acad Sci U S A, *95:* 993-998, 1998.
130. Bindra, R. S., Vasselli, J. R., Stearman, R., Linehan, W. M., and Klausner, R. D. VHL-mediated hypoxia regulation of cyclin D1 in renal carcinoma cells. Cancer Res, *62:* 3014-3019, 2002.
131. Zatyka, M., da Silva, N. F., Clifford, S. C., Morris, M. R., Wiesener, M. S., Eckardt, K. U., Houlston, R. S., Richards, F. M., Latif, F., and Maher, E. R. Identification of cyclin D1 and other novel targets for the von Hippel-Lindau tumor suppressor gene by expression array analysis and investigation of cyclin D1 genotype as a modifier in von Hippel-Lindau disease. Cancer Res, *62:* 3803-3811, 2002.
132. Gunaratnam, L., Morley, M., Franovic, A., de Paulsen, N., Mekhail, K., Parolin, D. A., Nakamura, E., Lorimer, I. A., and Lee, S. Hypoxia inducible factor activates the transforming growth factor-alpha/epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells. J Biol Chem, *278:* 44966-44974, 2003.
133. Iliopoulos, O., Kibel, A., Gray, S., and Kaelin, W. G., Jr. Tumour suppression by the human von Hippel-Lindau gene product. Nat Med, *1:* 822-826, 1995.

134. Kondo, K., Klco, J., Nakamura, E., Lechpammer, M., and Kaelin, W. G., Jr. Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell, *1:* 237-246, 2002.
135. Maranchie, J. K., Vasselli, J. R., Riss, J., Bonifacino, J. S., Linehan, W. M., and Klausner, R. D. The contribution of VHL substrate binding and HIF1-alpha to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell, *1:* 247-255, 2002.
136. Kondo, K., Kim, W. Y., Lechpammer, M., and Kaelin, W. G., Jr. Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. PLoS Biol, *1:* E83, 2003.
137. Zimmer, M., Doucette, D., Siddiqui, N., and Iliopoulos, O. Inhibition of hypoxia-inducible factor is sufficient for growth suppression of VHL-/- tumors. Mol Cancer Res, *2:* 89-95, 2004.
138. Mandriota, S. J., Turner, K. J., Davies, D. R., Murray, P. G., Morgan, N. V., Sowter, H. M., Wykoff, C. C., Maher, E. R., Harris, A. L., Ratcliffe, P. J., and Maxwell, P. H. HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. Cancer Cell, *1:* 459-468, 2002.
139. Carmeliet, P., Dor, Y., Herbert, J. M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C. J., Ratcliffe, P., Moons, L., Jain, R. K., Collen, D., and Keshert, E. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature, *394:* 485-490, 1998.
140. Ryan, H. E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J. M., and Johnson, R. S. Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res, *60:* 4010-4015, 2000.
141. Ravi, R., Mookerjee, B., Bhujwalla, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., Dillehay, L. E., Madan, A., Semenza, G. L., and Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev, *14:* 34-44, 2000.
142. Akakura, N., Kobayashi, M., Horiuchi, I., Suzuki, A., Wang, J., Chen, J., Niizeki, H., Kawamura, K., Hosokawa, M., and Asaka, M. Constitutive expression of hypoxia-inducible factor-1alpha renders pancreatic cancer cells resistant to apoptosis induced by hypoxia and nutrient deprivation. Cancer Res, *61:* 6548-6554, 2001.
143. Unruh, A., Ressel, A., Mohamed, H. G., Johnson, R. S., Nadrowitz, R., Richter, E., Katschinski, D. M., and Wenger, R. H. The hypoxia-inducible factor-1 alpha is a negative factor for tumor therapy. Oncogene, *22:* 3213-3220, 2003.
144. Kung, A. L., Wang, S., Klco, J. M., Kaelin, W. G., and Livingston, D. M. Suppression of tumor growth through disruption of hypoxia-inducible transcription. Nat Med, *6:* 1335-1340, 2000.
145. Chen, J., Zhao, S., Nakada, K., Kuge, Y., Tamaki, N., Okada, F., Wang, J., Shindo, M., Higashino, F., Takeda, K., Asaka, M., Katoh, H., Sugiyama, T., Hosokawa, M., and Kobayashi, M. Dominant-negative hypoxia-inducible factor-1 alpha reduces tumorigenicity of pancreatic cancer cells through the suppression of glucose metabolism. Am J Pathol, *162:* 1283-1291, 2003.
146. Sun, X., Kanwar, J. R., Leung, E., Vale, M., and Krissansen, G. W. Regression of solid tumors by engineered overexpression of von Hippel-Lindau tumor suppressor protein and antisense hypoxia-inducible factor-1alpha. Gene Ther, *10:* 2081-2089, 2003.
147. Yeo, E. J., Chun, Y. S., Cho, Y. S., Kim, J., Lee, J. C., Kim, M. S., and Park, J. W. YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. J Natl Cancer Inst, *95:* 516-525, 2003.
148. Jiang, B. H., Rue, E., Wang, G. L., Roe, R., and Semenza, G. L. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem, *271:* 17771-17778, 1996.
149. Killion, J. J., Radinsky, R., and Fidler, I. J. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. Cancer Metastasis Rev, *17:* 279-284, 1998.
150. Kaur, B., Khwaja, F. W., Severson, E. A., Matheny, S. L., Brat, D. J., and Van Meir, E. G. Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. Neuro-oncol, *7:* 134-153, 2005.

CHAPTER 2

The hypoxic response of tumors is dependent on the microenvironment

Barbara Blouw, Hanqiu Song, Tarik Tihan, Jenel Bosze, Napoleone Ferrara, Hans-Peter Gerber, Randall S. Johnson and Gabriele Bergers

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ARTICLE

The hypoxic response of tumors is dependent on their microenvironment

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Summary

To reveal the functional significance of hypoxia and angiogenesis in astrocytoma progression, we created genetically engineered transformed astrocytes from murine primary astrocytes and deleted the hypoxia-responsive transcription factor HIF-1α or its target gene, the angiogenic factor VEGF. Growth of HIF-1α- and VEGF-deficient transformed astrocytes in the vessel-poor subcutaneous environment results in severe necrosis, reduced growth, and vessel density, whereas when the same cells are placed in the vascular-rich brain parenchyma, the growth of HIF-1α knockout, but not VEGF knockout tumors, is reversed: tumors deficient in HIF-1α grow faster, and penetrate the brain more rapidly and extensively. These results demonstrate that HIF-1α has differential roles in tumor progression, which are greatly dependent on the extant microenvironment of the tumor.

Introduction

Every tissue, including solid tumors, is dependent on adequate oxygen delivery. Angiogenesis, the formation of new blood vessels, is a discrete step in tumor progression that is required for the expansion of tumor mass (Folkman, 2000). The stage in which angiogenesis is initiated, however, can vary among tumors, depending on the type of malignancy and the microenvironment in which the tumor develops.

Astrocytomas, a class of malignant brain tumors, are very oxygen dependent (Brat et al., 2003). Interestingly, low-grade astrocytomas first acquire their blood supply by coopting existing normal brain blood vessels, without initiating angiogenesis. However, when grade III astrocytomas progress to grade IV astrocytomas/glioblastomas (the most malignant form of astrocytoma, also known as glioblastoma multiformae, or GBM), they show features of hypoxic and necrotic palisades. These palisades are in part caused by vessel regression (Holash et al., 1999a) and increased tumor cell proliferation (Brat et al., 2002; Holland, 2000). The resultant hypoxia is then thought to induce new blood vessels that supply the tumor with the necessary metabolites (Brat et al., 2003). In fact, glioblastomas are in part defined by the appearance of proliferating endothelial cells and high blood vessel densities; these distinguish grade IV tumors from the lower grade astrocytomas histologically (Louis et al., 2001).

Hypoxic response is triggered to a large extent by the hypoxia inducible factor-1, or HIF-1, which is composed of a basic helix-loop-helix transcription factor dimer. This dimer has two components: HIF-1β, also known as ARNT, which is constitutively expressed, and HIF-1α, which is regulated posttranslationally by ubiquitination-triggered proteolysis (Semenza, 2002b). HIF-1 controls the expression of more than 40 target genes, whose protein products are implicated in angiogenesis, metabolism, and cell survival (Semenza, 2002a). Although three HIF-α proteins have been discovered, targeted deletion of the

SIGNIFICANCE

The role of hypoxia and angiogenesis in tumor progression has been modeled in numerous studies of malignancy in rodents; however, less well understood is the role that specific anatomic microenvironments play in determining hypoxic response and neovascularization in tumorigenesis. We show here that hypoxic response through the HIF-1α transcription factor plays dramatically different roles, depending on the tissue in which tumors are introduced. HIF-1α is a critical regulator of the production of angiogenic factors, including the vascular endothelial growth factor (VEGF); this study shows that target-directed therapies, including those that modulate hypoxic response, may have very different effects, dependent not on the cell type, or malignancy of the tumor, but on its location and microenvironment.

HIF-1α gene results in loss of hypoxic responsiveness of virtually every HIF-1-regulated transcript (Ryan et al., 2000).

Interestingly, HIF-1α is overexpressed in human glioblastoma biopsies, and the level of expression is correlated with highest grade of malignancy in human GBM (Zagzag et al., 2000). Among the most prominent target genes of HIF-1α is the angiogenic factor VEGF, which is also highly expressed in glioblastomas (Chaudhry et al., 2001). These data suggest that HIF-1α and VEGF may be tumor-promoting factors in astrocytoma progression.

Several laboratories have demonstrated that loss of the HIF-1α transcription factor results in reduced growth rates of various tumors, accompanied in some, but not all, models by decreased vascular density (Maxwell et al., 1997; Ryan et al., 1998, 2000). Other studies, however, have pointed in the opposite direction, describing HIF-1α as a tumor suppressor, whose loss gives rise to more aggressively growing embryonic stem cell-derived teratocarcinomas (Carmeliet et al., 1998). Thus, the role of the hypoxic response, and HIF-1α specifically, during tumor growth has been controversial.

One of the confounding variables in these studies has been the use of a range of cell types (e.g., hepatomas and fibrosarcomas) and different cell lines within those cell types (e.g., different embryonic stem [ES] cell lines). This makes comparative studies of the divergent tumors even more difficult to interpret, with respect to determining the actual role of hypoxic response during tumorigenesis in vivo.

One of the variables not extensively analyzed thus far in the experimental study of HIF-1α-deficient tumors is the relationship of site of malignancy to tumor progression. Most studies of tumor formation have been performed in immunocompromised mouse models at subcutaneous sites. Although this is a historic and well-studied site of tumor growth, the subcutaneous space has a number of intrinsic peculiarities that set it apart from sites elsewhere in the mammalian body. These include a lack of spatial constraints in the form of matrix or skeletal elements, relatively sparse vascularization, and the interface of a number of different tissue types (fat, muscle, connective tissue) found immediately under the skin.

The role of the tissue vasculature is particularly important when considering the hypoxic response of tumors. Tumors vary tremendously from tissue to tissue in degrees of invasiveness, levels of vascular density, and metastatic potential. How much of this variation is related to the state of the vasculature in the tissue itself? This is a particularly critical issue when studying the role of the HIF-1 transcription factor, which is activated by alterations in the microenvironment that are controlled by the local vasculature. It is important to note that many GBM studies still involve mouse models in which tumors grow subcutaneously, in a poorly vascularized environment (Huang et al., 1995; Ozawa et al., 1998; Prewett et al., 1999); this contrasts strongly with their natural habitat, the highly vascularized brain parenchyma.

We investigated whether HIF-1α activity and its downstream target, the angiogenic factor VEGF, are driving forces in astrocytoma progression by generating HIF-1α- and VEGF-deficient astrocytomas, and analyzed tumor propagation in two different microenvironments, the subcutaneous and intracranial habitat. Our study demonstrates that the HIF-1α-regulated hypoxic response to the microenvironment is highly dependent on the extant tissue microenvironment and involves VEGF-dependent and -independent mechanisms.

Results

Generation of HIF-1α-deficient (HIFko) astrocytomas

We isolated astrocytes from the hippocampus of mice homozygous for a HIF-1α/loxP allele (Figure 1A). To assure aggressive tumor formation, we immortalized and transformed them with SV40 large T antigen and the V12-H-ras oncogene, respectively (Figure 1A). V12H-ras was chosen because activation of the p21-ras pathway has been implicated in astrocytoma proliferation and angiogenesis (Feldkamp et al., 1999; Guha et al., 1997), and mouse strains expressing V12-H-ras under the control of a glial cell-specific promoter (GFAP) have been shown to develop high-grade astrocytomas (Ding et al., 2001). Similarly, SV40 large T antigen binds, and thereby inactivates, the two tumor suppressor proteins p53 and Rb that are commonly lost in astrocytoma progression (Louis et al., 2001; Xiao et al., 2002); in addition, transgenic mice expressing the Rb binding form of SV40 large T antigen in astrocytes also develop high-grade astrocytomas (Xiao et al., 2002).

To obtain HIF-1α-null (also known as HIFko) astrocytomas, we infected transformed HIF-1α loxP astrocytes with an adenovirus that expresses the bacteriophage recombinase Cre, or with a control virus expressing β-galactosidase. The recombinase efficiently deleted the floxed gene sequences present on the two conditional HIF-1α alleles, creating HIF-1α-deficient (HIFko) astrocytomas; real time-PCR analysis did not detect any nonrecombinant wild-type cells in adenoviral cre-infected cultures (data not shown). The adenovirus-treated control astrocytomas did not elicit any recombination and were defined as HIF-1α wild-type (also known as HIFwt) astrocytomas (Figure 1A). This multistep procedure allows a dissection of the role of HIF-1α in astrocytomas without the complications of using genetically altered and possibly diverged cell lines, because transformation is carried out prior to the removal of the HIF-1α gene by Cre recombinase activity; deletion then occurs at a very low passage number.

HIFko astrocytoma cells did not induce transcription of the HIF-1α-regulated genes phosphoglycerate kinase (PGK) and glucose transporter-1 (Glut-1) under hypoxic conditions, whereas HIFwt cells revealed 2- to 6-fold higher RNA levels of those genes when oxygen levels were decreased (Figure 1B). Slightly increased VEGF levels were still observed in HIFko cells because VEGF mRNA expression and stabilization is regulated by different mechanisms. Loss of hypoxic response through HIF-1α did not substantially affect cell growth of astrocytomas in vitro (Figure 1C), nor did it change the transformation status of those cells as evaluated in a soft agar assay (Figure 1D).

Subcutaneous growth of HIF-1α-deficient astrocytomas is impaired

We next assessed the role of HIF-1α transcriptional response in astrocytomas in a standard assay for tumorigenic potential in vivo, in which HIFwt and HIFko astrocytoma cell lines were injected subcutaneously into nude mice. Not only did HIFko astrocytomas grow more slowly than wild-type astrocytomas, reaching only 40% of the wild-type tumor weight at 21 days postinjection (Figure 2A), but there were also 30% fewer proliferating cells relative to wild-type astrocytomas (Figure 2B). Surprisingly, we did not observe any differences in the number of

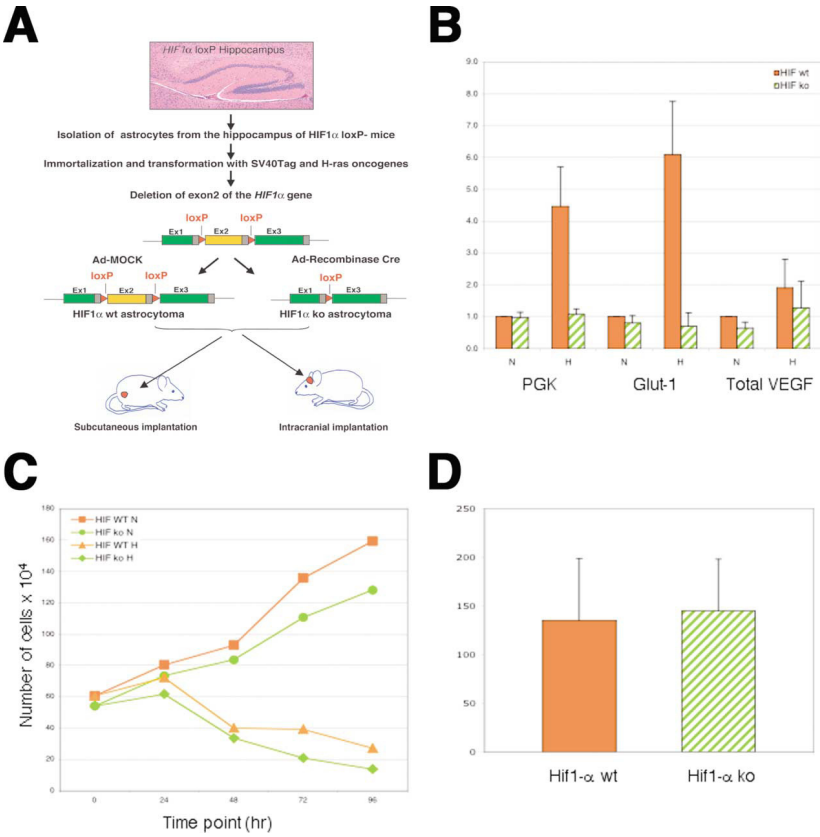

Figure 1. In vitro characterization of HIF-1α wild-type (HIFwt) and HIF-1α-deficient (HIFko) transformed astrocytes

A: Primary hippocampal astrocytes were isolated from HIF-1α loxP mice, immortalized, and transformed with SV40Tag and H-ras. HIF-1α-deficient transformed astrocytes were obtained with the bacteriophage recombinase Cre by deleting the floxed gene sequences of the HIF-1α alleles.
B: HIFwt and HIFko astrocytoma cells were cultured for 8 hr under hypoxic (0.5% pO_2) and normoxic (20% pO_2) conditions, harvested, and transcription levels of the hypoxia target genes *Pgk*, *Glut-1*, and *Vegf A* (all isoforms) determined by real-time PCR analysis. Induction of gene transcription was observed in HIFwt but not in HIFko astrocytes, under hypoxic conditions. Normoxia revealed similar expression levels of these genes in both cell lines. Error bars indicate SEM.
C: Growth rate of HIFwt and HIFko transformed astrocytes under normoxic (20% pO_2) and hypoxic (0.5% pO_2) conditions. The growth rates of HIF wt and HIFko cells did not differ under low and high oxygen conditions.
D: HIFwt and HIFko transformed astrocytes formed similar numbers and sizes of colonies in a soft agar colony formation assay. Error bars indicate standard deviation (SD).

apoptotic cells (Figure 2C). However, more than 70% of the small HIFko tumor nodules had hypoxic and necrotic areas, in contrast to wild-type control astrocytomas, which exhibited much smaller areas of hypoxic and necrotic palisades (Figures 2E and 2F). The morphology of the tumor cells in the two genotypes was indistinguishable (Figures 3A and 3B), and both wild-type and HIFko astrocytomas grew as encapsulated nodules (Figures 3C and 3D).

HIF-1α-deficient astrocytomas show increased cell proliferation in the brain but not in the subcutaneous habitat

We then injected HIFwt and HIFko astrocytomas intracranially into the brain parenchyma of nude mice, and compared the tumor phenotypes observed to the features of astrocytomas grown subcutaneously. Both HIFwt and HIFko astrocytomas showed typical features of high-grade gliomas: in particular,

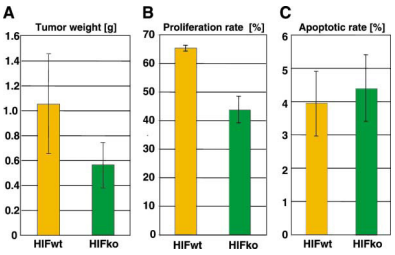

Figure 2. Growth comparison of HIFwt and HIFko astrocytoma in the subcutaneous space

A: Mice were inoculated subcutaneously with HIFwt and HIFko astrocytes, and tumors dissected after 21 days and weighed. HIFko astrocytomas had a reduced tumor weight of 60% compared to HIFwt tumors.
B: Proliferation index of HIFwt and HIFko tumors was assessed immunohistochemically by counting Ki-67 positive cells on tumor tissue sections. HIFko astrocytomas showed a 30% reduction in proliferation rate ($p < 0.0001$).
C: Apoptotic index of HIFwt and HIFko astrocytomas was quantified by TUNEL staining on tumor sections. There was no statistical difference in apoptotic cells between HIFwt and HIFko tumors ($p = 0.2345$). Error bars indicate standard deviation (SD).

they were fast growing, and elicited hypoxic and necrotic palisades. However, striking differences were observed. Mice bearing HIFwt or HIFko astrocytomas had similar survival times, of approximately 16–18 days (Figure 4C). This finding indicated that HIF-1α-deficient astrocytomas were not smaller than wild-type control tumors in the intracranial habitat which was confirmed by MRI (data not shown) and histopathological analyses (Figures 5A and 5B).

In contrast to subcutaneously grown astrocytomas, where the number of dividing cells declined by 30% in the absence of HIF-1α (Figure 2B), the number of proliferating cells in intracranial HIFko astrocytomas was 30% higher than that in wild-type tumors (Figure 4A). No differences in the apoptotic index were observed between wild-type and HIFko astrocytomas (Figure 4B).

Interestingly, HIFwt astrocytomas could often be distinguished morphologically from HIFko astrocytomas. Astrocytomas deficient in HIF-1α appeared more spindle-like than wild-type astrocytomas, which also contained mixed cell types (e.g., giant cells (Figures 5A and 5B). Most surprisingly, HIFko astrocytomas did not reveal large necrotic and hemorrhagic centers (Figures 5A–5D) but contained only small areas of hypoxic palisades, in contrast to the much larger areas seen in wild-type control astrocytomas (Figures 5C and 5D). It is important to note, however, that HIF-1α-deficient and wild-type astrocytomas were both defined as grade IV or mixed grade III/IV astrocytomas by our pathologist.

Loss of HIF-1α increases the invasive capacity of astrocytomas in the brain but not in the subcutaneous habitat

One important feature of both genotypes of astrocytomas was their diffuse growth into the brain parenchyma (Figures 5E–5H) that contrasted with the encapsulated phenotype of the same tumor lines grown subcutaneously (Figures 3C and 3D). The infiltrative behavior of the intracerebrally placed tumors in fact nicely mimics the phenotypes observed in human astrocytomas. The tumor cells grew along existing blood vessels in the perivascular (Virchow-Robin) spaces in the brain (Figures 6C and 6D) and tracked along leptomeninges. To our surprise, however, HIFko astrocytomas invaded even into the collateral hemisphere, being detectable at different locations in both hemispheres of the brain (Figure 6B), as visualized by SV40 large T antigen staining (which specifically detects the injected tumor cells).

Although HIFwt astrocytomas were also diffusely growing (Figure 6C), they stayed focally localized—even at large sizes as illustrated in Figure 6A—whereas HIFko tumors did not. We were able to observe small clusters or even single HIF-1α-deficient tumor cells percolating throughout the brain parenchyma (Figure 6D), a feature of the diffusely growing tumor gliomatosis cerebrii. Covisualization of tumor cells and blood vessels revealed that HIFko astrocytomas had become extremely invasive, tracking along blood vessels in the perivascular space and moving along leptomeninges in both hemispheres (Figure 6D). In summary, loss of HIF-1α in tumors implanted in the brain had an almost diametrically opposed phenotype compared to the phenotype resulting from subcutaneous implantation. HIFko tumors had become more proliferative, and invasive, without showing features of large necrotic centers once implanted intracranially.

The microenvironment radically alters vascular structure and density in experimental astrocytomas, in a HIF-1α-dependent fashion

Based on the results above, we suspected that loss of HIF-1α might affect tumor vascularization differentially in these two tumor sites. We sought therefore to visualize and analyze the vascular phenotype of both subcutaneous and intracranial astrocytoma-derived tumors by perfusing the circulatory system with a fluorescent lectin (Figure 7). We found that subcutaneous HIFko astrocytomas were rather poorly vascularized, with small patches of distorted blood vessels that surrounded hypoxic and necrotic areas (Figure 7D), and vessel densities of only 52 vessels/mm^2 (Figure 7E). Wild-type astrocytomas contained 50% more blood vessels per unit area than subcutaneous HIFko tumors (105 vessels/mm^2; Figure 7E), a differential likely responsible in part for the relatively robust growth of the wild-type tumor cells at this site (Figures 7C and 7E).

Surprisingly, HIFko astrocytomas in the brain were not only highly vascularized (Figure 7B), but they also exhibited about 50% more blood vessels than the wild-type control astrocytomas (Figure 7E); this is the exact opposite of the finding in subcutaneous growth. The vessel density of HIFko tumors, however, was still lower than the vessel density of normal brain, a highly vascularized organ (380 vessels/mm^2 in normal brain versus 269 vessels/mm^2 in HIFko astrocytomas) (Figure 7E). It is important to note in this context that most tumors, including astrocytomas, exhibit a lower vessel density than the corresponding normal tissue because their oxygen consumption is lower than that of normal tissue (Eberhard et al., 2000; Hlatky et al., 2002). The majority of HIFko astrocytoma vessels resembled the morphology of the vascular network in normal brain (Figure 7B and 7F), appearing slim and organized. This is in contrast to blood vessels of HIFwt astrocytomas, which exhibited typical features of a tumor vasculature; i.e., the vessels were

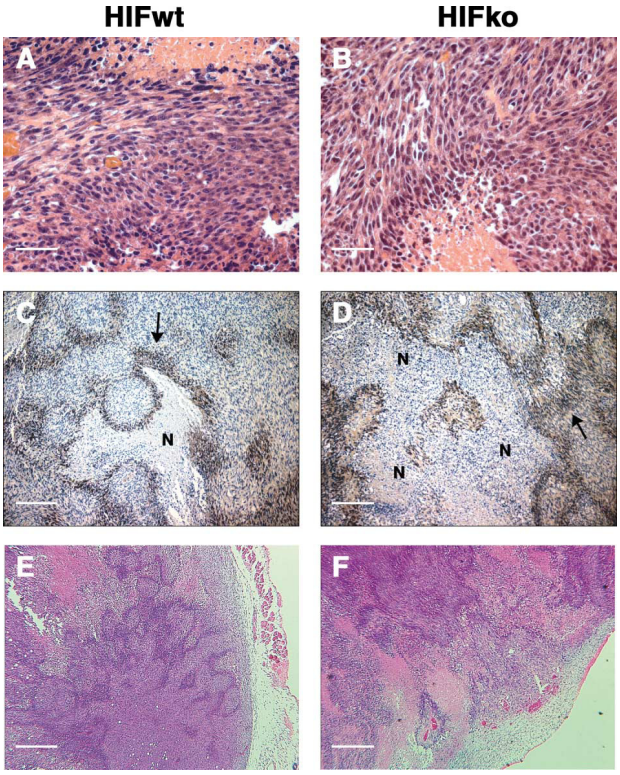

Figure 3. Illustrative histopathology of subcutaneous HIFwt and HIFko astrocytomas

Hematoxylin & eosin (H&E) staining of HIFwt (**A**) and HIFko (**B**) astrocytomas (Bar = 40 μm). Tumor cells are morphologically similar. **C and D:** Hypoxia and necrosis in HIFwt and HIFko astrocytomas. Hypoxic areas (in brown) were visualized in tumors of pimonidazole-treated mice using the hypoxyprobe-1 monoclonal antibody (black arrow); necrotic areas are indicated as "N" (bar = 85 μm). HIFko astrocytomas are much more necrotic than HIFwt astrocytomas. **E and F:** Subcutaneous astrocytomas are encapsulated as visualized by H&E staining (bar = 200 μm).

irregularly shaped, distorted, dilated, and leaky, as indicated by extravasation of fluorescent lectin into the brain parenchyma (Figure 7A). The most striking differences in vascular morphology and density were apparent between HIFko astrocytomas in the subcutaneous area and brain. Subcutaneous tumors reduced to less than 20% in vessel density compared to intracranial HIFko tumors, only exposing small patches of distorted blood vessels (Figures 7E, 7B, and 7D) in contrast to the dense and elongated vasculature of HIFko tumors in the brain parenchyma. HIFwt astrocytomas had similar vessel densities in both settings, but with differences in vessel branching and morphology of the vasculature between the necrotic areas that covered the tumor (Figures 7A and 7C). The vasculature of subcutaneous HIFwt astrocytomas appeared less distorted, and showed signs of decreased branching, in comparison to the intracranial HIFwt astrocytomas (Figures 7A and 7C). The surprising result, that the vasculature of intracranial HIFko tumors resembles normal brain blood vessels, suggests that these tumors have not undergone neovascularization yet, but coopted the existing normal brain vasculature. This would explain the increased degree of invasion of these fast-growing tumors, likely dictated by the necessity to retrieve sufficient oxygen and nutrients. If this hypothesis holds true, then one would expect differing levels of angiogenic factors in HIFwt and HIFko astrocytomas. What is the downstream factor of the HIF pathway that determines whether vessels will be coopted or neovascularization will be initiated?

Loss of VEGF limits tumor growth and regresses coopted blood vessels in the brain

VEGF-A (also known as VEGF) is highly expressed in grade IV astrocytomas/glioblastomas (GBM) and the most prominent angiogenic candidate, because its transcription is partly induced by the HIF-1 complex (Ferrara et al., 2003; Pugh and Ratcliffe, 2003). We analyzed VEGF RNA and protein levels in HIFwt and HIFko cell lines and tumors. VEGF RNA and protein was expressed in both cell lines under normoxic conditions, and was induced under hypoxic conditions, albeit to a lesser degree

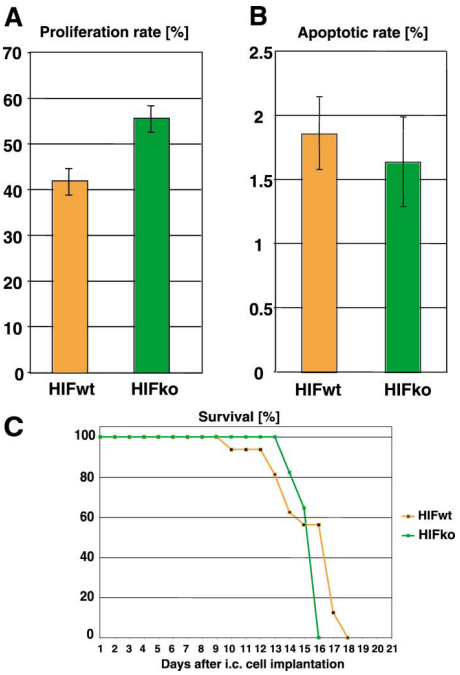

Figure 4. Growth comparison of HIFwt and HIFko astrocytoma in the brain parenchyma

A: Mice were injected intracranially with HIFwt or HIFko astrocytoma and monitored daily. Mice were sacrificed as soon as they showed side effects of tumor expansion such as lateral recumbency and weight loss. Survival curves of mice bearing either HIFwt or HIFko astrocytomas are plotted. Mice had similar survival means ($p = 0.129$)

B: Proliferation index of HIFwt and HIFko tumors was assessed immunohistochemically by counting Ki-67 positive cells on tumor tissue sections. HIFko astrocytomas showed a 30% relative increase in the proliferation rate ($p < 0.0001$).

C: Apoptotic index of HIFwt and HIFko astrocytomas was quantified by TUNEL staining in tumor sections. There was no significant statistical difference in apoptotic cells between HIFwt and HIFko tumors ($p = 0.1074$). Error bars indicate standard deviation (SD).

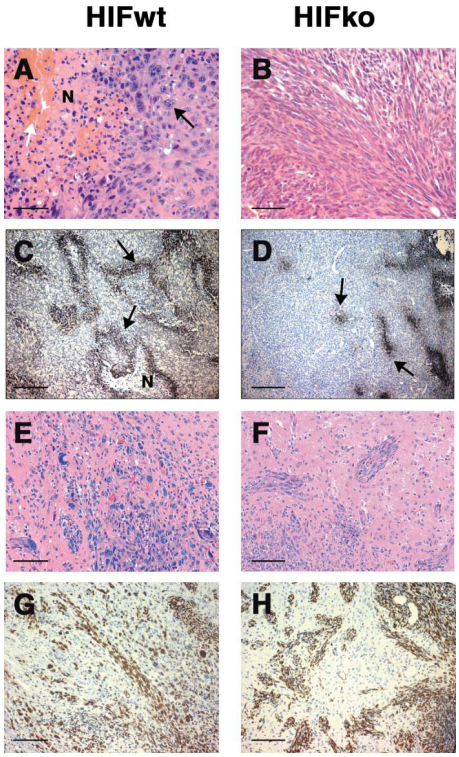

Figure 5. Illustrative histopathology of intracranial HIFwt and HIFko astrocytomas

H&E staining reveals hemorrhagic and necrotic areas (white arrow) and features of giant cells (black arrow) in HIFwt tumors (**A**), whereas HIFko tumors (**B**) often appear more spindle-like with little necrosis (bar = 45 μm). **C and D:** Detection of hypoxia and necrosis in HIFwt and HIFko astrocytomas. Hypoxic areas (in brown) were visualized in tumors of pimonidazole-treated mice using the hypoxyprobe-1 monoclonal antibody (black arrow); necrotic areas are indicated as "N." HIFko astrocytomas exhibit only small areas of hypoxia in contrast to HIFwt astrocytomas. H&E staining reveals a diffuse growth pattern of HIFwt (**E**) and HIFko astrocytomas (**F**) (bar = 95 μm) into the brain parenchyma that is confirmed by SV40 large T antigen staining (**G and H**) that detects injected tumor cells (bar = 95 μm).

in HIFko cells (Figures 1B and 7G). VEGF positive cells were also detected in intracranially and subcutaneously growing HIFwt and HIFko astrocytomas as revealed by immunohistochemical analysis (Supplemental Figures S1 and S2). Although the VEGF expression pattern was very heterogeneous at different areas within the tumors, intracranial HIFko tumors appeared to have overall lower levels of VEGF protein than the wild-type controls, whereas subcutaneous HIFwt and HIFko tumors both exhibited VEGF levels predominantly around perinecrotic palisades (Supplemental Figure S2).

In order to determine whether the differing phenotypes observed in HIFwt and HIFko astrocytomas are dependent on downstream activity of VEGF, we generated transformed astrocyte cell lines that were deficient in VEGF. Would tumors deficient in VEGF behave like those that are deficient in HIF-1α? We employed an approach similar to that described for the generation of HIFko astrocytomas (Figure 1A) by isolating astrocytes from VEGFloxP mice, wherein exon 3 of the *VEGF* gene is flanked by loxP recombination sites (Gerber et al., 1999).

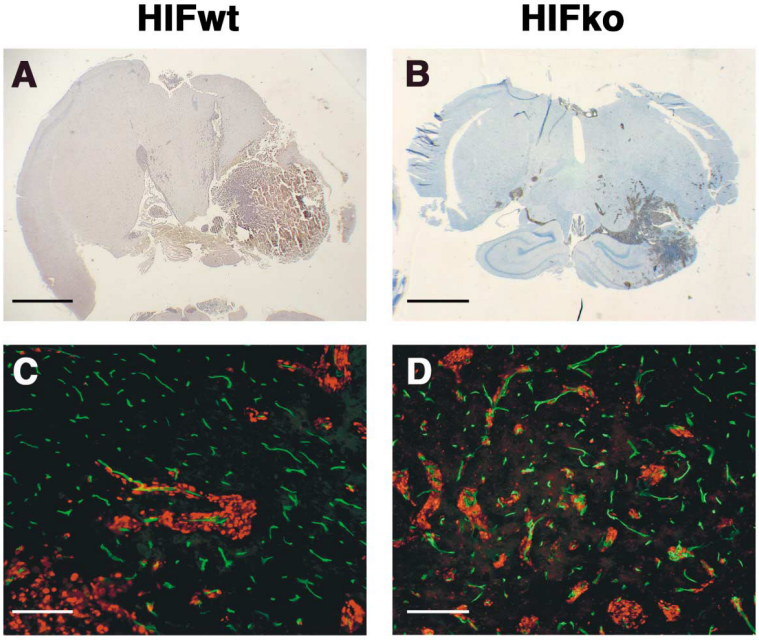

Figure 6. Comparison of invasive behavior in intracranial HIFwt and HIFko astrocytomas

A and B: Invading tumor cells in the brain were detected by SV40 large T antigen staining (in brown). HIFko astrocytomas infiltrate both hemispheres of the brain (**B**; bar = 1.6 mm), whereas HIFwt astrocytomas, even at large sizes, (**A**, Bar = 1.3 mm) stay focally localized and expand at the site of implantation. **C and D:** Tumor-bearing mice were injected i.v. with FITC-labeled tomato lectin (lycopersicon esculentum) to stain blood vessels in green, and then heart-perfused with 4% paraformaldehyde (PFA), followed by immunohistochemical staining with Cy3-labeled SV40 large T antigen antibody to label tumor cells in red. Tumor cells are in close contact with normal brain blood vessels, and grow and migrate in the perivascular space. HIFko cells are detected in wide areas of the normal brain parenchyma (**C**), whereas HIFwt tumor cells are only detected around blood vessels close to the tumor mass (**D**).

We then immortalized and transformed VEGFloxP astrocytes with SV40Tag and V12H-ras oncogenes, and in situ excised the floxed exon 3 of the *VEGF* gene with an adenovirus expressing recombinase Cre (VEGFko astrocytomas). Infection with a control adenovirus expressing β-galactosidase was carried out as control, resulting in VEGF wild-type astrocytomas (also known as VEGFwt).

Both cell lines were then implanted subcutaneously and intracranially, tumor growth was observed, and dissected tumors were analyzed for their morphology, proliferation and apoptotic rates, and vascular profile. Similar to HIF-1α-deficient tumors, VEGFko astrocytomas grew much slower subcutaneously than the control VEGFwt tumors, reaching only about 50% of the wild-type tumor weight at 21 days postinjection (Figure 8A). We observed, however, striking differences between HIFko and VEGFko tumors in the brain. VEGFko astrocytomas grew slower than the control astrocytomas in the brain parenchyma, and mice had a statistically significant prolonged mean survival of 30%, compared to mice bearing VEGFwt tumors ($p < 0.001$) (Figures 8B and 8C). VEGFko tumors in the subcutaneous habitat and brain elicited a significantly lower proliferation rate (Figure 8C), and exhibited a higher apoptotic rate than wild-type tumors (Figure 8D).

Histopathological analysis revealed more necrotic and hypoxic areas in subcutaneously growing VEGFko tumors compared to VEGFwt tumors (B.B., unpublished data) that can be partly explained by the 50% lower vascular density in VEGF-deficient subcutaneous astrocytomas (Figure 8E). These results are similar to those obtained from subcutaneous HIFko and HIFwt tumors (Figures 3A–3C and 7E). There were, however, significant differences between VEGFko and HIFko tumors in the brain parenchyma. VEGFko tumors (Figure 8Fb) did not form real tumor masses like VEGFwt (Figure 8Fa) or HIFko tumors (Figure 5B). Those tumors that were still defined as gradeIII/IV astrocytomas grew rather in migrating clusters, with necrosis in some of the centers that were encircled by vascular tumor nests (Figure 8Fb). Despite this diffusely growing phenotype, VEGF-deficient tumors were not detected in both brain hemi-

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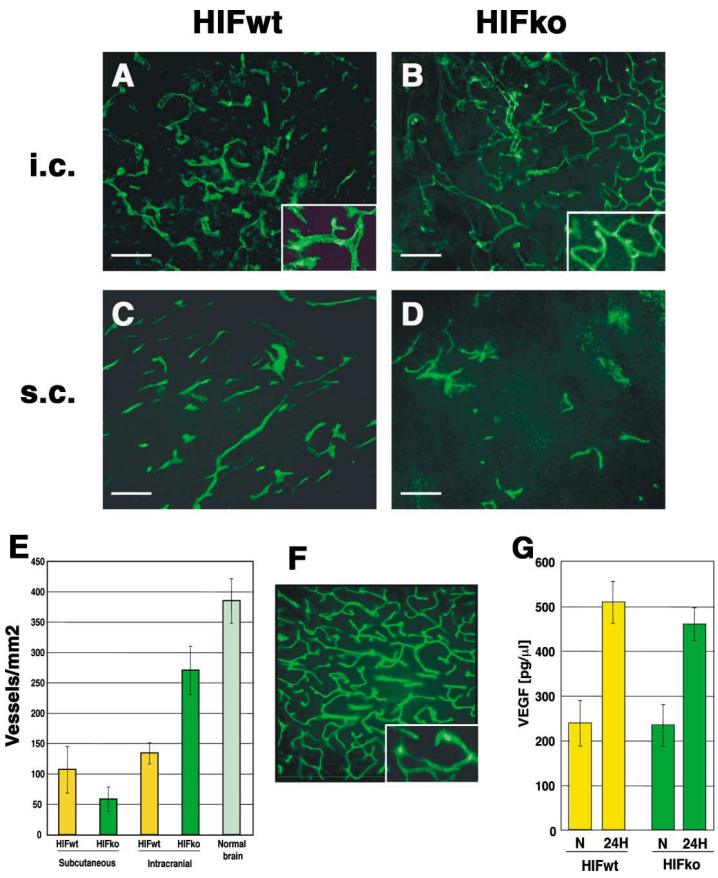

Figure 7. Comparison of vascular morphology and vessel density in HIFwt and HIFko astrocytomas grown in the subcutaneous and intracranial space

To visualize functional blood vessel, tumor-bearing mice were injected i.v. with FITC-labeled tomato lectin (lycopersicon esculentum) and then heart-perfused with 4% PFA. Brains were frozen in OCT medium and sectioned at 50 μm. The vasculature is visualized in green of intracranial (i.c.) HIFwt (**A**) and HIFko (**B**) astrocytomas, and subcutaneous (s.c.) HIFwt (**C**) and HIFko (**D**) astrocytomas (bar = 130 μm). **E:** Blood vessel density was assessed by counting vessels/mm^2 of 4–5 different tumors per group. Subcutaneous HIFko astrocytomas (**D** and **E**) show a 50% reduction in vessel density compared to s.c. HIFwt tumors ($p = 0.0317$) (**C** and **E**), exposing small patches of distorted and leaky vessels. In contrast, intracranial HIFko tumors are highly vascularized (5-fold higher than s.c. HIFko tumors) and reveal a 50% induction of vessel density in comparison to intracranial HIFwt astrocytomas ($p = 0.0159$) (**E**). Intracranial HIFko tumor vessels resemble normal brain blood vessels but show reduced vessel density in comparison to normal brain (**B** and **F**) in contrast to the distorted, dilated, and leaky vessels of intracranial HIFwt tumors in (**A**). **F:** Vasculature of normal brain. **G:** VEGF protein levels were assessed in conditioned medium of HIFwt and HIFko cells under normoxic and hypoxic (24 hr) conditions by an ELISA assay. VEGF protein levels are elevated in both cell lines under low oxygen levels.

spheres, but stayed focally localized, in striking contrast to the widespread dissemination of HIFko tumors. The distinct invasive patterns may be attributed to the differing vascular phenotype of both tumor types. Whereas HIFko tumors contained a dense network of more normal, elongated brain vessels (Figure 7B), VEGF-deficient tumors revealed a low density of distorted blood vessels; the vasculature was reduced by about 45% relative to VEGFwt tumors (Figures 8E, 8Fc, and 8Fd). The same relative

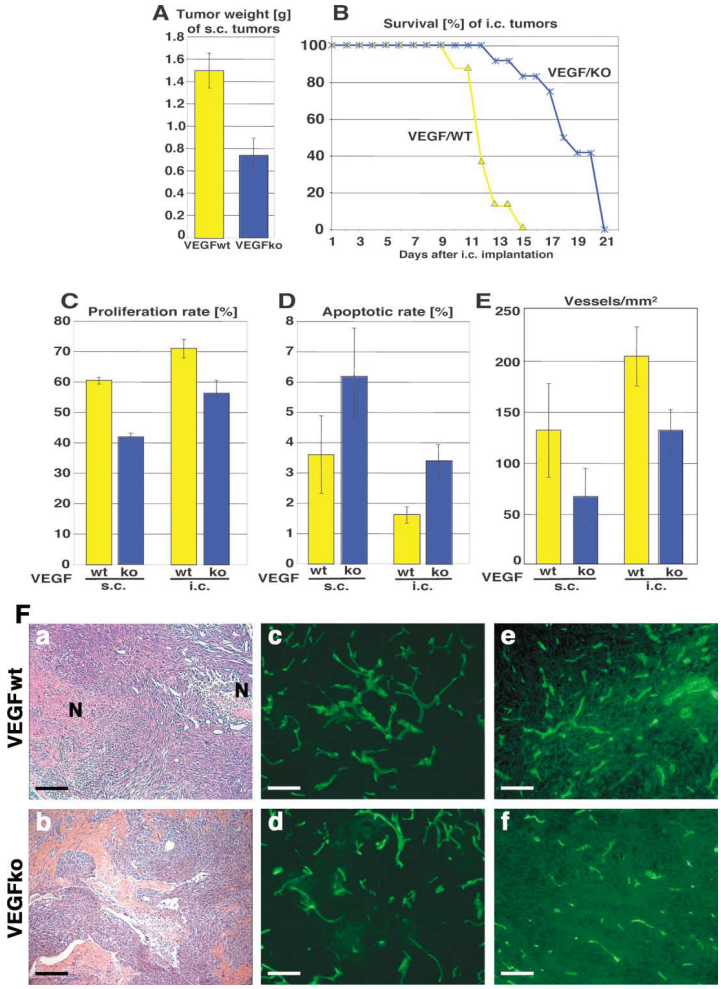

Figure 8. Comparison of cellular and vascular phenotypes of VEGFwt and VEGFko astrocytomas grown in subcutaneous (s.c.) and intracranial (i.c.) space

A: Mice were inoculated s.c. with VEGFwt and VEGFko astrocytomas, and tumors dissected after 21 days and weighed. The tumor weight of VEGFko astrocytomas was reduced by about 50% in comparison to VEGFwt tumors.

B: When mice were injected i.c. with VEGFwt and VEGFko tumors, mice bearing VEGFko tumors had a 35% longer survival mean than VEGFwt-tumor bearing mice ($p < 0.001$).

C and D: Proliferative (**C**) and apoptotic rate (**D**) of VEGFwt and VEGFko tumors was assessed immunohistochemically by counting Ki-67- or TUNEL-positive cells, respectively. The proliferation rate was significantly reduced in subcutaneously ($p < 0.0001$) and intracranially ($p < 0.0001$) grown VEGFko tumors. The apoptotic rate was increased in both s.c. ($p = 0.0012$) and i.c. ($p = 0.0008$) grown VEGFko tumors in comparison to VEGFwt tumors.

E: Blood vessel density was assessed by counting vessels/mm² of 4–5 fields of different tumors per group. Subcutaneous ($p = 0.0303$) and intracranial ($p = 0.0159$) VEGFko tumors showed a 40%–50% reduction in vessel density in comparison to the respective wt tumors. Error bars indicate standard deviation.

F: Illustrative histopathology and vascular morphology and density of VEGFwt and VEGFko astrocytomas. **a:** H&E staining reveals tumor masses of VEGFwt i.c. tumors with necrotic areas (N). **b:** VEGFko astrocytomas grow in migrating clusters without forming a real tumor mass. Blood vessels in i.c. (**c** and **d**) and s.c. (**e** and **f**) tumors are visualized with a FITC-labeled tomato lectin.

Table 1. Comparison of HIFko and VEGFko astrocytomas in the subcutaneous and intracranial environment

	HIFko versus HIFwt		VEGFko versus VEGFwt	
s.c.	Proliferation	↓	Proliferation	↓
	Apoptosis	○	Apoptosis	↑
	Vessel density	↓	Vessel density	↓
	Tumor growth	↓	Tumor growth	↓
	Invasion	no	Invasion	no
i.c.	Proliferation	↑	Proliferation	↓
	Apoptosis	○	Apoptosis	↑
	Vessel density	↑	Vessel density	↓
	Tumor growth	↑	Tumor growth	↓
	Invasion	↑↑	Invasion	↑

○, no change; ↑, induced; ↓, reduced.

vascular phenotype was observed in subcutaneously growing VEGFwt and VEGFko tumors; the vasculature of VEGFko tumors was also reduced by about 50% (Figures 8E, 8Fe, and 8Ff).

In summary, loss of VEGF in astrocytomas reduced subcutaneous and intracerebral tumor growth by severely impairing growth and survival of coopted tumor vessels. The tumors reacted differently to the regressing vessels: subcutaneous tumors became very necrotic, and showed increased apoptosis, whereas intracerebral tumors grew along blood vessels in migrating clusters, albeit only in close conjunction to the tumor (Table 1). Collectively, these results indicate that the hypoxic response of a tumor can determine initiation of new blood vessels, or cooption of existing blood vessels, but is highly dependent on the local environment in which those tumors arise.

Discussion

We have made the unexpected observation that HIF-1α can act as a negative or positive factor in astrocytoma progression, dependent on the microenvironment in which tumors grow. Loss of HIF-1α impairs astrocytoma growth subcutaneously, but increases proliferative and invasive properties of astrocytomas in the brain. These effects are not observed when VEGF, a downstream target of HIF-1α, is genetically ablated in astrocytomas (Table 1). VEGF-deficient astrocytomas exhibit a growth disadvantage in either microenvironment.

How can these opposite effects be explained? Hypoxia is a hallmark of grade IV astrocytomas/glioblastomas (GBM), and enables these tumors to partly induce angiogenesis in a HIF-1α-dependent manner. HIF-1α and its target VEGF are both highly upregulated in GBMs, and vascular proliferation is a prognostic marker in GBMs that distinguishes them from lower grade astrocytomas. In agreement with predicted behaviors of the tumors, our subcutaneous astrocytoma studies reveal that loss of HIF-1α, or VEGF, results in poorly vascularized tumors, and causes severe necrosis and restrained growth in the subcutaneous space. Thus, loss of HIF-1α or VEGF activity severely impaired astrocytoma growth. These data are in consensus with results of other laboratories: that absence of HIF-1α in ES cell-derived tumors resulted in reduced vascularization within the tumor due to a reduced capacity to induce VEGF expression by hypoxia (Carmeliet et al., 1998; Ryan et al., 1998; Tsuzuki et al., 2000). Similarly, genetic ablation of VEGF has been shown to cause a dramatic reduction in vascularity and growth of various tumors (Grunstein et al., 1999; Inoue et al., 2002; Tsuzuki et al., 2000).

The hypoxic response is dependent on the vascular microenvironment

What we did not expect was the radical change in tumor behavior when the site of implantation was changed from the commonly exploited subcutaneous site to a site much more reflective of the origin of these transformed cells, the intracranial space. In this microenvironment, HIF-1α-deficient tumors became more motile, moving along the perivascular spaces of normal blood vessels, and thereby infiltrating both hemispheres of the brain. Further, HIF-1α-deficient astrocytomas were better vascularized than the wild-type tumors, leading to a less necrotic phenotype in the brain. Nevertheless, the vessel density of HIFko tumors was lower than that of normal brain. It is important to note that it is a hallmark of most tumors that they exhibit a lower oxygen consumption rate, and therefore a lower vessel density, than the corresponding normal tissue, allowing tumor cells to accumulate between capillaries and thereby expanding the intercapillary distance (Eberhard et al., 2000; Hlatky et al., 2002). This also holds true for high-grade astrocytomas, because their microvessel density is lower than that of normal brain tissue (Eberhard et al., 2000).

Interestingly, low-grade astrocytomas first coopt existing brain vessels, and propagate along them; only when these tumors progress into grade IV astrocytomas/GBM do they become neovascularized. It is a peculiar feature of glioblastomas that they first regress the normal coopted blood vessels within the tumor mass before they form new tumor vessels; these are morphologically very distinct from the normal vasculature (Holash et al., 1999a, 1999b).

Blood vessel regression in astrocytomas is partly caused by Angiopoietin-2 (Ang2), a ligand of the receptor tyrosine kinase Tie-2, which is expressed in endothelial cells (Maisonpierre et al., 1997; Yancopoulos et al., 2000). Ang2 is induced early, when oxygen levels drop (Abdulmalek et al., 2001), and causes blood vessel regression in the absence of VEGF, but as soon as VEGF is induced, it will synergize with VEGF in new blood vessel formation (Hanahan, 1997). We have obtained preliminary data indicating that Ang2 levels are elevated in intracranial HIFwt, but not in HIFko astrocytomas (G.B. and Jocelyn Holash, Regeneron Inc., unpublished data). The high levels of Ang2 and the relatively low vessel density further support the notion that the vasculature of HIFwt tumors undergoes regression and remodeling as indicated by the appearance of more tortuous and distorted tumor blood vessels (Holash et al., 1999a, 1999b; Zagzag et al., 2000). In contrast, HIFko tumors express much lower levels of Ang 2 and consist of more densely packed elongated blood vessels, similar to vessels of normal brain, suggesting that these vessels have not undergone a transition to an angiogenic tumor vasculature.

Taken together, these observations suggest that HIF-1α-deficient astrocytomas are unable to induce angiogenesis, but that they adapt to this disadvantage by behaving like lower-grade astrocytomas; i.e., they take advantage of the highly vascularized environment, and migrate along existing normal blood vessels to propagate. Notably, HIFwt as well as HIFko astrocytomas were histopathologically both defined as grade III/IV or grade IV astrocytomas.

Based on these results, one would expect that VEGFko astrocytomas behave similarly to HIFko tumors, and exhibit increased invasive behavior in tumor cells, specifically around normal blood vessels in the brain parenchyma. Surprisingly,

VEGF-gene ablation in astrocytomas did not induce the phenotypes observed in HIFko astrocytomas when grown intracranially (summarized in Table 1). Whereas HIFko tumors grew faster than the wild-type tumors, VEGFko astrocytomas were at a growth disadvantage. This may be again attributed to their vascular phenotypes: in the brain, HIFko tumors were well vascularized, but VEGFko tumors were poorly vascularized. How can these discrepancies be explained? Loss of HIF-1α disables hypoxia-dependent VEGF induction, but we found that HIF-1α-deficient tumors still express VEGF, likely due to a combination of hypoxia-induced stabilization of VEGF mRNA, transcriptional induction by oncogenes such as H-ras, and VEGF expression of host cells within the tumor. The somewhat lower VEGF levels maybe sufficient to keep coopted brain vessels functional, but are not adequate to initiate angiogenesis. Moreover, other additional mechanisms, such as downregulation of angiogenic inhibitors like thrombospondin-1, might be necessary to induce the angiogenic switch (Watnick et al., 2003). The situation changes when VEGF is ablated in tumor cells: although tumors form nests around coopting blood vessels, those start to regress, which in turn keeps tumor cell proliferation limited. One may argue that the VEGF levels in intracranial VEGFko tumors, made by host cells, are not sufficient to support the existing vasculature of the growing tumor. The differing phenotypes of HIFko and VEGFko astrocytomas indicate that other factors than VEGF are likely to be involved in the adaptation of HIF-1α-deficient tumors to propagate without initiating angiogenesis. Importantly, the adaptation to become more invasive is also dependent on the microenvironment, suggesting an interaction between tumor cells and host factors in the extracellular matrix of the brain parenchyma.

Why do HIFko astrocytomas not coopt blood vessels in the subdermis, and become more invasive as they do in the brain? The subcutaneous environment is a very vessel-poor space that requires extensive recruitment and cooptation of vessels for tumor survival. In this context, loss of HIF-1α or VEGF, and subsequent loss of angiogenic capacity, is likely a negative factor, resulting in fewer vessels, less proliferation, and slower growth. This demonstrates that vessel recruitment and angiogenesis are parameters that affect tumor growth in highly variable ways. It is quite possible that the differing effects seen due to loss of HIF-1α in other laboratories are also tightly linked, not to the cell line involved, but to variations in the space in which transplanted tumors grow.

One could certainly argue that tumors deficient in HIF-1α may adapt other conduits to survive than the ones that we suggested above. Alternative paths by H-ras or p53-deficiency due to ectopic expression of SV40Tag can be likely excluded, because astrocytes were first transformed with these oncogenes before HIF-1α was deleted, leading to genetically identical cell lines, except for HIF-1α ablation. It is also unlikely that HIF-2α compensates for HIF-1α deficiency in the transformed astrocyte cells because we have analyzed HIF-2α protein levels in HIFwt and HIFko astrocytoma cell lines, and our preliminary data suggest that HIF-2α protein is present in our transformed astrocyte lines, but at low levels, and does not appear to be induced in the absence of HIF-1α (B.B., unpublished data).

Tumor-promoting effects of HIF-1α have been demonstrated by several groups (Maxwell et al., 1997; Ryan et al., 1998, 2000; Unruh et al., 2003). The role of HIF-1α as a potential "negative factor" in tumorigenesis has been implied by a smaller number of studies, although these have been exclusively embryonic stem cell-derived teratocarcinomas, injected subcutaneously (Carmeliet et al., 1998). Our study illustrates that the HIF-1α-mediated pathway can positively or negatively influence the fate and behavior of a tumor, such as an astrocytoma, but this influence is dependent upon the microenvironment. This is nicely demonstrated in the cell cycle regulation of HIFko astrocytomas. Loss of HIF-1α in astrocytomas in the brain revealed a higher proliferation index which is similar to the finding by Goda et al. that p53-deficient fibroblasts and B lymphocytes can be further pushed into cell cycle when HIF-1α is absent (Goda et al., 2003). The same transformed HIFko astrocytes, however, behave the opposite when transplanted into the subcutaneous space; i.e., they grow slower than the control astrocytomas, supporting the notion that the microenvironment is an additional crucial player in cell cycle regulation. Indeed, intracranial HIFko astrocytomas are not very hypoxic and necrotic and well vascularized, which is an environment that would favor tumor cell growth. In contrast, HIF-1α-deficient astrocytomas in the subcutaneous space are extremely hypoxic and necrotic and poorly vascularized, a harsh environment that limits tumor cell growth.

Implications for cancer therapy

Our observation of intracranial HIFko astrocytomas also has implications for therapy based on HIF-1α targeted inhibition. Our data suggest that inhibition of HIF-1α, thereby impairing the hypoxic response, might not be a desired strategy to treat astrocytomas, as they might adapt by becoming more invasive and infiltrative.

Interestingly, gene ablation of VEGF in astrocytomas exhibited a growth disadvantage both in the subcutaneous space and the brain, eliciting decreased proliferation rate and vessel density when compared to wild-type astrocytomas, arguing that regressing and impairing tumor vessels is a limiting step and a desired therapeutic approach for astrocytomas. This is in agreement with the findings that functional ablation of VEGF activity with a neutralizing antibody directed against VEGF resulted in vessel reduction, decreased tumor growth, and increased apoptosis in a rat model of glioblastomas (Rubenstein et al., 2000).

In summary, these results emphasize that the microenvironment has to be taken into consideration to better understand tumor behavior and adaptation in response to treatment modalities. Indeed, our findings are confirmed by several reports demonstrating that the microenvironment and tumor location functionally impacts tumor growth and metastasis. Many tumors have different behaviors when inoculated at sites different from their natural environment, most commonly in subcutaneous sites; they become encapsulated, lose their metastatic behavior, change their morphology, and, indeed, respond differently to treatment (Cuenca et al., 1996; MacDonald et al., 2001; Miyoshi et al., 2000; Taillandier et al., 2003).

Experimental procedures

Generation of *Hif1-α*^{WT} and *Hif1-α*$^{-/-}$, and *VEGFwt* and *VEGF*$^{-/-}$, transformed mouse astrocytes

Primary astrocytes were isolated from the hippocampus of 1- to 2-day-old HIF-1α$^{+/+f}$ (HIFloxP) or VEGF$^{+/+f}$ pups. HIF-1α$^{+/+}$ mice harbor flanked lox P sites of the second exon of the *Hif1-α* allele (Ryan et al., 2000), whereas VEGF$^{+/+f}$ mice contain flanked lox P sites within exon 3 of the VEGF gene

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(Gerber et al., 1999). Astrocytes of both mouse strains were isolated as follows: briefly, after removal of the meninges, the hippocampus was digested with papain (Worthington Biochemical Corporation, Lakewood, NJ) at a final concentration of 45 units/ml in a standard enzyme solution containing 50 mM EDTA, 150 mM $CaCl_2$, 100 mM L-cysteine, and 0.1 μg/ml Dnase in a solution consisting of 1 M HANKS, 10 mM HEPES (Invitrogen, Carlsbad, CA), and 7.5% $NaHCO_3$. The enzyme solution was aspirated and the digested tissue was washed in astrocyte tissue culture medium: BME supplemented with 10% FBS, 1% penicillin/streptromycin, 1% sodium pyruvate (all from Invitrogen), 0.6% glucose (Fisher, Los Angeles, CA), and 0.1% mito-serum extender (Becton Dickinson Biosciences, La Jolla, CA). Single-cell suspensions were cultured in the medium described and grown at 37°C in a humidified incubator containing 5% CO_2. After one week, primary astrocytes were purified by shaking the flasks on a rotator at RT at 300 rpm (Innova 2000, New Brunswick Scientific, Edison, NJ) and purity confirmed with an anti-GFAP antibody. Next, cells were immortalized by stable transfection with a SV-40 Large T antigen DNA construct. This was carried out by electroporation of pMC1neo Poly A (Stratagene, La Jolla, CA) and SV-40 Large T antigen using a BioRad Gene Pulser 2, set at 975 μF, ∞ resistance and 250 volts. Starting 48 hr posttransfection, stably transfected cells underwent G418 selection (300 μg/ml; Invitrogen). Resistant colonies were pooled and transformed by transfection of a retrovirus containing the H-Ras oncogene. Thereby, cells were plated in 6-well plates (Corning) at a density of 7×10^4 cells/well in BME and virus was added to the cells in combination with polybrene (Sigma, St. Louis, MO) at 4 μg/ml and medium. 48 hr postinfection, stably transfected cells were selected by Puromycin (Sigma) at a concentration of 1 μg/ml. After about 7 to 9 days, clones appeared, and they were pooled to obtain a heterogenous group of stably transformed cells, which were grown in the same medium, containing 5% FBS (Invitrogen). Next, the *Hif1*-α exon 2 was deleted by transfecting the cells transiently with an adenovirus expressing either cre recombinase or β-galactoside (MOCK). To determine the efficiency of *Hif1*-α deletion, genomic DNA was isolated from the cells and the recombination frequency was analyzed by TaqMan Real Time PCR analysis.

Growth curve and soft agar assay

For the growth curve, cells were plated in 12-well plates (Corning) in triplicates at a density of 1×10^5 cells/well in the described tissue culture medium for transformed astrocytes. Cells were exposed to normoxia 20% pO_2 and hypoxia 0.5% pO_2 for up to 96 hr. Cell counts were performed in duplicate at each time point using a hemacytometer. For the soft agar assay, cells were plated in triplicates in a 6-well plate at 5×10^5 cells/well, mixed in 0.35% LMP agarose (Invitrogen, Carlsbad, CA). After culturing the cells for 21 days, the wells were stained with 0.005% crystal violet for 4 hr and analyzed for colony number and size.

Real-time PCR assay

Cells were grown in 10 cm plates (Falcon) at 7×10^6 cells/plate and under normoxic and hypoxic conditions. After 8 hr incubation, total RNA was isolated using a Qiagen RNeasy kit according to the manufacturer's instructions. From 1 μg of total RNA, first-strand cDNA was synthesized using SUPERSCRIPT First-Strand (Invitrogen) according to the instructions of the manufacturer. For real-time PCR detection, 5 ng of input cDNA was analyzed in triplicate per primer pair per sample and the corresponding threshold cycle (Ct) values expressed as the mean ± s.e.m. All reactions were performed using 2× Taq Master Mix (Perkin Elmer Applied Biosystems), 900 nM of the forward and the reverse PCR primers, and 250 nM of a fluorescently tagged primer pair-specific probe in a total volume of 25 μl using default cycling parameters on an ABI Prism 7200 Sequence Detector. The following primer and probe sequences were used:

PGK-1: (F) 5′-CAGGACCATTCCAAACAATCTG-3′; (R) 5′-CTGTGGTACTGAGAGCAGCAAGA-3′; (probe) 5′-(6FAM)TAGCTCGACCCACAGCCCTCGGCATAT-(TAMRA)-3′.

Glut-1: (F) 5′-ACGAGGAGCACCGTGAAGAT-3′; (R) 5′-GGGCATGTGCTTCCAGTATGT-3′; (probe) 5′-(6FAM) CAACTGTGCGGCCCCTACGTCTTC-(BHQ)-3′.

VEGF total: (F) 5′- ATCCGCATGATCTGCATGG-3′; (R) 5′-AGTCCCATGAAGTGATCAAGTTCA-3′; (probe) 5′- (6-FAM) TGCCCACGTCAGAGAGCAACATCAC-(BHQ)-3′.

Subcutaneous implantation of *HIFwt* and *HIF*$^{-/-}$, and *VEGFwt* and *VEGF*$^{-/-}$ transformed astrocytes

22 athymic mice (4–6 weeks of age; Simonsen laboratory, San Jose, CA) were implanted subcutaneously with either 100 μl of 1×10^7 HIFwt, or 8 athymic mice with 1×10^7 VEGFwt transformed astrocytes. 21 athymic mice were injected subcutaneously with 100 μl of 1×10^7 HIFko or 8 mice with 1×10^7 VEGFko transformed astrocytes. 21 days postimplantation, tumors were harvested, weighed, and processed as described below for immunohistochemical analyses. All implantation experiments were repeated up to two times.

Intracranial implantation of HIFwt and HIF$^{-/-}$, and *VEGFwt* and *VEGF*$^{-/-}$ transformed astrocytes

35 athymic mice (4–6 weeks of age; Simonsen laboratory, San Jose, CA) were implanted intracranially with either 2.5 μl of 0.7×10^6 HIFwt or with 2.5 μl of 0.7×10^6 HIFko-transformed astrocytes. Similarly, six mice were injected with VEGFwt astrocytomas and eight mice with VEGFko-transformed astrocytes. Transplantation site was exactly 2 mm deep into the brain parenchyma and 3 mm to the right of the midline behind the breegma using a Hamilton syringe. All the mice were sacrificed between 15 to 21 days postimplantation when they showed side effects of tumor expansion such as lateral recumbency and weight loss. Mice were anesthetized, heart-perfused with 4% paraformaldehyde (PFA), and then brains either embedded into paraffin or frozen into OCT medium. All implantation experiments were repeated up to three times.

Visualization of the vasculature

To visualize blood vessels in tumors and normal tissue, mice were first anesthetized and injected (i.v.) with 0.05 mg FITC-labeled tomato lectin (*lycopersicon esculentum*; Vector Laboratories, Burlingame, CA) and then heart-perfused with 4% paraformaldehyde (PFA). Brains were frozen in OCT, sectioned at 50 μm, and appropriate sections mounted.

Detection of hypoxic areas in tumors

To allow assessment of the hypoxic regions within tumors, mice were injected i.p. with 60 mg/kg (in w/v PBS) pimonidazole (Hydroxyprobe-1TM, Natural Pharmacia International Inc.), 1.5 hr prior to sacrifice. Tumors were resected, processed, and embedded into paraffin. 5 μm sections were treated with 0.01% pronase for 40 min. at RT, washed, and incubated with Hydroxyprobe-1 mouse monoclonal antibody 1(MAb1; Natural Pharmacia International Inc) at a 1:50 dilution for 40 min at RT. A secondary biotinylated goat-anti mouse IgG antibody was applied, and staining visualized using the DAB chromophore (Vector ABC; DAB). Sections were rinsed and counterstained with hematoxylin.

Immunohistochemical analysis

HIFwt or HIFko astrocytoma cells in the brain were identified with a rabbit anti-SV40 Tag antibody. Briefly, slides were deparaffinized and incubated with the anti-Tag antibody (1:500; gift from Douglas Hanahan at UCSF) followed by treatment with a biotinylated goat anti-rabbit IgG antibody (1:200; Vector laboratories, Burlingame, CA). Antibody reactions were revealed with an ABC kit, using the chromophore 3,3-diaminobenzidine (DAB) substrate. For fluorescent detection, frozen tumor sections were stained with Tag antibodies and tumor cells visualized with a CY3-labeled goat anti rabbit IgG antibody (1:200; Jackson Immuno Research Laboratories, Inc).

In order to visualize tumor cells and the vasculature simultaneously, fluorescent immunohistochemistry was performed with the Tag antibody on brain cryosections of mice whose vascular system had been perfused FITC-Lectin prior to euthanasia.

Apoptotic index was assessed by TUNEL staining (Naik et al., 1996), and proliferating cells were detected with a rat anti-mouse Ki-67 antibody (1:100; DAKO Corporation, Carpinteria, CA) followed by incubation with a biotinylated rabbit-anti rat IgG antibody (1:200; Vector laboratories, Burlingame, CA) and DAB treatment as described above.

VEGF ELISA

HIFwt and HIFko transformed astrocyte cell lines were cultured under normoxia (20% pO_2) and incubated for 24 hr under hypoxic conditions (10% pCO_2; 0.5% pO_2). Conditioned medium was removed and VEGF levels were assayed by a sandwich DuoSet ELISA kit (DY493, R&D Systems, San Diego,

California), according to the manufacturer's instructions. Each assay condition was tested in triplicates.

Statistical analysis

The number of animals was calculated with support from the biostatistician Dr. Alex McMillan at UCSF. Statistical analyses were performed with a two-tailed, unpaired Mann-Whitney test. Error bars indicate standard deviations except for the quantitative RT-PCR analyses, which show SEM. P values less than 0.05 are considered statistically significant.

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References

Abdulmalek, K., Ashur, F., Ezer, N., Ye, F., Magder, S., and Hussain, S.N. (2001). Differential expression of Tie-2 receptors and angiopoietins in response to in vivo hypoxia in rats. Am. J. Physiol. Lung Cell. Mol. Physiol. *281*, L582–L590.

Brat, D.J., Castellano-Sanchez, A., Kaur, B., and Van Meir, E.G. (2002). Genetic and biologic progression in astrocytomas and their relation to angiogenic dysregulation. Adv. Anat. Pathol. *9*, 24–36.

Brat, D.J., Kaur, B., and Van Meir, E.G. (2003). Genetic modulation of hypoxia induced gene expression and angiogenesis: relevance to brain tumors. Front. Biosci. *8*, D100–D116.

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.

Chaudhry, I.H., O'Donovan, D.G., Brenchley, P.E., Reid, H., and Roberts, I.S. (2001). Vascular endothelial growth factor expression correlates with tumour grade and vascularity in gliomas. Histopathology *39*, 409–415.

Cuenca, R.E., Takita, H., and Bankert, R. (1996). Orthotopic engraftment of human lung tumours in SCID mice for the study of metastasis. Surg. Oncol. *5*, 85–91.

Ding, H., Roncari, L., Shannon, P., Wu, X., Lau, N., Karaskova, J., Gutmann, D.H., Squire, J.A., Nagy, A., and Guha, A. (2001). Astrocyte-specific expression of activated p21-ras results in malignant astrocytoma formation in a transgenic mouse model of human gliomas. Cancer Res. *61*, 3826–3836.

Eberhard, A., Kahlert, S., Goede, V., Hemmerlein, B., Plate, K.H., and Augustin, H.G. (2000). Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res. *60*, 1388–1393.

Feldkamp, M.M., Lau, N., Rak, J., Kerbel, R.S., and Guha, A. (1999). Normoxic and hypoxic regulation of vascular endothelial growth factor (VEGF) by astrocytoma cells is mediated by Ras. Int. J. Cancer *81*, 118–124.

Ferrara, N., Gerber, H.P., and LeCouter, J. (2003). The biology of VEGF and its receptors. Nat. Med. *9*, 669–676.

Folkman, J. (2000). Tumor angiogenesis. In Cancer Medicine, Robert C. Bast, Jr., ed. (Hamilton, Ontario: BC Decker).

Gerber, H.P., Hillan, K.J., Ryan, A.M., Kowalski, J., Keller, G.A., Rangell, L., Wright, B.D., Radtke, F., Aguet, M., and Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. Development *126*, 1149–1159.

Goda, N., Ryan, H.E., Khadivi, B., McNulty, W., Rickert, R.C., and Johnson, R.S. (2003). Hypoxia-inducible factor 1α is essential for cell cycle arrest during hypoxia. Mol. Cell. Biol. *23*, 359–369.

Grunstein, J., Roberts, W.G., Mathieu-Costello, O., Hanahan, D., and Johnson, R.S. (1999). Tumor-derived expression of vascular endothelial growth factor is a critical factor in tumor expansion and vascular function. Cancer Res. *59*, 1592–1598.

Guha, A., Feldkamp, M.M., Lau, N., Boss, G., and Pawson, A. (1997). Proliferation of human malignant astrocytomas is dependent on Ras activation. Oncogene *15*, 2755–2765.

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. Science *277*, 48–50.

Hlatky, L., Hahnfeldt, P., and Folkman, J. (2002). Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J. Natl. Cancer Inst. *94*, 883–893.

Holash, J., Maisonpierre, P.C., Compton, D., Boland, P., Alexander, C.R., Zagzag, D., Yancopoulos, G.D., and Wiegand, S.J. (1999a). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science *284*, 1994–1998.

Holash, J., Wiegand, S.J., and Yancopoulos, G.D. (1999b). New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene *18*, 5356–5362.

Holland, E.C. (2000). Glioblastoma multiforme: the terminator. Proc. Natl. Acad. Sci. USA *97*, 6242–6244.

Huang, P., Allam, A., Taghian, A., Freeman, J., Duffy, M., and Suit, H.D. (1995). Growth and metastatic behavior of five human glioblastomas compared with nine other histological types of human tumor xenografts in SCID mice. J. Neurosurg. *83*, 308–315.

Inoue, M., Hager, J.H., Ferrara, N., Gerber, H.P., and Hanahan, D. (2002). VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer Cell *1*, 193–202.

Louis, D.N., Holland, E.C., and Cairncross, J.G. (2001). Glioma classification: a molecular reappraisal. Am. J. Pathol. *159*, 779–786.

MacDonald, T.J., Taga, T., Shimada, H., Tabrizi, P., Zlokovic, B.V., Cheresh, D.A., and Laug, W.E. (2001). Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. Neurosurgery *48*, 151–157.

Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N., et al. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science *277*, 55–60.

Maxwell, P.H., Dachs, G.U., Gleadle, J.M., Nicholls, L.G., Harris, A.L., Stratford, I.J., Hankinson, O., Pugh, C.W., and Ratcliffe, P.J. (1997). Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc. Natl. Acad. Sci. USA *94*, 8104–8109.

Miyoshi, T., Kondo, K., Ishikura, H., Kinoshita, H., Matsumori, Y., and Monden, Y. (2000). SCID mouse lymphogenous metastatic model of human lung cancer constructed using orthotopic inoculation of cancer cells. Anticancer Res. *20*, 161–163.

Naik, P., Karrim, J., and Hanahan, D. (1996). The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to progression from angiogenic progenitors. Genes Dev. *10*, 2105–2116.

Ozawa, T., Wang, J., Hu, L.J., Lamborn, K.R., Bollen, A.W., and Deen, D.F. (1998). Characterization of human glioblastoma xenograft growth in athymic mice. In Vivo *12*, 369–374.

Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L., et al. (1999). Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody

inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res. *59*, 5209–5218.

Pugh, C.W., and Ratcliffe, P.J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. Nat. Med. *9*, 677–684.

Rubenstein, J.L., Kim, J., Ozawa, T., Zhang, M., Westphal, M., Deen, D.F., and Shuman, M.A. (2000). Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption. Neoplasia *2*, 306–314.

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.

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

Semenza, G.L. (2002b). Involvement of hypoxia-inducible factor 1 in human cancer. Intern. Med. *41*, 79–83.

Taillandier, L., Antunes, L., and Angioi-Duprez, K.S. (2003). Models for neuro-oncological preclinical studies: solid orthotopic and heterotopic grafts of human gliomas into nude mice. J. Neurosci. Methods *125*, 147–157.

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.

Unruh, A., Ressel, A., Mohamed, H.G., Johnson, R.S., Nadrowitz, R., Richter, E., Katschinski, D.M., and Wenger, R.H. (2003). The hypoxia-inducible factor-1α is a negative factor for tumor therapy. Oncogene *22*, 3213–3220.

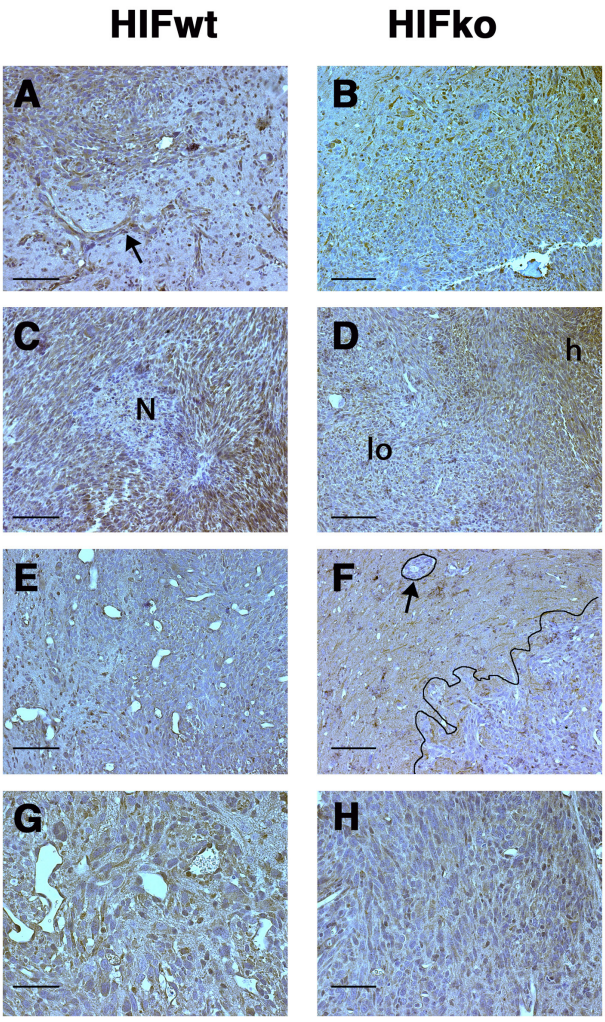
Watnick, R.S., Cheng, Y.N., Rangarajan, A., Ince, T.A., and Weinberg, R.A. (2003). Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis. Cancer Cell *3*, 219–231.

Xiao, A., Wu, H., Pandolfi, P.P., Louis, D.N., and Van Dyke, T. (2002). Astrocyte inactivation of the pRb pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN mutation. Cancer Cell *1*, 157–168.

Yancopoulos, G.D., Davis, S., Gale, N.W., Rudge, J.S., Wiegand, S.J., and Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. Nature *407*, 242–248.

Zagzag, D., Zhong, H., Scalzitti, J.M., Laughner, E., Simons, J.W., and Semenza, G.L. (2000). Expression of hypoxia-inducible factor 1α in brain tumors: association with angiogenesis, invasion, and progression. Cancer *88*, 2606–2618.

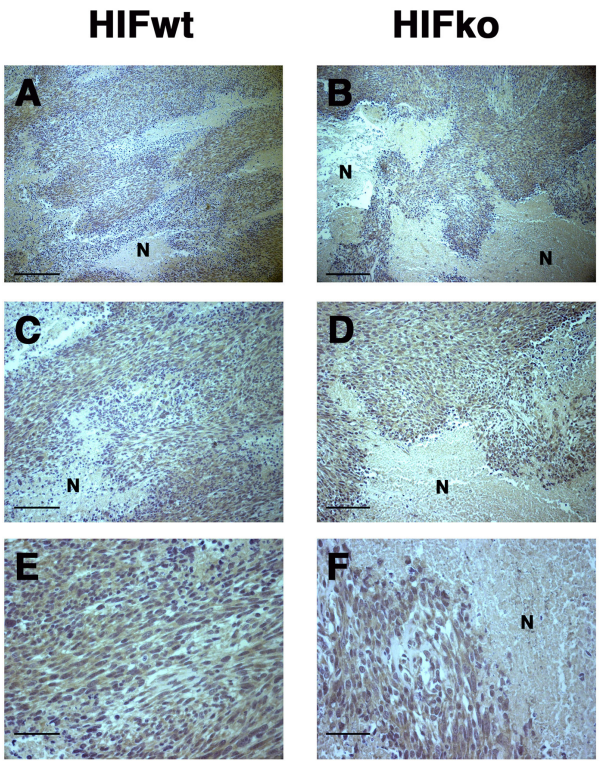
Supplemental data for Blouw et al., Cancer Cell *4*, pp. 133–146



Supplemental Figure S1: VEGF protein expression analysis of intracranial HIFwt and HIFko astrocytomas

Briefly, paraffin sections were deparaffinized, microwaved in citrate buffer (Antigen retrieval), incubated with a rabbit polyclonal anti-VEGF antibody (Calbiochem, San Diego), and subsequently applied with a secondary biotinylated goat-anti rabbit IgG Ab (Vector laboratories, Burlingame, CA). VEGF staining was visualized with a DAB chromophore (Vector laboratories) and sections counterstained with Hematoxylin.
Substantial heterogeneity in VEGF levels was observed in HIFwt and in HIFko astrocytomas but appeared to be somewhat higher in HIFwt tumors. **A**, **C**, and **E** represent different fields of HIF wt astrocytomas expressing high (**A** and **C**) as well as low levels of VEGF (**E**) whereas **B**, **D**, and **F** illustrate areas of HIFko tumors with varying VEGF levels (bar = 45 μm). HIFwt tumors appeared to express high levels of VEGF at some tumor borders (**A**), around co-opted blood vessels (**A**) and around necrotic areas (**C**; N = necrosis), but could also contain low levels of VEGF as illustrated in **E**. In addition, VEGF levels varied in different areas of the same tumor (**A** and **E**).
HIFko tumors exhibited the same variation in VEGF protein concentrations ranging from very low levels (**F**; line shows the tumor border) to substantial VEGF levels (**D**). Notably, HIFko astrocytomas did not appear to express detectable VEGF levels in various co-opted blood vessel areas in the brain (**F**, circle and arrow) in contrast to HIFwt astrocytomas (**A**, arrow). Further, VEGF was also identified in host cells (probably reactive astrocytes, neurons, etc.) specifically in the surrounding brain parenchyma of both HIFwt (**A** and **E**) and HIFko (**B** and **F**) astrocytomas. In both tumor types, VEGF expression intensity varied in different areas of the same tumor (HIFwt, compare **A** with **E**; HIFko, **D**: lo = low VEGF levels; h = high VEGF levels). High magnification images identified only a subset of tumor cells that express VEGF (**G**, **H**, bar = 22.5 μm).

SUPPLEMENTAL DATA



Supplemental Figure S2: VEGF protein expression analysis of subcutaneous HIFwt and HIFko astrocytomas

VEGF immunohistochemistry was performed as described in Supplemental Figure S1. HIFwt as well as HIFko astrocytomas demonstrated substantial VEGF levels around necrotic areas (N) but varied in VEGF intensity in different areas of the same tumor (**A** and **B**, and **E** and **F**). Three different magnifications were taken to illustrate the VEGF expression profile and the heterogeneous distribution of VEGF (**A** and **B**, bar = 90 μm; **C** and **D**, bar= 45 μm; **E** and **F**, bar = 22.5 μm).

CHAPTER 3

Loss of VEGF expression reduces vascularization, but not growth, of tumors lacking the Von Hippel-Lindau tumor suppressor gene

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ABSTRACT

Individuals bearing germ line mutations in the Von Hippel Lindau tumor suppressor gene are predisposed to the development of highly angiogenic tumors. This is correlated with an increased expression of the angiogenic factor VEGF in these tumors, which is in part caused by elevated expression of the HIF-1 hypoxia inducible transcription factors. We created malignant astrocytes with genetic deletions of the VHL gene and implanted them in subcutaneous and intracranial sites, of vessel poor and vessel rich sites, respectively. When grown in a vessel poor site, VEGF expression in VHL null cells was important for both vascularization and tumor growth. However, when the same cells are grown in the vessel rich intracranial environment, loss of VEGF expression reduces vascularity, but does not affect tumor growth. This indicates that anti-angiogenic therapies for tumors that express high levels of angiogenic factors such as VEGF may vary in their efficacy, with potentially lowered effectiveness in sites such as the brain that inherently vessel rich.

Keywords: HIF, VEGF, angiogenesis, hypoxia, astrocytoma

INTRODUCTION

Mutations in the von Hippel Lindau tumor suppressor gene give rise to tumors in a variety of organs including the kidney and the central nervous system. These tumors are a result of inactivation of the WT allele in individuals that are heterozygous for a germ line mutation in *VHL*. pVHL regulates the hypoxia inducible transcription factors HIF-1α and HIF-2α by acting as an E3 ubiquitin ligase, targeting both proteins for degradation under normal oxygen conditions. Hence, inactivation of pVHL leads to upregulation of both HIF-1α, HIF-2α and the transcription of their target genes. (reviewed by Kaelin 2002). It remains unknown how exactly loss of pVHL promotes tumor growth. Studies have shown that inactivation of pVHL did not increase tumor formation *in vivo* (Mack *et al.*, 2003; Mack *et al.*, 2005).

In order to develop effective therapy to treat VHL disease, it is important to identify the contribution of HIF-1α and VEGF to the angiogenesis and growth of tumors lacking pVHL. Angiogenesis is a result of the balance between pro-angiogenic and anti-angiogenic factors which are released by the tumor and surrounding cells and is therefore highly dependent on the microenvironment (Bergers & Benjamin, 2003). One of the limitations of the subcutaneous tumor implantation model is that the interaction between the malignant cells and the natural stromal microenvironment cannot be recapitulated adequately which may explain the lack of the drastic vascular phenotype associated with loss of pVHL in this model. We addressed this obstacle by analyzing different parameters associated with tumor angiogenesis in a murine astrocytoma implantation model in which transformed astrocytes containing deletions in respectively, pVHL alone (VHL KO) or in combination with HIF-1α (HIF-1α/VHL KO) or VEGF (VEGF/VHL KO) were implanted subcutaneously and in the brain of immunocompromised mice. This allows comparisons of tumor growth derived from the same cell line in different micro-environments, and was shown to successfully recapitulate the relevant host-tumor cell interactions during tumorigenesis in the orthotopic setting (Blouw *et al.*, 2003). We report that the VHL KO tumors grow independently of HIF-1α, but its contribution to tumor vascularization depends on the microenvironment in which the cells are grown. Loss of VEGF in the VHL KO background causes a striking reduction in tumor vascularity and angiogenesis, but this only correlates with a reduction in tumor growth when these cells are grown subcutaneously and not in the brain. Our data indicates that VEGF is a critical factor for tumor angiogenesis, but not for tumor growth associated with loss of VHL.

MATERIALS AND METHODS

Generation of VHL KO, HIF-1α/VHL KO and VEGF/VHL KO transformed astrocytes

Primary astrocytes were isolated from 1-2 day old pups that were homozygous for either $VHL^{+f/+f}$ alone (Haase *et al.*, 2001), or in combination with $HIF^{+f/+f}$ (Ryan *et al.*, 1998) or $VEGF^{+f/+f}$ (Gerber *et al.*, 1999). From these cells, stably transformed cell lines were created that lacked the floxed alleles as described in Blouw *et al.* 2003. The WT or null status was confirmed as described in Tang *et al.*, 2006. HIF-1α expression and target gene up regulation was carried out according to Seagroves *et al.*, 2003.

Tissue Culture

Cells were cultured in DMEM supplemented with 10% FBS, 1% Pen/Strep, 1% Sodium Pyruvate and 1% Hepes (all Invitrogen, Carlsbad, CA) in a standard humidified incubator with pO_2 20% and CO_2 5%. For the hypoxia treatment cells were transferred to a hypoxia chamber (Sanyo, Bensenville, IL) and cultured for 6 hr at pO_2 0.5%.

Tumor Implantation experiments

All tumor implantation experiments were performed on nu/nu mice at 4 to 6 weeks of age (Simonsen Laboratory, San Jose, CA). For the subcutaneous implantation, 3×10^6 cells in 100 µl PBS were grown in the flank of the mice (10 -13 mice per group) for 21 days. The intracranial implantation, was carried according to Blouw *et al.*, 2003. RNA was extracted from 3-5 tumors per group and VEGF expression was tested according to Tang *et al.*, 2004.

Histological analysis

Sections were stained with Anti-Rat CD31 primary antibody (Pharmigen BD Biosciences, San Jose, CA) followed by a FITC conjugated Anti-Rat secondary antibody (Pierce, Rockford, IL). Vessel density was calculated using ImageJ software analysis of photographs taken with the 20X objective of 5 randomly chosen fields from 7 tumors per group and expressed as number of vessels per mm^2. Endothelial cell proliferation was visualized and calculated as described in Tang *et al.*, 2004. Sections were stained for CD31 as is described above and with Anti-Rabbit Active Caspase 3 primary antibody (R&D Systems, Minneapolis, MN) followed by Alexa Fluor 555 secondary Anti-Rabbit (Invitrogen, Carlsbad, California). Vessel apoptosis was determined by calculating the ratio of vessels that stained for both CD31 and Caspase 3 per total number of vessels per mm^2 from pictures taken with the 20X objective of 5 randomly chosen fields from 7 tumors per group. Sections were stained with goat anti-mouse VEGF

(R&D Systems, Minneapolis, MN) followed by Texas Red conjugated anti-mouse secondary antibody (Invitrogen, Carlsbad, California), with rabbit α-fibronectin, and rabbit α-laminin (both Sigma, St. Louis, MO) respectively. Both were followed by a secondary antibody biotinylated anti-rabbit (Vectorlabs, Burlingame, CA). The magnifications of the photographs of the histology are indicated in each figure.

Statistical Analysis

Results are expressed as mean values with 95% confidence interval. Unless otherwise noted, the statistical significance of differences between experimental and control groups was determined by a two-tailed unpaired Student's *t* test. P-values less or equal to 0.05 are considered statistically significant.

RESULTS

The subcutaneous tumor growth and vascularity of VHL KO astrocytes is independent of HIF-1α

Transformed astrocytes lacking pVHL alone or in combination with HIF-1α were created via the transformation of primary astrocytes isolated from from transgenic mice homozygous for specific exons flanked by LoxP sites in the VHL and HIF-1α gene (VHL^{f+f} and $HIF\text{-}1\alpha^{f+f}/VHL^{f+f}$), as described in Materials and Methods. Taq Man Real Time PCR analysis showed over 95% deletion efficiency of the exons (supplemental figure 1A). Functional loss of VHL alone or in combination with HIF-1α was confirmed by a HIF-1α western blot and by the expression of its target genes PGK and Glut-1 of cells growing at normoxia and hypoxia (S1 B-C).

To explore the effects of HIF-1α deletion in the VHL KO background on subcutaneous tumor growth, the VHL KO, HIF-1α /VHL KO and their WT control astrocytes were grown in the flanks of immunocompromised mice as described in Materials and Methods. As is shown in figure 1A loss of HIF-1α in the VHL background reduced tumor mass, although just to the border of significance ($p = 0.06$). Hematoxylin & Eosin analysis of these tumors revealed lack of hemangiomas (1B). Furthermore, the size and number of necrotic regions was similar and all the tumor types formed well defined borders and did not metastasize to other organs (not shown). The vessel density was analyzed as is described in Materials and Methods. As shown in figure 1C both the VHL KO and HIF-1α /VHL KO tumors displayed a 2.6 fold increase in vessel density compared to WT. Additional characteristics of increased angiogenesis or vascular lesions associated with VHL disease were not detected in the subcutaneous tumors.

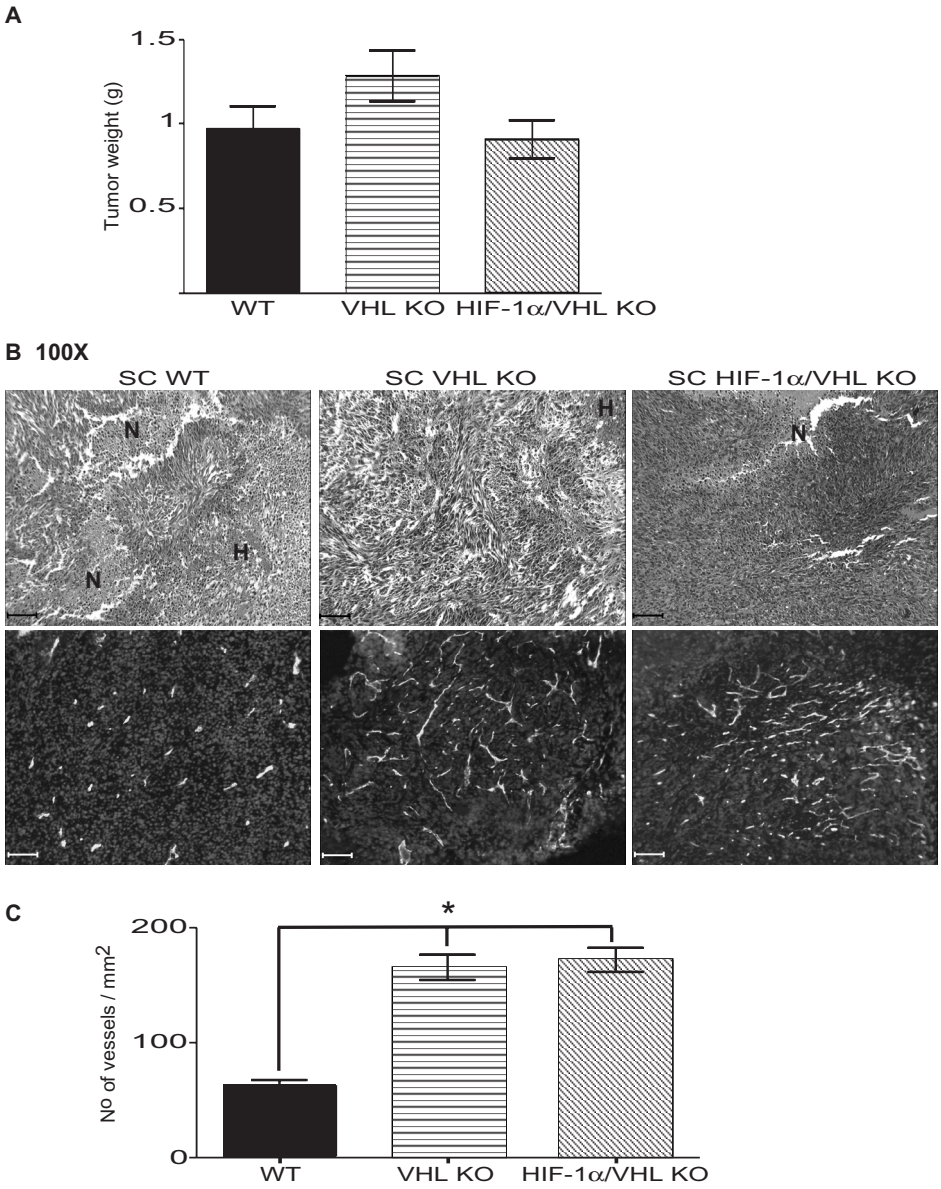

Figure 1. The vascularity in subcutaneously growing VHL KO astrocytes is independent of HIF-1α A. VHL KO and HIF-1α/VHL KO transformed astrocytes were grown in the flank of mice as described in Materials and Methods. *Columns* represent the average tumor weights (n=13 mice per group); *error bars* represent 95% confidence intervals. B. Top panel: H&E stains of subcutaneously grown tumors of VHL KO and HIF-1α/VHL KO astrocytes. N = necrosis; H = hemorrhage. Lower panel: Blood vessels were visualized by immunofluorescence for CD31 (green) and counterstained for DAPI (blue). Magnifications are indicated; scale bar is 100 μm. C. Bar graph of vessel density. Calculations were performed as is described in Materials and Methods. *Columns*, mean; *error bars* represent the 95% confidence intervals ($p < 0.05$).

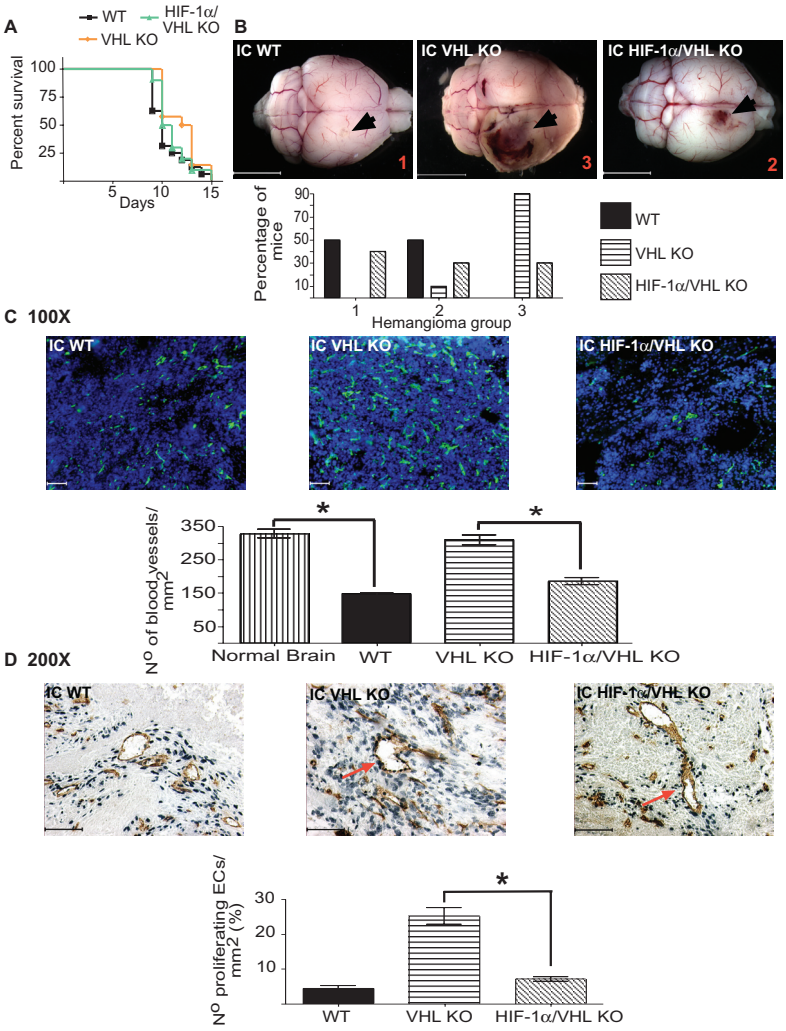

Figure 2. VHL KO astrocytes form a highly vascular tumor in HIF-1α dependent fashion when grown in the brain
A. Survival curves of mice in which the VHL KO and HIF-1α/VHL KO astrocytes were grown as brain tumors; n=13 per group. B. Top panels: Photographs of the brains implanted with the astrocytes upon dissection. Black arrow indicates the injection sites. Scale bars = 5 mm. Lower panel: Incidence of hemangiomas in the brain tumors. *Columns*, percentage of mice without a hemangioma (group 1), with a hemangioma that takes up a narrow area around the injection site (group 2) and with a large hemangioma that take up at least 30-50% of the hemisphere in which the cells were implanted (group 3). Red numbers in upper panels indicate the group numbers. Each photograph shown is representable for the entire group (n= 13). C. Upper panels: Blood vessels were visualized by immunofluorescence as described earlier. Magnifications are indicated, scale bars = 100 µm. Lower panel: Bar graph of vessel density. Calculations were performed as is described earlier. *Columns*, mean; *error bars*, 95% confidence intervals. ($p < 0.05$). D. Upper panels: Endothelial cell proliferation was visualized by a double stain using CD31 (developed with DAB-brown) and Brdu (Vector SG-blue). Proliferating vessels stain double for Brdu and CD31. Magnifications are indicated; scale bar = 100 µm. Compared to the HIF-1α/VHL KO brain tumors, the VHL KO astrocytoma displayed vessels with multiple Brdu positive nuclei (compare arrows in center and right panel). Lower panel: Bar graph of the endothelial cell proliferation. Calculations were done as described in Materials and Methods. *Columns*, mean; *error bars*, 95% confidence intervals ($p < 0.05$).

VHL null transformed astrocytes implanted in the brain form highly vascular tumors in a HIF-1α dependent manner

The lack of additional vascular lesions associated with loss of VHL in the subcutaneous tumor model made it difficult to establish the contribution of HIF-1α to the angiogenesis in tumors lacking VHL. Therefore, the cells were also implanted in the brain, which is their natural habitat and recapitulates the interactions between the tumor cells and natural environment, which are important for angiogenesis. There was no significant difference in the survival of the mice between the VHL KO and HIF-1α/VHL KO tumor groups (figure 2A, $p = 0.32$). It was therefore surprising that the brain tumors derived from the VHL KO cells, displayed a striking HIF-1α dependent increase in vascularity (fig 2B-D). Loss of VHL caused large hemangiomas in 90% of the brain tumors, which was reduced to 35% upon deletion of HIF-1α in this background ($p=0.01$ by χ^2 test; figure 2B). Compared to the VHL KO, the vessel density was a 41% reduced, in the HIF-1α /VHL KO brain tumors (figure 2C lower panel). Similarly, loss of VHL in the brain tumors causes a 25% increase in the percentage of proliferating endothelial cells compared to the wild type controls, which was reduced to 7% upon loss of HIF-1α in this background (2D, lower panel). Furthermore, vessels of the VHL KO brain tumors contained multiple nuclei that were positive for Brdu (black arrow shown in upper center panel, 2D), indicating vessel hyperplasia which is associated with the vascular lesions in VHL disease (Hasselblatt *et al.*, 2005). This aspect of angiogenesis was also dependent on HIF-1α, as vessel hyperplasia was absent in the HIF-1α /VHL KO brain tumors (compare vessels indicated by black arrows in center –VHL KO and right- HIF-1α/VHL KO panels, 2D).

Vessel apoptosis is HIF-1α dependent in VHL KO cells grown in the brain

It was reported that the increase in vascularity of subcutaneously growing tumors lacking pVHL was correlated with dysregulation of fibronectin assembly (Mack *et al.*, 2003; Mack *et al.*, 2005; Rathmell *et al.*, 2004). In an attempt to explain the site-specific contribution of HIF-1α to the angiogenesis of the VHL KO tumors, the cells and tumors were analyzed for fibronectin expression and assembly. Fibronectin was not expressed *in vitro* in the transformed astrocytes (not shown). Immunohistochemical analysis showed reactivity of αfibronectin with blood vessels (Supplemental figure 2A and B), but did not reveal any difference in the expression level or assembly between the different tumor types grown in the different microenvironments. Similar data was found for laminin, another molecule involved in vessel maturation (S2 C-D).

Because angiogenesis is a result of the balance between endothelial cell proliferation and apoptosis (Bergers & Benjamin, 2003) we next analyzed vessel apoptosis in the tumors as described in Materials and Methods. In the subcutaneous tumors, only a small percentage of the vessels were apoptotic (6% for the WT, and 3% for the HIF-1α /VHL KO tumors, figure

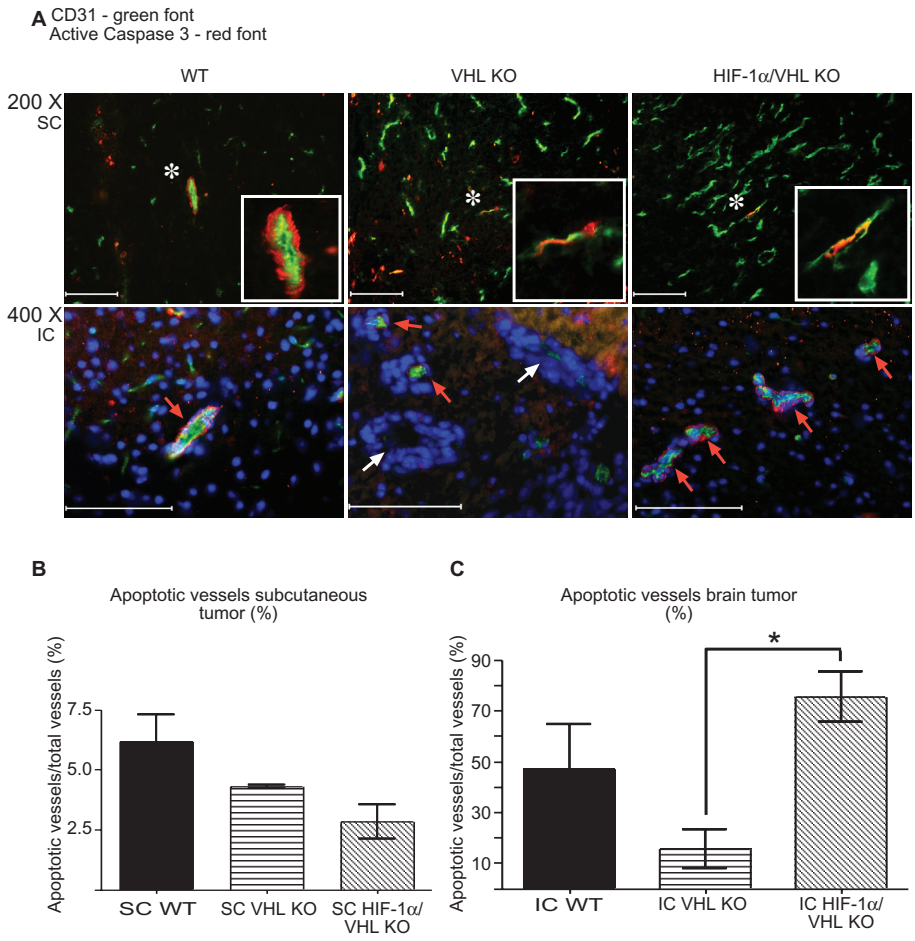

Figure 3. Vessel apoptosis is HIF-1α dependent in VHL KO astrocytes grown in the brain. A. Vessel apoptosis was tested using a double stain for CD31 (green) and for Active Caspase 3 (red). Apoptotic vessels stain double for both these markers, as shown by the insets of the vessels indicated by the asterixes in the upper panels (inset is 600X magnification). Lower panels represent the double stain in the brain tumors, where also a background stain with DAPI was performed to indicate tumor cells co-opting the blood vessels. Red arrows point to co-opted vessels that are apoptotic, white arrows point to those that are not apoptotic. Magnifications are indicated; scale bar = 100 μm. B-C Bar graphs showing the rate of apoptotic vessels in respectively the subcutaneous tumors (B) and the brain tumors (C). Apoptotic rate was determined as is described in Materials and Methods. *Columns*, mean; *error bars*, 95% confidence intervals ($p < 0.05$).

3A&B). In contrast, the percentage of apoptotic blood vessels was significantly increased in the brain tumors. This was particularly the case in the vessels of the HIF-1α /VHL KO brain tumors that were co-opted by tumor cells, indicated by red arrows in lower panels of fig 3A. Compared to the VHL KO tumors, the apoptotic rate of the vessels in the HIF-1α /VHL KO tumors was almost a 5- fold increased (figure 3C). These data suggest that the reduced vascularity observed in the HIF-1α/VHL KO brain tumors results from increased blood vessel apoptosis due to loss of HIF-1α.

Expression of VEGF in VHL KO transformed astrocytes is dependent on HIF-1α

It has been shown by many studies that that tumor cell-secreted VEGF is a critical factor for vascular function (Grunstein *et al.*, 1999), and that loss of VEGF in tumors derived from a glioma cell line results in an increase in vessel apoptosis (Benjamin & Keshet, 1997). Given the difference in vessel apoptosis between the HIF-1α /VHL KO tumors grown in the brain and

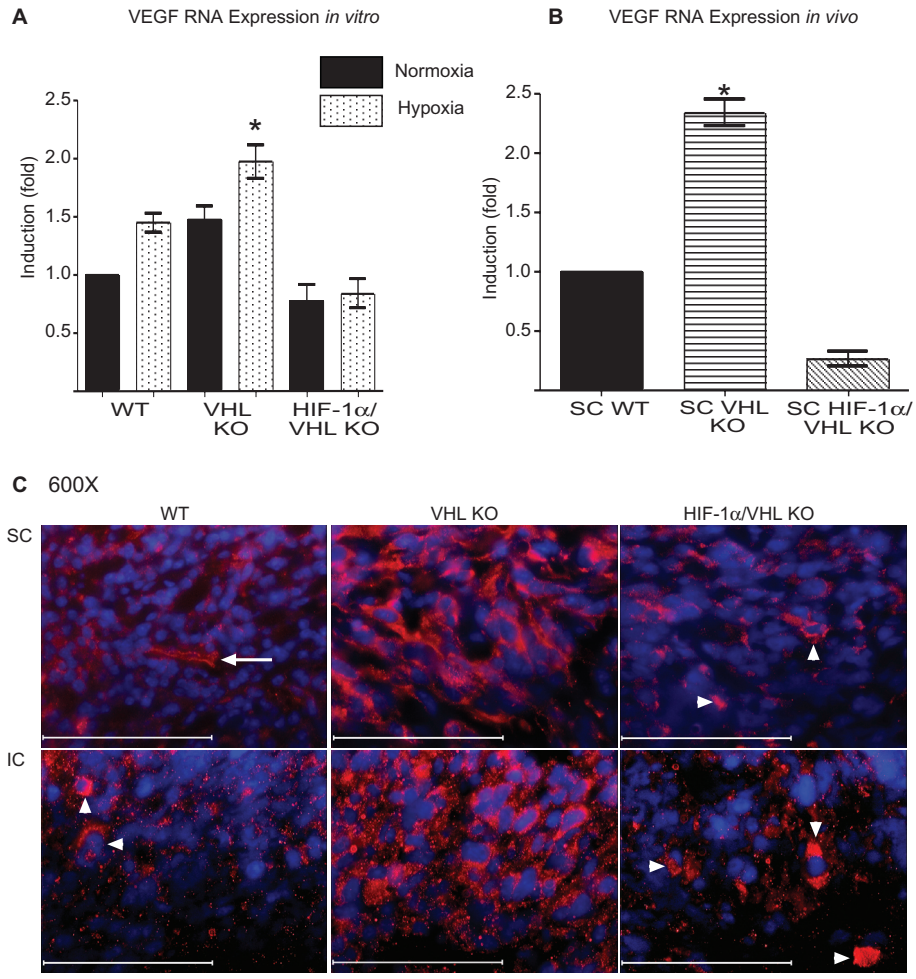

Figure 4. VEGF expression in VHL KO astrocytes is dependent on HIF-1α

A. VHL KO and HIF-1α/VHL KO astrocytes were grown at normoxia and hypoxia (pO_2=0.5%). After harvest, VEGF RNA expression determined as described in Materials and Methods.*Columns*, mean; *error bars*, 95% confidence intervals, * p= 0.05. B. RNA was extracted from subcutaneously grown tumors and tested for VEGF expression as described in Materials and Methods. *Columns*, mean; *error bars*, 95% confidence intervals. * p=0.05; n = 3. C. Sections were stained for VEGF by immunofluorescence followed by a background stain with DAPI. White arrowheads indicate the fraction of cells expressing VEGF, white arrow indicates a blood vessel. Magnifications are indicated and were takend with a 2 second exposure time for VEGF and 0.03 seconds for DAPI; scale bar = 100 µm.

subcutaneously, we asked whether the expression of VEGF in the VHL KO tumors might be dependent on the microenvironment.

As is shown in figure 4A-B there is a small but significant HIF-1α-dependent increase (p= 0.05) in VEGF expression in the VHL KO cells growing under normoxia and hypoxia conditions or subcutaneously (respectively 1.5-, 2-, 2.3-fold). Similar values were obtained for the VHL KO brain tumor. These data were confirmed by immunofluorescence staining for VEGF of both subcutaneous and brain tumors (figure 4C). Therefore, we conclude that in the VHL KO transformed astrocytes, VEGF expression is dependent on HIF-1α regardless of the microenvironment in which the cells are grown. These data suggest that VEGF is not the key factor in causing the different vascularity of the HIF-1α/VHL KO tumors growing in the brain versus subcutaneous site.

Tumor vascularity of VHL KO transformed astrocytes is dependent on VEGF

Loss of VHL causes the up regulation of many growth factors, which may compensate for a reduction in VEGF expression, and allow tumor growth to occur (Kaelin, 2002). To test whether the VHL KO tumor growth and vascularity was dependent on expression of tumor cell derived VEGF, we next implanted astrocytes in which VEGF is deleted in the VHL KO background (VEGF/VHL KO) in the subcutis and brain. These cells were created in similar fashion to the VHL KO and HIF-1α /VHL KO cell lines.

As shown in figure 5A, loss of VEGF in the VHL KO tumors decreases tumor growth by 63% compared to the VHL KO tumors ($p<0.05$). Surprisingly, when the same cels were grown in the brain, the survival was not altered compared to mice having the VHL KO brain tumors (figure 6A, p= 0. 24).

Independent of the implantation site however, the vascularity of the VHL tumors was dependent on VEGF. Compared to the VHL KO, the vessel density was reduced by 54% in the subcutaneous tumor, and by 47% in the brain tumor respectively, upon loss of VEGF in the VHL KO background (figure 5B-C and 6D). In the subcutaneous site, vessels appeared necrotic (encircled area in figure 5B, right panel). Along those lines compared to the VHL KO tumors, vessel apoptosis was approximately a 10-fold increased in the subcutis, and a 5-fold in the brain tumors (figures 5D-E and 6E). Endothelial cell proliferation was decreased in both sites (not shown). Further, loss of VEGF eradicated the appearance of large hemangiomas associated with loss of VHL in the brain tumors (6B-C).

Taken together, our data indicates that VEGF is a critical factor in tumor angiogenesis associated with loss of pVHL, regardless of the microenvironment. Our data implies that even though inhibition of VEGF reduces tumor angiogenesis, it may not be sufficient to inhibit tumor growth in VHL disease.

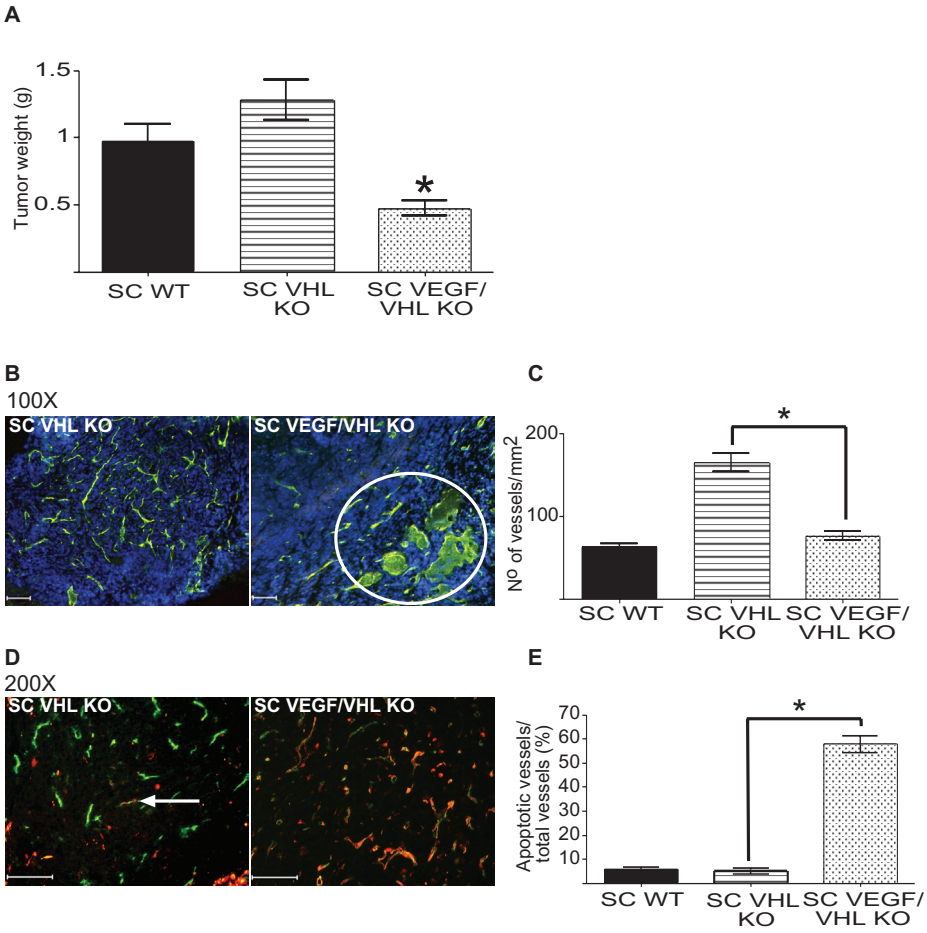

Figure 5. Vascularity of VHL KO astrocytes grown subcutaneously is dependent on VEGF. A.VEGF/VHL KO (dark grey bar) and WT (black bar) cells were grown subcutaneously in mice for 21 days as described in Materials and Methods. *Columns*, mean; *error bars*, 95% confidence intervals, $p < 0.05$. B. Vessels in the subcutaneous tumors were visualized as described earlier. Encircled area in right panel indicates necrotic vessels in the VEGF/VHL KO tumor. Magnifications are indicated, scale bar = 100 µm. C. Bar graph of vessel density. Calculations were done as is described earlier. *Columns*, mean; *error bars*, 95% confidence intervals ($p < 0.05$). D. Vessel apoptosis in the subcutaneous tumors was determined as described earlier. White arrow indicates a single apoptotic vessel in SC VHL KO. Magnifications are indicated, scale bar = 100 µm.E. Bar graphs showing the apoptotic rate of the blood vessels. Apoptotic rate was determined as described earlier. *Columns*, mean; *error bars*, 95% confidence intervals ($p < 0.05$).

DISCUSSION

Determining the contribution of the HIF/VEGF pathway to the the tumor growth and angiogenesis associated with loss of VHL is important for novel therapeutic approaches. In the well studied VHL null cell line 786-O, HIF-1α is not expressed and VEGF expression is mainly driven by HIF-2α (Carroll & Ashcroft, 2006). However, in other tumor types associated with

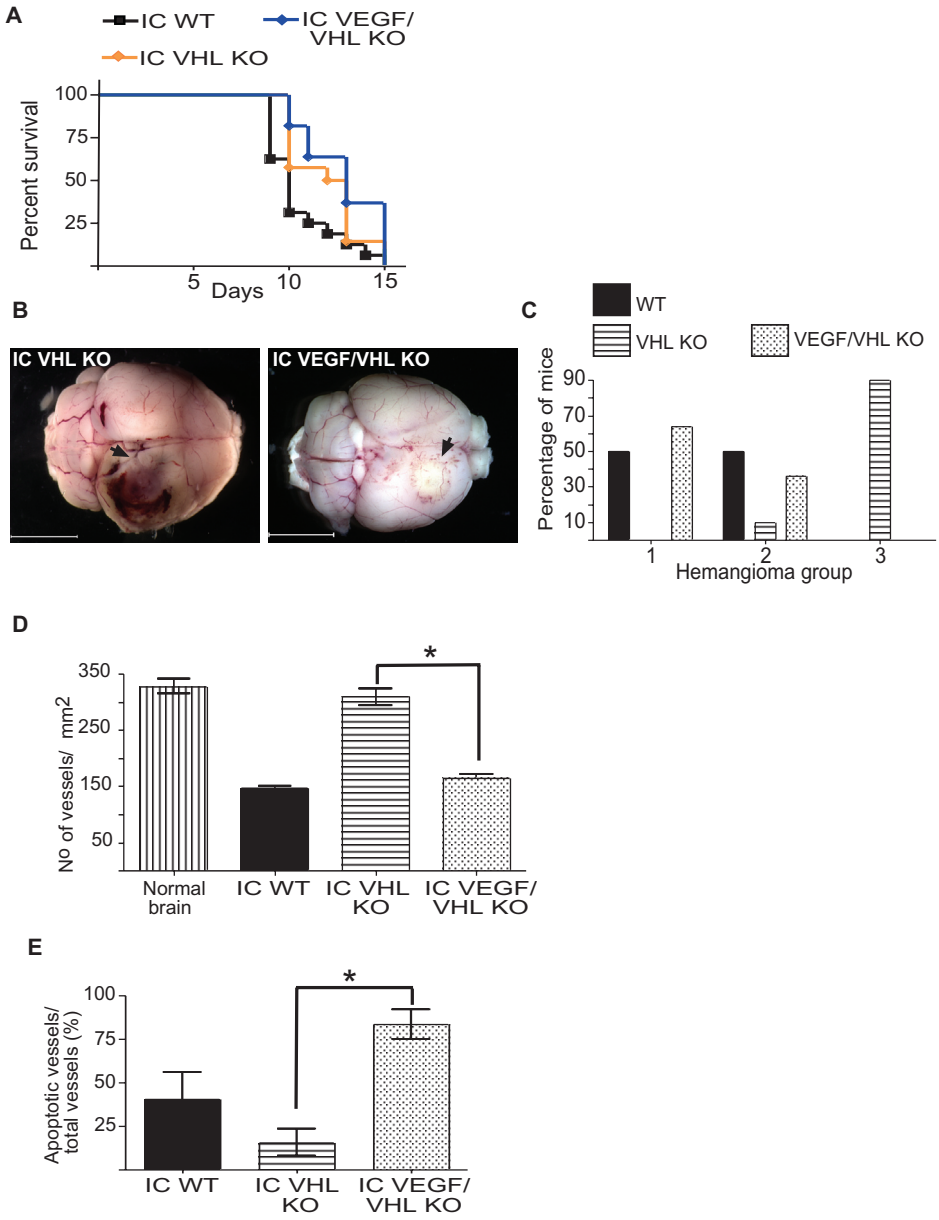

Figure 6. Tumor vascularity of VHL KO astrocytes grown in the brain is dependent on VEGF. A.Survival curves of mice in which VHL KO and VEGF/VHL KO astrocytes were grown as brain tumors; n=13. B. Photographs of brains implanted with respectively VHL KO and VEGF/VHL KO astrocytes. Black arrowheads indicate the tumor area. Each photograph shown is representable for the entire group (n= 13). Scale bars = 5 mm. C. Incidence of hemangiomas in the brain tumors derived from VHL KO and VEGF/VHL KO astrocytes as is described earlier. The reduction in hemangioma formation in the VEGF/VHL KO compared to the that in the VHL KO tumors was significant as revealed by the χ^2 Test, p= 0.01 (n=13). D. Bar graph of vessel density. *Columns*, mean; *error bars*, 95% confidence intervals ($p < 0.05$). E. Bar graphs showing the rate of apoptotic blood vessels. *Columns*, mean; *error bars*, 95% confidence intervals ($p < 0.05$).

VHL disease such as hemangioblastoma, HIF-1α is over-expressed and correlates with the expression of VEGF (Flamme *et al.*, 1998; Zagzag *et al.*, 2000). In addition, both HIF-1α and HIF-2α are expressed in lesions of the kidney in VHL disease (Mandriota *et al.*, 2002). These observations underscore the importance of understanding the relative contributions of each protein to different aspects in tumor formation associated with VHL disease.

In this work we examined the contribution of HIF-1α and its target VEGF to tumor growth and angiogenesis upon loss of pVHL in a genetic mouse implantation model. Transformed astrocytes lacking pVHL alone or in combination with HIF-1α (HIF-1α/VHL KO), and VEGF (VEGF/VHL KO), were grown in heterotopic and orthotopic sites in mice. This model requires the transformation of primary cells that are obtained from the transgenic mice (in this work SV 40 Large T and H-Ras), in order to generate tumor growth by these cells *in vivo*. Even though this may lead to side effects that potentially influence the growth proliferation of these cells unrelated to the deletion of the specific gene, this method is frequently used when investigating the role of a particular gene during tumor growth *in vivo* using primary mouse cells (Ryan *et al.*, 2000; Unruh *et al.*, 2003; Mack *et al.*, 2005). We primarily chose this model because it was shown to recapitulate relevant tumor-host interactions in the orthotopic setting which are important for angiogenesis (Blouw *et al.*, 2003).

We report astrocytoma cells lacking pVHL implanted subcutaneously formed tumors with an increased vessel density but additional vascular lesions associated with loss of pVHL were not observed (Kaelin, 2002). This result is consistent with what has been reported for subcutaneously growing fibrosarcomas lacking pVHL (Mack *et al.*, 2005). Possibly, cells must grow in a highly vascular- or their natural environment in order to recapitulate the vascular features in tumors associated with loss of pVHL. This is supported by our model, where loss of pVHL in transformed astrocytes formed a highly angiogenic tumor type when grown in the brain with a vessel pathology that resembled that of human hemangioblastoma (Hasselblatt *et al.*, 2005; Hansel, 2006).

In our model, the contribution of HIF-1α to the vascularity of astrocytoma lacking pVHL was dependent on the micro-environment. Compared to the wild type control tumors, the vessel density was increased in both the VHL KO and HIF-1α /VHL KO subcutaneous tumors. In the brain, the vascularity of the HIF-1α /VHL KO tumors was reduced comparable to that of the WT. These data did not correlate with the expression of VEGF; irrespective of the micro-environment, loss of HIF-1α in the VHL KO background reduced the expression of VEGF. This is consistent with reports of MEFs and MCF7 cells that show a HIF-1α dependent increase expression of VEGF upon hypoxia exposure (Ryan *et al.*, 2000; Mack *et al.*, 2005; Carroll & Ashcroft, 2006). We did not detect differences in HIF-2α expression in the astrocytes under hypoxia or normoxia (not shown) (Blouw *et al.*, 2003). In a conditional mouse model where pVHL is deleted in the kidney and liver, VEGF expression in these tissues was dependent on HIF-1β (Rankin *et al.*, 2005). This indicates that the contribution of either HIF-1α or HIF-2α, or

both, to the expression of VEGF and angiogenesis might depend on the different tumor types associated with VHL disease.

The angiogenesis of the VHL KO astrocytoma depends on the expression of tumor cell derived VEGF. Loss of VEGF in the VHL KO background causes hemangiomas to disappear, combined with a striking increase in vessel apoptosis in both the subcutaneous and brain tumor. This correlated with a reduction in tumor growth only when cells were growing in the subcutaneous site and not in the brain. Although the orthotopic tumor growth is measured by the survival of the mice, which may be an outcome of both tumor formation and edema and may not necessarily reflect tumor growth alone, we previously we reported that deletion of VEGF in otherwise wild type transformed astrocytes reduced tumor growth independent of the implantation site (Blouw *et al.*, 2003). This apparent discrepancy can be explained by studies showing that loss of pVHL causes the up regulation of genes that are important in angiogenesis and in invasion such as CXCR4, SDF-1 (Staller *et al.*, 2003; Zagzag *et al.*, 2005), matrix metalloproteinase 2 and 9, the Met receptor (Koochekpour *et al.*, 1999) and components of the urokinase plasminogen activator pathway (Los *et al.*, 1999). Consistent with these observations, the VEGF/VHL KO astrocytes were capable of invading different parts of the brain when grown intra-cranially (not shown). This indicates that although loss of VEGF in the VHL KO background reduces tumor angiogenesis, the up regulation of other pro-invasive and pro-angiogenic factors may compensate and be sufficient to sustain tumor growth and tumor cell survival.

Tumors that arise in VHL disease are in general benign and have a low metastatic rate. The treatment is often surgical removal of the tumor (Lonser *et al.*, 2003). Nevertheless, many patients still develop severe disease complications, such as blindness or brain damage. Therefore it was proposed that a VEGF blockade, would be of great value to patients with VHL syndrome (Harris, 2000). Since that time, clinical trials have been carried out with various angiogenesis inhibitors (Staehler *et al.*, 2005). Our data supports the idea exploring anti-angiogenesis therapeutics for this disease; but suggests that this could be strongly influenced by the tissue in which the disease is manifesting itself.

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REFERENCES

Benjamin, L.E. & Keshet, E. (1997). *Proc Natl Acad Sci U S A*, **94,** 8761-6.

Bergers, G. & Benjamin, L.E. (2003). *Nat Rev Cancer*, **3,** 401-10.

Blouw, B., Song, H., Tihan, T., Bosze, J., Ferrara, N., Gerber, H.P., Johnson, R.S. & Bergers, G. (2003). *Cancer Cell*, **4,** 133-46.

Carroll, V.A. & Ashcroft, M. (2006). *Cancer Res*, **66,** 6264-70.

Flamme, I., Krieg, M. & Plate, K.H. (1998). *Am J Pathol*, **153,** 25-9.

Gerber, H.P., Hillan, K.J., Ryan, A.M., Kowalski, J., Keller, G.A., Rangell, L., Wright, B.D., Radtke, F., Aguet, M. & Ferrara, N. (1999). *Development*, **126,** 1149-59.

Grunstein, J., Roberts, W.G., Mathieu-Costello, O., Hanahan, D. & Johnson, R.S. (1999). *Cancer Res*, **59,** 1592-8.

Haase, V.H., Glickman, J.N., Socolovsky, M. & Jaenisch, R. (2001). *Proc Natl Acad Sci U S A*, **98,** 1583-8.

Hansel, D.E. (2006). *Histol Histopathol*, **21,** 437-44.

Harris, A.L. (2000). *Oncologist*, **5 Suppl 1,** 32-6.

Hasselblatt, M., Jeibmann, A., Gerss, J., Behrens, C., Rama, B., Wassmann, H. & Paulus, W. (2005). *Neuropathol Appl Neurobiol*, **31,** 618-22.

Kaelin, W.G., Jr. (2002). *Nat Rev Cancer*, **2,** 673-82.

Koochekpour, S., Jeffers, M., Wang, P.H., Gong, C., Taylor, G.A., Roessler, L.M., Stearman, R., Vasselli, J.R., Stetler-Stevenson, W.G., Kaelin, W.G., Jr., Linehan, W.M., Klausner, R.D., Gnarra, J.R. & Vande Woude, G.F. (1999). *Mol Cell Biol*, **19,** 5902-12.

Lonser, R.R., Glenn, G.M., Walther, M., Chew, E.Y., Libutti, S.K., Linehan, W.M. & Oldfield, E.H. (2003). *Lancet*, **361,** 2059-67.

Los, M., Zeamari, S., Foekens, J.A., Gebbink, M.F. & Voest, E.E. (1999). *Cancer Res*, **59,** 4440-5.

Mack, F.A., Patel, J.H., Biju, M.P., Haase, V.H. & Simon, M.C. (2005). *Mol Cell Biol*, **25,** 4565-78.

Mack, F.A., Rathmell, W.K., Arsham, A.M., Gnarra, J., Keith, B. & Simon, M.C. (2003). *Cancer Cell*, **3,** 75-88.

Mandriota, S.J., Turner, K.J., Davies, D.R., Murray, P.G., Morgan, N.V., Sowter, H.M., Wykoff, C.C., Maher, E.R., Harris, A.L., Ratcliffe, P.J. & Maxwell, P.H. (2002). *Cancer Cell*, **1,** 459-68.

Rankin, E.B., Higgins, D.F., Walisser, J.A., Johnson, R.S., Bradfield, C.A. & Haase, V.H. (2005). *Mol Cell Biol*, **25,** 3163-72.

Rathmell, W.K., Hickey, M.M., Bezman, N.A., Chmielecki, C.A., Carraway, N.C. & Simon, M.C. (2004). *Cancer Res*, **64,** 8595-603.

Ryan, H.E., Lo, J. & Johnson, R.S. (1998). *Embo J*, **17,** 3005-15.

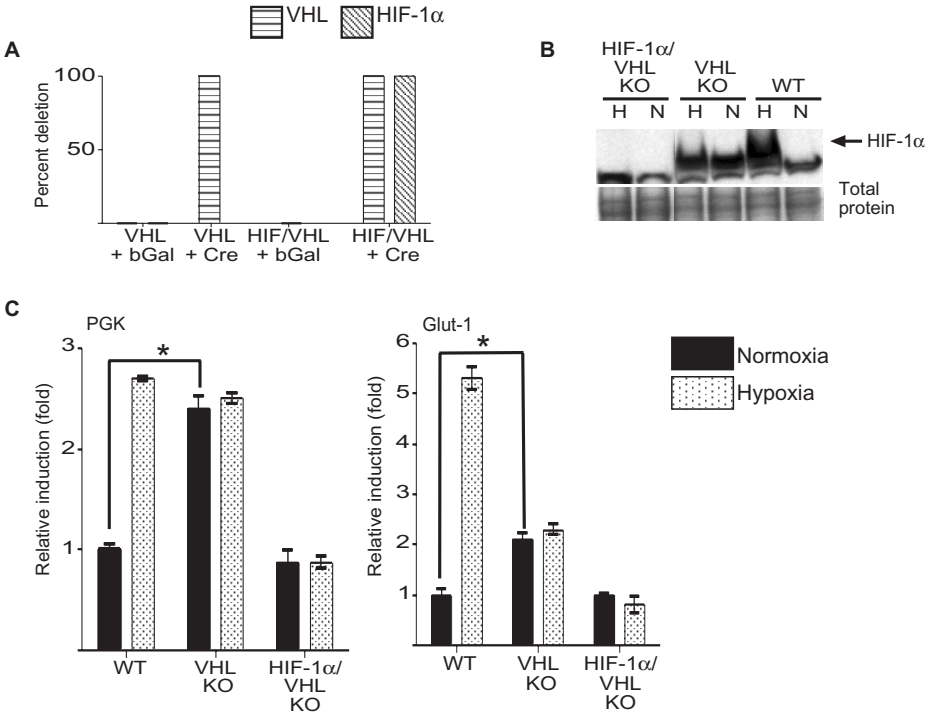
Ryan, H.E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J.M. & Johnson, R.S. (2000). *Cancer Res*, **60,** 4010-5.

Staehler, M., Rohrmann, K., Haseke, N., Stief, C.G. & Siebels, M. (2005). *Curr Drug Targets*, **6,** 835-46.

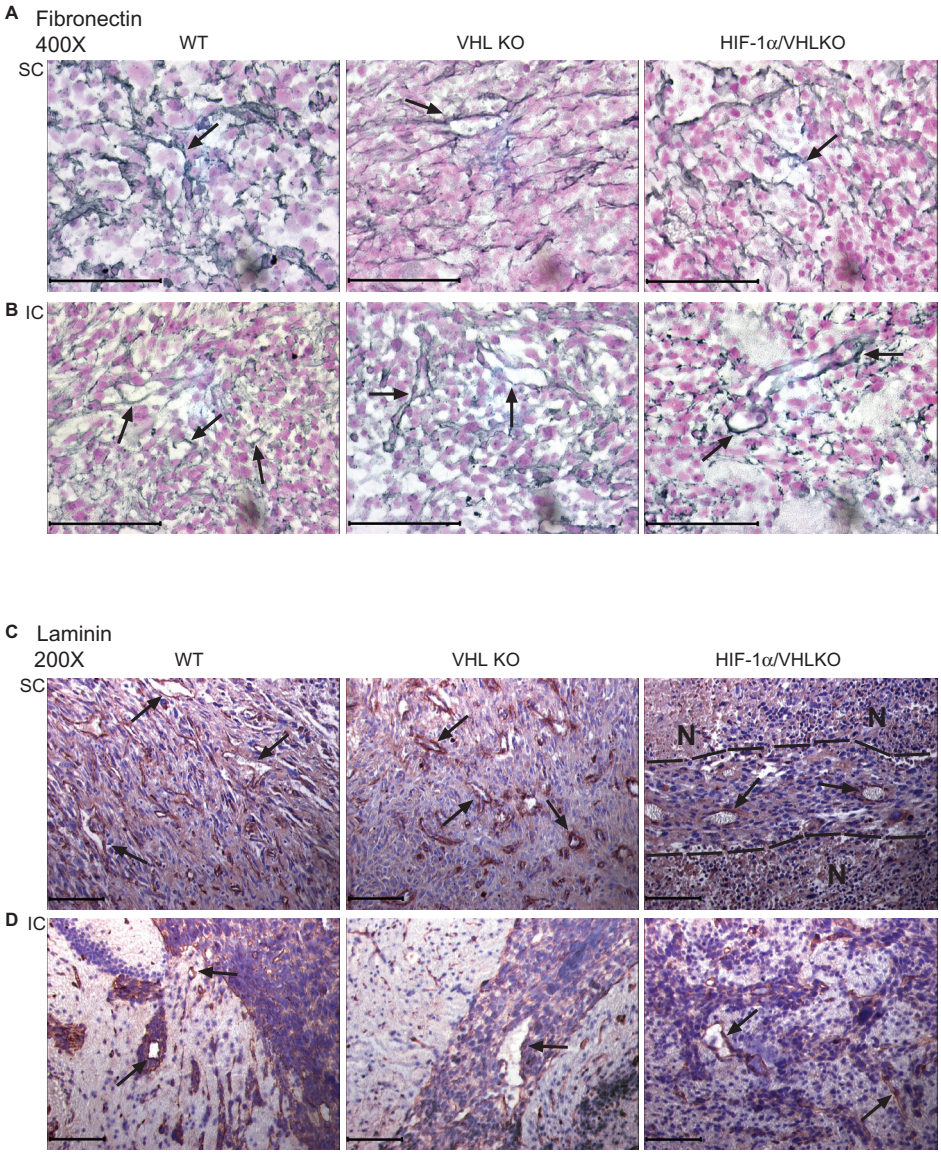
Staller, P., Sulitkova, J., Lisztwan, J., Moch, H., Oakeley, E.J. & Krek, W. (2003). *Nature*, **425,** 307-11.

Zagzag, D., Krishnamachary, B., Yee, H., Okuyama, H., Chiriboga, L., Ali, M.A., Melamed, J. & Semenza, G.L. (2005). *Cancer Res*, **65,** 6178-88.

Zagzag, D., Zhong, H., Scalzitti, J.M., Laughner, E., Simons, J.W. & Semenza, G.L. (2000). *Cancer*, **88,** 2606-18.



Supplemental figure 1. Hypoxic response in VHL KO transformed astrocytes is dependent on HIF-1α. A. Deletion efficiency of respectively VHL and the combination of HIF-1α and VHL in transformed astrocytes was determined by TaqMan Real Time PCR on genomic DNA obtained from transformed mouse astrocytes that were homozygous for respectively VHL^{+f+f} and $HIF\text{-}1\alpha^{+f+f}/VHL^{+f+f}$ which were infected with adenovirus expressing Cre recombinase, or βGalactosidase. Relative values were normalized to the house-keeping gene cJun and expressed as a percentage deletion compared to that of the cells infected with βGalactosidase. B. Upper panel: nuclear extract was assayed by Western blot for the HIF-1α protein, obtained from the VHL KO and HIF-1α/VHL KO astrocytes cultured at normoxia (N) and hypoxia (H- pO_2=0.5%) for 6 hr. Lower panel: to demonstrate equivalent loading, total protein was stained with Amido black followed by destaining and rehydration, prior to blotting. C. VHL KO and HIF-1α/VHL KO transformed astrocytes were cultured at normoxia (black bars) and hypoxia (white bars) in triplicates for 6 hr, harvested and the expression of target genes was normalized to 18S RNA. After normalization, the relative expression of each gene was expressed as a percentage compared to that of wild type cells growing at normoxia. *Columns*, mean; *error bars* represent the 95% confidence intervals. p= 0.05.



Supplemental figure 2. Vascularity of VHL KO tumors does not correlate with defects in the assembly of extracellular matrix molecules fibronectin and laminin. A-B Frozen sections of both sc and brain tumors were stained with an antibody directed against Fibronectin, developed in Vector SG (blue-grey) and counterstained with Nuclear Fast Red. Irrespective of the micro-environment, endothelial cells stained positive (arrows), and there were no differences in expression level or assembly. Photographs were taken at a magnification of 400X, scale bar is 100 µm. C-D. Sections were stained with an antibody directed against laminin, developed in NovaRed and counterstained with Hematoxylin. In both micro-environments only the vessels stained positive for laminin (arrows) and there were no differences in the level of expression or assembly. In top left panel: dashed line demarcates the border between tumor and necrotic area (N). Photographs were taken a magnification of 200X, scale bar is 100 µm.

CHAPTER 4

Loss of pVHL sensitizes transformed mouse astrocytes to growth inhibition mediated by 2-deoxy-D-glucose *in vitro*

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ABSTRACT

The presence of hypoxia in solid tumors is associated with poor prognosis and a more aggressive tumor type. The physiological adaptation of cells to hypoxia is orchestrated by the Hypoxia Inducible Factors (HIFs) and involves a number of genetic alterations that promote angiogenesis, migration and cell death. One of the most remarkable actions of HIFs as mediators of this adaptation, is the switch to anaerobic metabolism by the regulation of genes involved in the glycolytic pathway, which is the only source of ATP when cells are exposed to hypoxia. We investigated whether cells that overexpress HIF-1α are sensitized to the inhibition of the glycolytic pathway, by exposing transformed astrocytes lacking pVHL to the glycolytic inhibitor 2-deoxy-D-glucose (2DG) *in vitro* and when grown as subcutaneous tumors *in vivo*. We report that *in vitro* loss of pVHL sensitizes to the growth inhibition mediated by 2DG. However when grown as subcutaneous tumors, 2DG had no significant effect on tumor growth.

Keywords: Hypoxia, glycolysis, glycolytic inhibitors, VHL

INTRODUCTION

As a result of the high proliferation rate of the tumor cells which causes them to outgrow the existing blood supply, and of the abnormal vasculature in the tumor itself, tumors become hypoxic (1). For many different types of cancer, hypoxia is correlated with a worse prognosis (2-5). In response to hypoxia, certain physiological changes occur that allow the cells to survive in an oxygen poor environment. These adaptations are orchestrated by the members of the Hypoxia Inducible Transcription Factors (HIFs) which include HIF-1α, HIF-2α and HIF-3α, respectively. HIF-1α is the best characterized family member and is thought to be the major regulator of the hypoxic response (6).

One of the adaptations to hypoxia involves the switch from oxidative phosphorylation (or the tricarboxyclic acid – TCA - cycle) to glycolysis to generate ATP, a phenomenon known as the Pasteur's effect (7). Several lines of evidence have shown HIF-1α to be a critical factor in regulating this process. First, it was demonstrated that HIF-1α regulates the expression of many of the enzymes in the glycolytic pathway such as Phosphofructo kinase (PFK), Aldolase A (ALDA), Phosphoglycerate kinase 1 (PGK1), Enolase (ENO), Pyruvate Kinase (PKM) and Lactate dehydrogenase (LDH A) (8), as well as the expression of the glucose transporters Glut-1 and Glut-3 (9) which mediate cellular glucose uptake. Later, our lab demonstrated that cells lacking HIF-1α fail to undergo the switch to glycolysis and as a result generate less ATP, and show a reduced proliferation rate under hypoxic conditions (10). The regulation of HIF-1α is complex and occurs through oxygen-dependent and independent pathways. The oxygen-dependent regulation is mediated by the tumor suppressor protein Von Hippel Lindau and by a hydroxylase known as Factor Inhibiting HIF (FIH).

Under normal oxygen conditions and in the presence of iron and 2-oxoglutarate, HIF-1α is hydroxylated by FIH and by different prolyl hydroxylases (11, 12). The activity of FIH inhibits the transcriptional activation of HIF-1 by preventing it to bind to the co-activators CBP/p300 (11, 13). The prolyl hydroxylation is required for HIF-1α to bind to the Von Hippel Lindau tumor suppressor (pVHL) (14). pVHL is the recognition subunit of a multiprotein complex that functions as a E3 ubiquitin ligase (15, 16) and as a result, under normal oxygen conditions HIF-1α is constantly degraded through the proteasomal pathway and the transcription of its target genes is inhibited (17-20). When oxygen levels are reduced however, the hydroxylation does not take place, as a consequence HIF-1α escapes recognition by pVHL, upon which it stabilizes and binds to HIF-1β (constitutively expressed). The heterodimer translocates to the nucleus where it binds to hypoxia response elements (HREs) in the DNA. In addition, the co-activators CBP/p300 bind to HIF-1α (now released from the transcriptional inhibition by FIH), leading the transcription of HIF-1 target genes (21, 22).

Recently it was reported that under different simulated conditions of hypoxia, cells were more sensitive to the growth inhibitory effects of glycolytic inhibitors such as 2-deoxy-D-glucose (2DG), compared to normal oxygen conditions (23-25) . 2DG is a synthetic

analogue of glucose in which the hydroxy group at the second position carbon is replaced by a hydrogen and inhibits the enzyme phosphohexose isomerase, the enzyme that converts phosphoglucose (G-6-P) to phosphofructose (F-6-P). As a result, the glycolysis is inhibited at the initial stage (26). The basis for this increased sensitivity under hypoxic conditions is that the cells must rely primarily on glycolysis to produce ATP. When exposed to a glycolytic inhibitor under these conditions, the ATP production is blocked, the cells cease to proliferate and eventually succumb.

Given the crucial role of HIF-1α in regulating the glycolysis, the observations described above led us to hypothesize that cells overexpressing HIF-1α might be sensitized to glycolytic inhibitors. We tested this possibility by exposing transformed astrocytes lacking pVHL (VHL KO), which were previously shown to constitutively overexpress HIF-1α, to 2DG *in vitro* and *in vivo*. We report that VHL KO cells exposed to 2DG show a greater growth inhibition compared to the wild type cells *in vitro*. When grown as subcutaneous tumors however, this growth inhibitory effect was not recapitulated. Our data indicates that 2DG can be used as an effective inhibitor of cell growth in cells that overexpress HIF-1α when grown *in vitro*, however its effects *in vivo* need further evaluation.

MATERIALS AND METHODS

Cell culture

Ras transformed Wild type (WT) and pVHL *null* (VHL KO) mouse astrocytes were generated as follows: primary astrocytes were isolated from 1 to 2 days old pups that were homozygous for the VHL allele in which the second exon is flanked by Lox P sites ($VHL^{+f/+f}$ or VHL DF hereafter) (27). Cells were immortalized with SV 40 Large T and transformed with H-Ras and polyclonal cultures were infected with Adenovirus expressing Cre recombinase, leading to excision of exon 2 of the VHL allele and thereby loss of pVHL function (VHL KO). The isolation of astrocytes, immortalization, transformation and adenovirus infection was carried out according to Blouw *et al*., 2003 (28). Simultaneously, control cells were obtained by infecting the VHL DF astrocytes with adenovirus expressing βGalactosidase (WT). Cells were cultured in DMEM supplemented with 10% FBS, 1% Pen/Strep, 1% Sodium Pyruvate and 1% Hepes (all Invitrogen, Carlsbad, CA) in a standard humidified incubator at 37°C with O_2 21 % and CO_2 5%.

2-Deoxyglucose dose-response

Cells were plated at 5 X 10^4 cells/ml in triplicates in 6-wells plates. After 24 hr, 2DG from a stock solution of 300 mg/ml dissolved in PBS (Invitrogen, Carlsbad, CA) was added to the cells in concentrations of respectively 0.1, 0.5, 1.0 and 2.0 mg/ml in a final volume of 2ml, whereas

the control cells received medium alone. After 72 hr, cells were harvested and trypan blue exclusion counts were performed using a hemocytometer. Cell survival was determined by the cell count ratio of treated to the respective untreated control sample, and the percentage was calculated for each.

Measurement of free ATP

A luciferase assay developed by Roche, the ATP Bioluminescence Assay Kit CLS II (Mannheim, Germany) was utilized to measure the effect of 2DG on the production of free ATP. WT and VHL KO astrocytes were plated in triplicates at 5 X 10^4 cells/ml in 6 wells plates. After a 24 hr, 2 DG was added to the cells at 2 mg/ml in a final volume of 2 ml. After 72 hours, cells were harvested on ice, washed in ice-cold PBS and centrigugated at 4°C. The pellets were respspended in ice-cold PBS to a concentration of either 10^5 or 10^6 in a total volume of 50 µl. Cells were lysed at 100°C in a buffer consisting of 100 mM Tris and 4 mM EDTA at pH 7.75, and returned on ice until analysis. To measure luciferase activity, 100 µl luciferase reagent provided in the CLS II kit was added to 50 µl of whole cell extract and read immediately at 562 in a Lumnistar luminometer at 10-s integration. In order to normalize the ATP to cellular protein, the protein content of 150 µl of each extract was determined via a Bradford assay (Bio-Rad) according to the manufacturer's instructions. The molar amount of ATP corresponding to each sample was determined based on a log-plot of the ATP standards (10^{-5} to 10^{-9} M ATP) versus the relative luciferase units. Next, the molar amount of ATP per microgram of protein produced by each cell line was determined. For each cell line and treatment condition, these data were averaged, and the average free ATP values ± the standard error of the mean were determined.

2 DG treatment of subcutaneous tumors

3 X 10^6 WT and VHL KO astrocytes resuspended in 100 µl serum free medium were injected in the flank of immunocompromised mice (10 mice per group). After 9 days, palpatable tumors were observed and the mice were randomized over 2 subgroups, one being the treated and the other being the control group. Mice were injected daily in the morning with either 2DG at 2 mg/ml in dPBS or with dPBS alone (control group) at a final volume of 200 µl. After 21 days mice were sacrificed and tumors were dissected out and processed for histological analysis and RNA isolation.

RNA isolation and Glut-1 Real Time PCR

RNA was extracted from 100 mg tumor tissue from which the necrotic areas were removed using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manu-

facturer. One µg RNA was used as template for cDNA synthesis and used to measure Glut-1 expression by Quantitative Real Time PCR as described previously (28). The primers had the following primer sequences:

F 5'-ACGAGGAGCACCGTGAAGAT-3'

R 5'- GGGCATGTGCTTCCAGTATGT-3'

Probe 5'- (6FAM) CAACTGTGCGGCCCCTACGTCTTC-(BHQ)-3'.

Hypoxia detection and Glut-1 immunohistochemistry

The detection of hypoxia within the tumors was carried out as is described previously (28). For the Glut-1 immunohistochemistry, paraffin sections were deparaffinized and after rehydration antigen retrieval was performed by boiling the sections in citrate buffer. After the sections had cooled down to RT, they were blocked in 10% normal goat serum/PBS-T for 1 hr followed by an incubation with a primary rabbit-anti Mouse Glut-1 antibody at 1:200 (Alpha Diagnostics, San Antonio, Texas) for 1 hr in the block solution. A secondary biotinylated goat anti Rabbit antibody was applied. Next, after washes and incubation with the ABC reagent, the signal was developed using DAB and sections were counterstained with hematoxylin (secondary antibody for both the hypoxia and Glut-1 immunohistochemistry, the DAB and hematoxylin were purchased from Vectorlabs, Burlingame, CA).

RESULTS

Transformed astrocytes lacking pVHL are sensitized to glycolytic inhibitors *in vitro*

In order to test whether cells that overexpress HIF-1α are sensitized to glycolytic inhibitors, stably transformed mouse astrocytes in which pVHL was deleted (VHL KO) were generated, resulting in the constitutive expression of HIF-1α. The isolation of the astrocytes, immortalization, transformation and subsequent deletion of pVHL was carried out as is described in Materials and Methods. The deletion of pVHL was confirmed by TaqManReal Time PCR on genomic DNA from the VHL KO and WT cells, and constitutively HIF-1α expression together with the transcription of its target genes under normal growth conditions in the VHL KO astrocytes was verified by a western blot for HIF-1α and TaqMan Real Time PCR (BB- *Oncogene* in press).

The sensitivity of the WT and VHL KO cells to the glycolytic inhibitor 2DG, was determined by cell viability assays, using the Trypan Blue exclusion method on treated and control cells. VHL KO and WT were plated in triplicates. After 24 hours, varying amounts of 2DG were added to the medium and cells were incubated under normal growth conditions for 72 hours, until the untreated cells reached confluency. At this stage, cells were trypsinized and after cen-

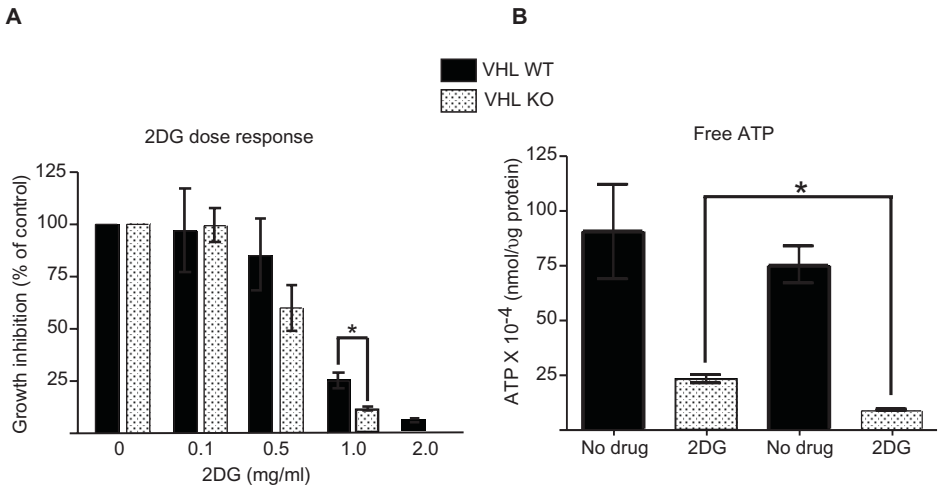

Figure 1. Loss of pVHL in transformed astrocytes increases sensitivity to growth inhibition by 2DG. A. WT and VHL KO astrocytes were plated in triplicates and exposed to various concentrations of 2DG for 72 hours. Cell survival was calculated as described in Materials and Methods. The VHL KO astrocytes are significantly more senstive to growth inhibition by 2DG compared to the WT counterparts, starting at a concentration of 1.0 mg/ml 2DG; p= 0.03 (student's t-test). B. WT and VHL KO astrocytes were plated, and treated with 2.0 mg/ml 2DG for 72 hr. Cells were harvested and the amount of free ATP was measured as described in Materials and Methods. The reduction in ATP yield was significantly increased in the treated VHL KO astrocytes versus the treated WT, p = 0.02 (student's t-test). A and B; *Columns* mean, *bars*, ± SD.

trifugation, resuspended in trypan blue and the blue-negative cells were scored. Cell survival was calculated as the percentage of blue negative cells in the 2DG treated wells compared to the untreated controls. As shown in figure 1A, both the WT and VHL KO cells display a dose-dependent growth inhibition to 2DG. When exposed to 1.0 mg/ml 2DG, the magnitude of growth inhibition was larger in the VHL KO cells compared to the WT controls (10.5 % ± 1.5 of the VHL KO cells are viable, versus 24.7 % ± 6.8 for the WT), which was significant by student's t-test, p=0.03. At 2.0 mg/ml the WT and VHL KO cells showed comparable toxicity to 2DG, with a small fraction of cells viable in the wild type (5.2 % ± 1.7) and no viable cells in the VHL KO cultures. We concluded that at 2 mg/ml 2DG growth inhibition was maximal in both cell types and used this as the final concentration for the subsequent assays.

The growth inhibitory effect of 2DG is thought to be a result of blockage of the glycolytic pathway by inhibiting phosphohexose isomerase, the enzyme that converts posphoglucose (G-6-P) to phosphofructose (F-6-P). As a result, exposure to 2DG will lead to growth inhibition and a reduction in ATP yield in the situation where cells are forced to use glycolysis as a source of energy (23-25). To test whether this correlation also existed in our system, an ATP assay was carried out on the WT and VHL KO transformed astrocytes exposed to 2 mg/ml 2DG for 72 hours.

As shown in figure 1D both the WT and VHL KO cells display a reduction in ATP yield upon exposure to 2DG. However, the magnitude of the reduction in ATP yield was significantly

greater in the VHL KO cells upon exposure to 2DG when compared to the WT (a reduction of 88% in the treated VHL KO, versus 75% in the treated WT cells; p=0.02 by student's t-test).

These data indicate that under normal *in vitro* culturing conditions, cells in which HIF-1α is constitutively expressed show an increased sensitivity to the growth inhibitory effects of the glycolytic inhibitor 2DG, which is correlated with a reduction in ATP yield.

Loss of pVHL in transformed astrocytes does not sensitize to growth inhibition by 2DG when grown subcutaneously

To asess whether the increased sensitivity to 2DG in the VHL KO astrocytes was also observed *in vivo*, cells were grown as subcutaneous tumors and treated with 2DG as described in Materials and Methods. 21 days post implantation the mice were sacrificed, the tumors were dissected out, weighed and processed for analysis. Prior to sacrifice mice were i.p. injected with pimonidazole, which forms adducts in hypoxic areas that can be detected with a specific antibody using immunohistochemistry. As shown in figure 2A, compared to the untreated control group for each cell type, the growth appeared to be reduced upon treatment with 2DG. However, for neither the VHL KO tumors, nor for the WT controls this reduction was significant (p = 0.20 for the VHL KO untreated vs treated, and p = 0.38 for the same comparison in the WT group, student's t-test).

The data described above indicate that only when grown *in vitro*, loss of pVHL increases the sensitivity to 2DG, but this effect is not apparent when these cells are grown in the subcutis of mice.

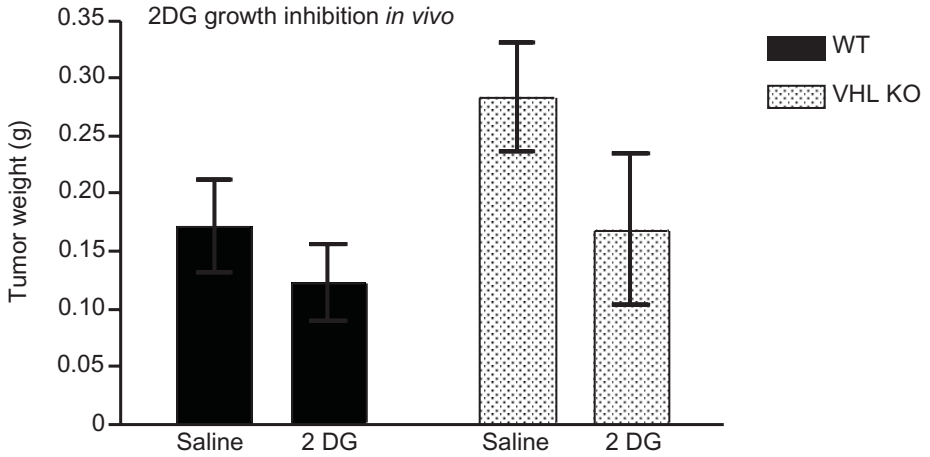

Figure 2. Loss of pVHL does not sensitize to growth inhibitory effects of 2DG when grown as subcutaneous tumors. WT and VHL KO astrocytes were implanted in the subcutis of immunocompromised mice at 10 mice per group, after 9 days randomized over 2 subgroups for each cell line and treated as described in Materials and Methods. There is no significant difference in tumor mass between the VHL KO treated versus untreated, p= 0.20 by student's t test.

Astrocytes lacking pVHL show up-regulation of the glucose transporter Glut-1 when grown subcutaneously

2DG and glucose are substrates for the glucose transporter, Glut-1 and previously it was demonstrated in human pancreatic cell lines that the increased expression of HIF-1α correlates with an increased expression of Glut-1 and sensitivity to 2DG (29). Transformed astrocytes lacking pVHL displayed an increased expression of Glut-1 under normal oxygen conditions *in vitro* (BB *Oncogene* in press). Depending on the specific growth conditions however, such as *in vitro* cell culture or subcutaneous tumor growth, the same cell type can express different genes as was shown for human glioma cell lines (30). This could imply that under different growth conditions cells may express different HIF-1α target genes.

To determine whether the expression of Glut-1 in the transformed astrocytes lacking pVHL was also increased when growing *in vivo*, tumors were lysed and the RNA was used for Quantitative Real Time PCR. When normalized to 18S RNA, VHL KO tumors displayed a 3 fold increase in Glut-1 RNA expression, compared to the wild type controls, figure 3A. This was confirmed by immunohistochemistry for Glut-1 on paraffin sections obtained from the tumors (3B lower left panel). In order to verify the presence of hypoxia and up regulation with Glut-1 expression, sections were stained for Glut-1 and hypoxia. As is shown in figure 3B in right panels, in both tumor types hypoxia is detected in the tumor cells that are close to areas of necrosis (N), but is absent in the cells close to blood vessels (arrow in upper right panel). In the WT tumors, Glut-1 staining follows the pattern of hypoxia, in such that Glut-1 is most intense near the necrotic areas, whereas mild staining is detected near the blood vessels (arrow in upper left panel in figure 3B). In contrast, in the VHL KO tumors, Glut-1 is up regulated in nearly all the cells, and irrespective of presence of hypoxia.

Taken together, our data indicates that when grown *in vitro*, 2DG appears to be an effective compound to reduce the growth of cells in which HIF-1α is up regulated, but this effect is not observed when grown *in vivo*. This discrepancy could not be explained by differences in Glut-1 expression or hypoxia. Therefore, the effects of 2DG on *in vivo* growth inhibition of tumors overexpressing HIF-1α needs to be further investigated.

DISCUSSION

Previously it was reported that cells growing under hypoxic conditions showed an increased sensitivity to various glycolytic inhibitors, including 2DG (31). Upon exposure to hypoxia, cells undergo a switch from oxidative phosphorylation to glycolysis, in order to generate ATP. When then challenged with an inhibitor of this pathway, such as 2DG, the cells will be depleted of ATP, leading eventually to cell death. It has been demonstrated extensively in different cell lines that the transcription factor HIF-1α is the main regulator of glycolytic

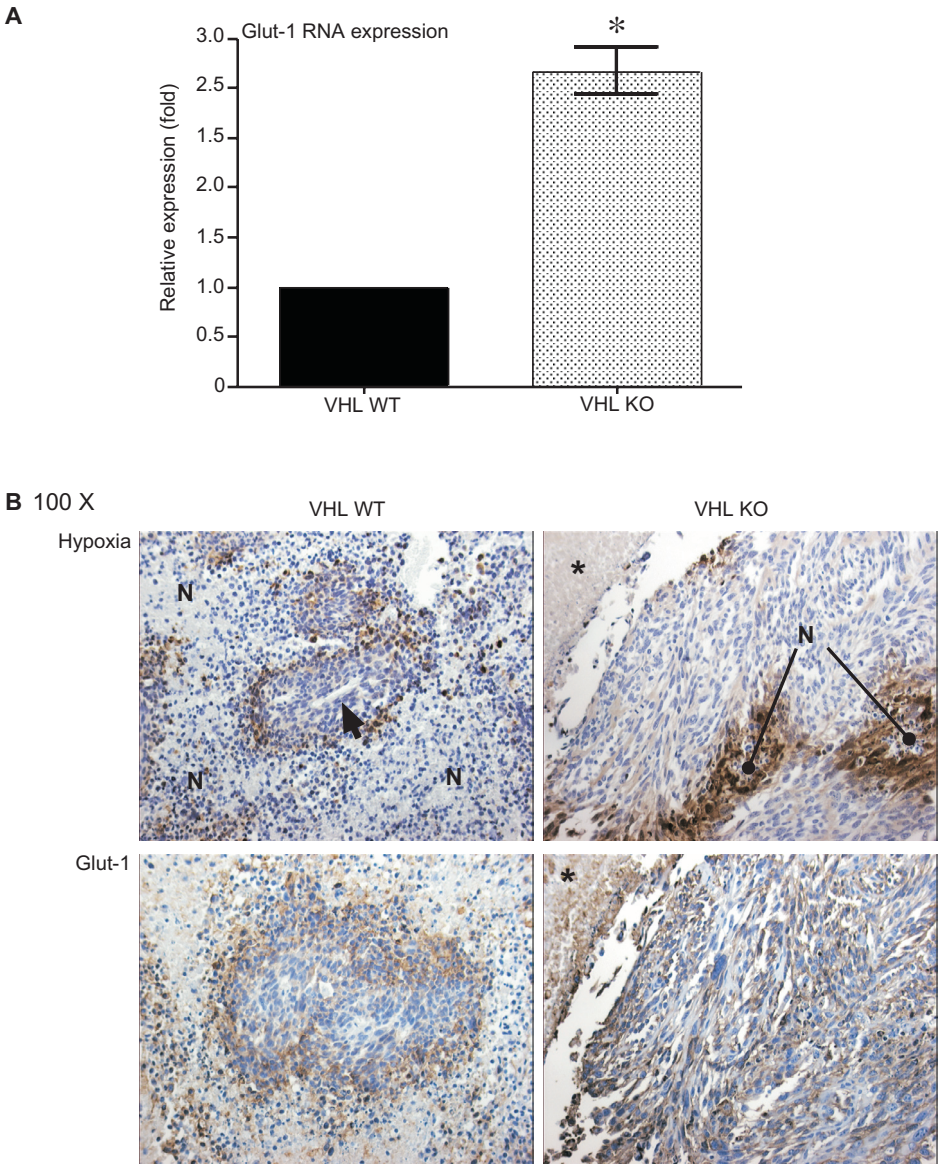

Figure 3. Loss of pVHL correlates with the up regulation of Glut-1 expression. A. Glut-1 expression was determined by TaqMan Real Time PCR on RNA obtained from the tumor tissue. Values were normalized to 18 S RNA. * = 0.001 by student's t-test. *Columns* mean, *bars* ± s.e.m. B. Tumor sections were stained with an antibody detecting Glut-1 (bottom panels) and with an antibody detecting hypoxia (top panels). Both antibodies were developed in DAB (brown). Left panels: in the WT tumors, Glut-1 staining followed the same pattern as hypoxia, being most intense in cells bordering necrosis (N). Arrow points to blood vessel (top left panel). Right panels: in the VHL KO tumor, Glut-1 staining (lower right panel) was equally intense, irrespective of presence of hypoxia. * indicates similar area of consequtive sections. Magnifications are 200X.

switch, and it was shown to regulate the expression of many of the genes in this pathway (6). Collectively, these data led us to hypothesize that cells in which HIF-1α is overexpressed display an increased sensitivity to the growth inhibitory effects of 2DG. This was tested by exposing transformed astrocytes nullizygous for the negative HIF-1α regulator pVHL, to the glycolytic inhibitor 2DG both under *in vitro* and *in vivo* growth conditions.

When grown *in vitro*, the astrocytes lacking pVHL showed an increased sensitivity to 2DG compared to the WT cells, which was accompanied by a reduction in ATP. When grown as subcutaneous tumors however, there was no significant difference in the sensitivity to 2DG between the WT and VHL KO cells, compared to their untreated counterparts. It has been reported that differences in sensitivity to glycolytic inhibitors can be explained by varying efficiencies in glucose uptake along with differences the expression levels of the glucose transporter Glut-1 (29). Although we did not measure the glucose uptake in the astrocytes *in vitro* nor *in vivo*, the VHL KO cells showed an increase expression of Glut-1 compared to WT cells (Figure 3A and B). Alternatively, the concentration of 2DG (2.0 mg/ml) for the *in vivo* experiment was too low. That seems unlikely however, because at higher concentrations the mice showed mild signs of toxicity such as a hunched back and we concluded that 2.0 mg/kg was the maximum tolerable dose. Furthermore, this dosage has been used in a previous study to investigate the effects on subcutaneously growing human xenografts in the same type of mice (32).

Possibly, *in vivo* the tumors were not hypoxic enough to drive the cells to glycolysis. The pattern and the fraction of the tumor staining hypoxic was similar in the WT and VHL KO tumors. Although it has been shown that HIF-1α downregulates the mitochondrial oxygen consumption (33), it is possible that when growing *in vivo*, the VHL KO cells still may have used to some extend the oxidative phosphorylation pathway, which could explain the resistance to 2DG *in vivo*.

It has been shown that cells lacking pVHL show up-regulation different proteins that can stimulate tumor growth, such as PDGF, bFGF, VEGF and TGF-α (34). Previously we observed an increased expression of VEGF in the VHL KO cells when growing as subcutaneous tumors (BB- *Oncogene* in press). It is possible that the overexpression of VEGF and the additional growth factors may have compensated for the reduction in ATP synthesis, thereby allowing the tumor to grow.

Alternatively, 2DG alone may have less efficacy in reducing tumor growth of cells growing *in vivo*, and may be more effective when used in combination with other chemotherapy drugs in this setting. This was supported by a previous report showing that treatment of subcutaneously growing human osteosarcoma cells with 2DG alone did not alter tumor growth compared to the untreated controls, whereas in combination with Adriamycin this was significantly reduced (32).

Collectively, our data indicates that under *in vitro* growth conditions 2DG seems to be an effective growth inhibitor in cells that overexpress HIF-1α, but its effects *in vivo* need to be further evaluated.

REFERENCES

1. Harris, A. L. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer, *2:* 38-47, 2002.
2. Brizel, D. M., Sibley, G. S., Prosnitz, L. R., Scher, R. L., and Dewhirst, M. W. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int J Radiat Oncol Biol Phys, *38:* 285-289, 1997.
3. Buchler, P., Reber, H. A., Lavey, R. S., Tomlinson, J., Buchler, M. W., Friess, H., and Hines, O. J. Tumor hypoxia correlates with metastatic tumor growth of pancreatic cancer in an orthotopic murine model. J Surg Res, *120:* 295-303, 2004.
4. Hockel, M., Schlenger, K., Aral, B., Mitze, M., Schaffer, U., and Vaupel, P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res, *56:* 4509-4515, 1996.
5. Hockel, M., Schlenger, K., Hockel, S., and Vaupel, P. Hypoxic cervical cancers with low apoptotic index are highly aggressive. Cancer Res, *59:* 4525-4528, 1999.
6. Semenza, G. L. Targeting HIF-1 for cancer therapy. Nat Rev Cancer, *3:* 721-732, 2003.
7. Racker, E. History of the Pasteur effect and its pathobiology. Mol Cell Biochem, *5:* 17-23, 1974.
8. Semenza, G. L., Roth, P. H., Fang, H. M., and Wang, G. L. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem, *269:* 23757-23763, 1994.
9. Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F., and Maity, A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem, *276:* 9519-9525, 2001.
10. Seagroves, T. N., Ryan, H. E., Lu, H., Wouters, B. G., Knapp, M., Thibault, P., Laderoute, K., and Johnson, R. S. Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol Cell Biol, *21:* 3436-3444, 2001.
11. Mahon, P. C., Hirota, K., and Semenza, G. L. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev, *15:* 2675-2686, 2001.
12. Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. Embo J, *20:* 5197-5206, 2001.
13. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science, *295:* 858-861, 2002.
14. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science, *292:* 464-468, 2001.
15. Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R. C., Conaway, J. W., Klausner, R. D., and Pause, A. Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. Proc Natl Acad Sci U S A, *96:* 12436-12441, 1999.
16. Lisztwan, J., Imbert, G., Wirbelauer, C., Gstaiger, M., and Krek, W. The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. Genes Dev, *13:* 1822-1833, 1999.
17. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature, *399:* 271-275, 1999.
18. Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R. C., and Conaway, J. W. Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. Proc Natl Acad Sci U S A, *97:* 10430-10435, 2000.
19. Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol, *2:* 423-427, 2000.

20. Tanimoto, K., Makino, Y., Pereira, T., and Poellinger, L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. Embo J, *19:* 4298-4309, 2000.
21. Dames, S. A., Martinez-Yamout, M., De Guzman, R. N., Dyson, H. J., and Wright, P. E. Structural basis for Hif-1 alpha /CBP recognition in the cellular hypoxic response. Proc Natl Acad Sci U S A, *99:* 5271-5276, 2002.
22. Freedman, S. J., Sun, Z. Y., Poy, F., Kung, A. L., Livingston, D. M., Wagner, G., and Eck, M. J. Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha. Proc Natl Acad Sci U S A, *99:* 5367-5372, 2002.
23. Hu, Y., Moraes, C. T., Savaraj, N., Priebe, W., and Lampidis, T. J. Rho(0) tumor cells: a model for studying whether mitochondria are targets for rhodamine 123, doxorubicin, and other drugs. Biochem Pharmacol, *60:* 1897-1905, 2000.
24. Liu, H., Hu, Y. P., Savaraj, N., Priebe, W., and Lampidis, T. J. Hypersensitization of tumor cells to glycolytic inhibitors. Biochemistry, *40:* 5542-5547, 2001.
25. Liu, H., Savaraj, N., Priebe, W., and Lampidis, T. J. Hypoxia increases tumor cell sensitivity to glycolytic inhibitors: a strategy for solid tumor therapy (Model C). Biochem Pharmacol, *64:* 1745-1751, 2002.
26. Kang, H. T. and Hwang, E. S. 2-Deoxyglucose: an anticancer and antiviral therapeutic, but not any more a low glucose mimetic. Life Sci, *78:* 1392-1399, 2006.
27. Haase, V. H., Glickman, J. N., Socolovsky, M., and Jaenisch, R. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci U S A, *98:* 1583-1588, 2001.
28. Blouw, B., Song, H., Tihan, T., Bosze, J., Ferrara, N., Gerber, H. P., Johnson, R. S., and Bergers, G. The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell, *4:* 133-146, 2003.
29. Maher, J. C., Savaraj, N., Priebe, W., Liu, H., and Lampidis, T. J. Differential sensitivity to 2-deoxy-D-glucose between two pancreatic cell lines correlates with GLUT-1 expression. Pancreas, *30:* e34-39, 2005.
30. Camphausen, K., Purow, B., Sproull, M., Scott, T., Ozawa, T., Deen, D. F., and Tofilon, P. J. Influence of in vivo growth on human glioma cell line gene expression: convergent profiles under orthotopic conditions. Proc Natl Acad Sci U S A, *102:* 8287-8292, 2005.
31. Maher, J. C., Krishan, A., and Lampidis, T. J. Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions. Cancer Chemother Pharmacol, *53:* 116-122, 2004.
32. Maschek, G., Savaraj, N., Priebe, W., Braunschweiger, P., Hamilton, K., Tidmarsh, G. F., De Young, L. R., and Lampidis, T. J. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. Cancer Res, *64:* 31-34, 2004.
33. Papandreou, I., Cairns, R. A., Fontana, L., Lim, A. L., and Denko, N. C. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab, *3:* 187-197, 2006.
34. Staehler, M., Rohrmann, K., Haseke, N., Stief, C. G., and Siebels, M. Targeted agents for the treatment of advanced renal cell carcinoma. Curr Drug Targets, *6:* 835-846, 2005.

CHAPTER 5

Summarizing Discussion

SUMMARIZING DISCUSSION

Cancer cells respond to anaerobic conditions by the transcription of specific genes that are involved in apoptosis, cell proliferation, glycolysis, angiogenesis and invasion. This adaptation allows the tumor cells to survive, and to metastasize to a distant organ. As a consequence, tumor hypoxia is correlated with an increased malignancy and a diminished response to anti-cancer treatment. The transcriptional regulation of the genes that are induced upon hypoxia are orchestrated by the members of the Hypoxia Inducible Factors; HIF-1α, HIF-2α and HIF-3α. HIF-1α is the main regulator of the response to hypoxia and is up regulated in many different solid tumor types. In cancer HIF-1α expression can be increased as a result of reduced oxygen levels, activation of the PI(3)K pathway, or loss of its negative regulator the Von Hippel Lindau tumor suppressor gene. Similar to hypoxia, a high expression of HIF-1α correlates with a diminished prognosis in most tumor types.

One type of cancer where the activation of the hypoxic pathway is particularly important in determining its malignancy and treatment difficulties is the grade IV astrocytoma, or Glioblastoma Multiforme. Chapter 2 aims to achieve more insight into the role of HIF-1α and its target and angiogenic factor VEGF in astrocytoma formation, using a mouse astrocytoma implantation model. This model was created by implanting transformed astrocytes in which HIF-1α or VEGF were conditionally deleted, in the subcutis and brain of immunocompromised mice. In this chapter it is described that differences in the vascularity of the implantation site, or micro-environment, in which transformed astrocytes lacking HIF-1α are grown, can lead to diametrically opposing tumor growth characteristics. Transformed astrocytes in which HIF-1α was deleted (HIF KO) formed smaller tumors compared to the wild type controls when grown in the subcutaneous space. When the same cells were grown in their natural habitat, the brain, their growth was not impaired. This was revealed by the similar survival times of mice in which the wild type, or HIF-1α null astrocytes, were grown. The vessel morphology of the HIF KO astrocytoma growing in different areas (subcutaneously and in the brain) indicated that these tumors had not initiated angiogenesis. Moreover, in the brain, the HIF KO astrocytes were growing in clusters around the blood vessels, invading areas of the brain away from the initial implantation site. In the relatively poorly vascularized subcutaneous implantation site, the lack angiogenesis led to a growth disadvantage for the HIF KO astroyctes. In contrast, in the vessel rich environment of the brain this was not a limiting factor for tumor growth, and these cells survived by surrounding the existing blood vessels. VEGF, the main inducer of angiogenesis, is a well described target of HIF-1α. Therefore, it was proposed that the deficient angiogenesis in the HIF KO tumors could have been a result of a reduced expression of VEGF. Indeed, when grown in the subcutaneous area, ablation of VEGF in transformed astrocytes (VEGF KO) caused a similar reduction in tumor growth and vascularity as deletion of HIF-1α. In the brain VEGF KO astrocytes grew also along blood vessels, but these did not remain viable, and they became necrotic. This resulted eventually in a reduced tumor growth and invasion,

compared to the HIF KO grown in this site. These contrasting results between the HIF KO and VEGF KO astrocytes grown in the brain can be explained by the reduced, but still sufficient, levels of VEGF expression in the HIF KO cells. It has been described that during brain tumor angiogenesis co-opted blood vessels in the brain will survive in presence of sufficient levels of VEGF, whereas ablation of VEGF will induce apoptosis in these vessels (1).

Besides the vascularity of the tumor micro-environment, other factors can also lead to the activation of compensatory mechanisms upon the inactivation of the hypoxic pathway in cancer cells, and thereby influence the anti-tumor efficacy of inhibiting HIF-1α. The astrocytes used in the experiments described above were transformed with H-Ras, which activates the PI(3)K pathway. Certain human cancer cell lines used in other studies, have been passaged in tissue culture for many years. For both the Ras transformed astrocytes, and the human cancer cell lines it is unclear which additional mutations they have acquired or how this may affect the expression of HIF-1α, and hence the tumor growth upon its modulation. Nevertheless, the different additional mutations may help to explain the cell type specific tumor growth response to inhibition of HIF-1α, even if the tumor cells are implanted in their natural habitat. For instance, in human gastric cancer and breast cancer cell lines, a reduction in HIF-1α expression led to a reduced tumor growth and vascularization when grown in both the subcutaneous area and in the orthotopic implantation site (2, 3). Evidence of the induction of a compensatory angiogenic pathway when HIF-1α is inactivated, was demonstrated by Mizukami *et al.* (2005). They showed that human colon cancer cell lines in which HIF-1α expression was reduced by siRNA (HIF^{kd}), displayed a reduction in subcutaneous tumor growth but not in tumor vascularity, compared to the wild type controls. Microarray analysis of the tumors revealed that the HIF^{kd} tumors showed a 2-fold increase of pro-angiogenic factor interleukin-8 (IL-8). Neutralization of IL-8 in the HIF-1α deficient but not in the wild type tumors respectively, led to inhibition of angiogenesis and tumor growth, providing evidence that in these cells upon loss of HIF-1α, other factors were able to induce angiogenesis (4).

Given the strong correlation between the expression of HIF-1α and tumor progression, different pharmacological HIF-1α inhibitors have been tested for anti-tumor growth efficacy. These include Geldanamycin (GA), Topotecan and small molecule inhibitors of HIF-1α. GA inhibits HIF-1α function by preventing the interaction between Heat Shock Protein 90 (Hsp90) and the bHLH domain of HIF-1α, thereby promoting its ubiquitin mediated degradation (5). It was shown that GA can reduce HIF-1α mediated *in vitro* migration in different human glioma cell lines in a dose-dependent manner under hypoxic conditions (6). Human neuroblastoma cells exposed to Topotecan and inhibitors of the PI(3)K pathway, showed a reduction in the expression of HIF-1α and its target genes PGK and VEGF when exposed to hypoxia *in vitro* (7, 8). Further, tumor size and vascularity was reduced in subcutaneously grown glioma treated with Topotecan (8). The small molecule inhibitors of HIF-1α, PX-478 and YC-1 had a marked anti-tumor activity on different human cancer cell lines that were grown subcutaneously (9, 10). More recently, several novel small molecule inhibitors of HIF-1α were identified using a

screen of 2,000 compounds (the National Cancer Institute Diversity Set) that were tested for their ability to inhibit the hypoxic activation of different reporter genes under the control of an HRE promoter. These inhibitors were able to reduce the expression of HIF-1α and its target genes VEGF and Glut-1 in different human cancer cell lines grown under hypoxic conditions (11, 12).

These compounds show promising anti-cancer effects using *in vitro* and subcutaneously *in vivo* tumor growth assays. However, as can be concluded from the experiments described in Chapter 2, and those described above for the orthotopic implantation experiments of the human cancer cell lines in which HIF-1α expression is reduced, further evaluation of these compounds using a large panel of different human cancer cell lines that are grown orthotopically in mice, is desirable. In addition, mice in which HIF-1α deficient tumor cells are grown orthotopically, and that are treated with these compounds may confirm whether the effects on tumor growth are specifically due to HIF-1α inhibition.

Because the astrocytoma mouse implantation model was able to recapitulate some aspects of the interactions between the tumor cells and microenvironment that appeared important in tumor angiogenesis, this model was also used to study the contribution of HIF-1α and VEGF to the tumor growth and vascularity associated with Von Hippel Lindau disease, chapter 3. In this chapter it is described that transformed astrocytes lacking VHL (VHL KO) grown in the brain recapitulated some aspects of the angiogenic histopathology that are also observed in tumors of patients of VHL disease, such as the occurrence of hemangiomas and endothelial cell proliferation. With the exception of an increased vessel density, VHL KO astrocytes grown in the subcutaneous area did not recapitulate these aspects. In contrast to the subcutaneously grown tumors, these angiogenic characteristics were reduced upon deletion of HIF-1α in the VHL null astrocytes (HIF-1α/VHL KO) grown in the brain. This did not affect tumor growth however; mice in which the VHL KO and HIF-1α/VHL KO cells were grown had similar survival times. This site specific effect upon loss of HIF-1α on the vascularity of the VHL KO tumors was independent of the expression of VEGF, which was reduced in the HIF-1α/VHL KO tumors in both microenvironments. This led to the hypothesis that the VHL KO tumors could grow independently of VEGF expression. That appeared only to be the case in the brain tumor model; mice in which astrocytes lacking both VEGF and VHL (VEGF/VHL KO) were grown, had similar survival times as the mice implanted with the VHL KO astrocytes. In contrast, when the VEGF/VHL KO cells were grown in the subcutis, tumor growth was impaired compared to the VHL KO. Regardless of the micro-environment however, the tumor vascularity associated with loss of VHL was dramatically reduced upon loss of VEGF.

The reduction in tumor vascularity in the HIF-1α/VHL KO and VEGF/VHL KO tumors grown in the brain, did not modulate their tumor growth compared to the VHL KO astrocytes. Possibly when grown in the brain, the additional growth and invasion factors that are up regulated in cells that are deficient for VHL, may have provided compensatory growth mechanisms, thereby contributing to the similar brain tumor growth of the VHL KO, HIF-1α/VHL KO and

VEGF/VHL KO astrocytes. In this regard it was described that loss of VEGF in otherwise wild type cells, decreased the tumor formation in the brain compared to the VEGF/VHL KO cells (chapter 2). The factors that were shown to be upregulated in human cancer cells nullizygous for VHL include TGF-α/β, PDGF, bFGF and CXCR4, and some of those have also shown to be targets of HIF-2α. A thorough evaluation of HIF-2α function and target gene expression in the different astrocytes, and creating an astrocytoma implantation model in which HIF-2α is deleted in the VHL null background, may clarify whether this may have played a role in the brain tumor growth of these cells.

Tumors that arise in patients that have VHL disease, are in general benign and have a low metastatic rate. Depending on the organ where the lesions arise, the treatment of choice is surgical removal. However, many patients still are likely to develop a relapse of the tumor, or severe disease complications, such as blindness, heart damage or brain damage. Therefore it was proposed that a chronic VEGF blockade would be of great value to patients with VHL syndrome. Since that time, clinical trials have been carried out with either direct VEGF inhibitors, or inhibitors of the VEGF receptors. These treatments have had limited clinical results so far (13). The findings described in chapter 3 imply that the inhibition of VEGF effectively targets the tumor vascularity associated with loss of VHL. Examination of the VEGF/VHL KO cells grown in their native site revealed however, that inhibition of VEGF alone in these cells may not reduce overall tumor growth, which is consistent with the reports of the clinical trials.

One of the physiological adaptations to hypoxia is the switch from oxidative phosphorylation to glycolysis as a source to generate ATP (the Pasteur's effect). The observation that human cancer cells exposed to hypoxia displayed an increased growth inhibition when treated with the glucose analogue and glycolytic inhibitor 2-Deoxy-Glucose (2DG) (14, 15), led to the hypothesis that cells overexpressing HIF-1α would be sensitized to the growth inhibitory effects of 2DG. Chapter 4 describes that transformed astrocytes lacking VHL, which were shown to overexpress HIF-1α (chapter 3), show a dose-dependent sensitivity to 2DG when grown *in vitro*. This was correlated with a similar reduction in ATP synthesis. When grown as subcutaneous tumors however, there was no significant difference in the sensitivity to 2DG between the wild type and VHL KO cells, compared to their untreated counterparts. The reason of this discrepancy is not clear. The expression of the glucose transporter and HIF-1α target gene, Glut-1, was up regulated both *in vitro* (chapter 3) and *in vivo* in the VHL KO astrocytes (chapter 4). Despite this observation, one cannot rule out the possibility that the glucose uptake in the VHL KO astrocytes was not increased, which needs to be tested further. Alternatively, the up regulation of the many different growth factors, in these cells may have compensated for the reduction in ATP synthesis, thereby allowing the tumor to grow. In that regard, inhibition of the glycolytic pathway may not be a limiting factor for tumor growth of VHL KO cells in the subcutaneous implantation site. As is indicated by the findings described in chapter 2 and 3, inhibition of the angiogenic pathway can reduce tumor growth more effectively in VHL KO

cells grown in this area. Taken together, under *in vitro* growth conditions 2DG seems to be an effective growth inhibitor in cells that overexpress HIF-1α, but its effects *in vivo* need to be further evaluated.

The xenograft mouse implantation model has been a valuable tool for cancer research. It is frequently used to test novel anti-cancer drugs, or to elucidate the role of a tumor suppressor gene or oncogene in tumor growth (16-18). The findings described in this thesis demonstrate that anti-cancer therapies, such as those targeting the hypoxic and angiogenic pathway, may affect tumor growth in unexpected and different ways, depending on the implantation site used. Therefore, the microenvironment needs to be taken into consideration to achieve a better understanding of tumor growth behavior in a tissue specific context.

REFERENCES

1. Benjamin, L. E. and Keshet, E. Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc Natl Acad Sci U S A, *94:* 8761-8766, 1997.
2. Stoeltzing, O., McCarty, M. F., Wey, J. S., Fan, F., Liu, W., Belcheva, A., Bucana, C. D., Semenza, G. L., and Ellis, L. M. Role of hypoxia-inducible factor 1alpha in gastric cancer cell growth, angiogenesis, and vessel maturation. J Natl Cancer Inst, *96:* 946-956, 2004.
3. Li, J., Shi, M., Cao, Y., Yuan, W., Pang, T., Li, B., Sun, Z., Chen, L., and Zhao, R. C. Knockdown of hypoxia-inducible factor-1alpha in breast carcinoma MCF-7 cells results in reduced tumor growth and increased sensitivity to methotrexate. Biochem Biophys Res Commun, *342:* 1341-1351, 2006.
4. Mizukami, Y., Jo, W. S., Duerr, E. M., Gala, M., Li, J., Zhang, X., Zimmer, M. A., Iliopoulos, O., Zukerberg, L. R., Kohgo, Y., Lynch, M. P., Rueda, B. R., and Chung, D. C. Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. Nat Med, *11:* 992-997, 2005.
5. Mabjeesh, N. J., Post, D. E., Willard, M. T., Kaur, B., Van Meir, E. G., Simons, J. W., and Zhong, H. Geldanamycin induces degradation of hypoxia-inducible factor 1alpha protein via the proteosome pathway in prostate cancer cells. Cancer Res, *62:* 2478-2482, 2002.
6. Zagzag, D., Nomura, M., Friedlander, D. R., Blanco, C. Y., Gagner, J. P., Nomura, N., and Newcomb, E. W. Geldanamycin inhibits migration of glioma cells in vitro: a potential role for hypoxia-inducible factor (HIF-1alpha) in glioma cell invasion. J Cell Physiol, *196:* 394-402, 2003.
7. Beppu, K., Nakamura, K., Linehan, W. M., Rapisarda, A., and Thiele, C. J. Topotecan blocks hypoxia-inducible factor-1alpha and vascular endothelial growth factor expression induced by insulin-like growth factor-I in neuroblastoma cells. Cancer Res, *65:* 4775-4781, 2005.
8. 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. Schedule-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation, angiogenesis, and tumor growth by topotecan in U251-HRE glioblastoma xenografts. Cancer Res, *64:* 6845-6848, 2004.
9. Welsh, S., Williams, R., Kirkpatrick, L., Paine-Murrieta, G., and Powis, G. Antitumor activity and pharmacodynamic properties of PX-478, an inhibitor of hypoxia-inducible factor-1alpha. Mol Cancer Ther, *3:* 233-244, 2004.
10. Yeo, E. J., Chun, Y. S., Cho, Y. S., Kim, J., Lee, J. C., Kim, M. S., and Park, J. W. YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. J Natl Cancer Inst, *95:* 516-525, 2003.
11. Tan, C., de Noronha, R. G., Roecker, A. J., Pyrzynska, B., Khwaja, F., Zhang, Z., Zhang, H., Teng, Q., Nicholson, A. C., Giannakakou, P., Zhou, W., Olson, J. J., Pereira, M. M., Nicolaou, K. C., and Van Meir, E. G. Identification of a novel small-molecule inhibitor of the hypoxia-inducible factor 1 pathway. Cancer Res, *65:* 605-612, 2005.
12. Chau, N. M., Rogers, P., Aherne, W., Carroll, V., Collins, I., McDonald, E., Workman, P., and Ashcroft, M. Identification of novel small molecule inhibitors of hypoxia-inducible factor-1 that differentially block hypoxia-inducible factor-1 activity and hypoxia-inducible factor-1alpha induction in response to hypoxic stress and growth factors. Cancer Res, *65:* 4918-4928, 2005.
13. Staehler, M., Rohrmann, K., Haseke, N., Stief, C. G., and Siebels, M. Targeted agents for the treatment of advanced renal cell carcinoma. Curr Drug Targets, *6:* 835-846, 2005.
14. Maher, J. C., Krishan, A., and Lampidis, T. J. Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions. Cancer Chemother Pharmacol, *53:* 116-122, 2004.
15. Liu, H., Savaraj, N., Priebe, W., and Lampidis, T. J. Hypoxia increases tumor cell sensitivity to glycolytic inhibitors: a strategy for solid tumor therapy (Model C). Biochem Pharmacol, *64:* 1745-1751, 2002.

16. Rangarajan, A. and Weinberg, R. A. Opinion: Comparative biology of mouse versus human cells: modelling human cancer in mice. Nat Rev Cancer, *3:* 952-959, 2003.
17. Sausville, E. A. and Burger, A. M. Contributions of human tumor xenografts to anticancer drug development. Cancer Res, *66:* 3351-3354, discussion 3354, 2006.
18. Sharpless, N. E. and Depinho, R. A. The mighty mouse: genetically engineered mouse models in cancer drug development. Nat Rev Drug Discov, *5:* 741-754, 2006.

Samenvatting

SAMENVATTING

Tijdens de ontwikkeling en groei van een solide tumor, staan de tumorcellen bloot aan een gebrek aan zuurstof (hypoxie). Dit is het gevolg van de snelle groei van de tumor, waardoor het netwerk van bloedvaten onvoldoende in staat is om de cellen van zuurstof te voorzien. Daarnaast functioneren de nieuwe bloedvaten die door de tumor zelf zijn gevormd meestal minder goed, waardoor de cellen eveneens met een zuurstof gebrek te maken krijgen. Een gebrek aan zuurstof kan leiden tot het afsterven van een cel, maar cellen hebben ook mechanismen ontwikkeld waardoor ze onder zuurstofarme omstandigheden kunnen overleven. Deze aanpassingen bestaan uit veranderingen in de hoeveelheid eiwitten die als het product van bepaalde genen worden gemaakt ('hypoxia target genes'). De verhoogde expressie van deze eiwitten kan de aanmaak van nieuwe bloedvaten stimuleren (angiogenese), of ze kunnen het losmaken van een cel uit de omgeving vergemakkelijken waarbij deze kan migreren. Bij de ontwikkeling van kanker, kunnen deze aanpassingen bijdragen aan de verspreiding van de cellen door het lichaam (metastase). Zo is bij veel verschillende soorten solide tumoren een lage zuurstof concentratie gecorreleerd met een meer agressief tumor type.

Sinds het vaststellen van dit verband, is intensief onderzoek gedaan naar welke factoren deze veranderingen reguleren. In de jaren '90 heeft dit geleid tot de identificatie van een aantal eiwitten die de expressie van de 'hypoxia target genes' reguleren. Deze eiwitten worden de hypoxie ('Hypoxia') Induceerbare ('Inducible') Factoren ('Factors') of HIFs, genoemd. Er zijn 3 HIFs geïndentificeerd, HIF-1α, HIF-2α en HIF-3α. Hoewel al deze factoren worden geïnduceerd in een cel bij een verlaging van de concentratie van zuurstof, speelt HIF-1α de belangrijkste rol in het reguleren van de 'hypoxia target gene' expressie.

Ongeacht de zuurstofspanning wordt er constant HIF-1α eiwit gemaakt in een cel. Echter, onder een normale concentratie van zuurstof wordt HIF-1α direkt weer afgebroken, doordat het zich bindt aan een ander eiwit; Von Hippel Lindau (VHL). VHL is een speciaal type eiwit dat ervoor zorgt dat de andere eiwitten die het bindt, uiteindelijk worden afgebroken. Zo heeft het binden van HIF-1α aan VHL tot gevolg dat HIF-1α direkt wordt afgebroken. Deze interactie is schematisch aangegeven in in figuur 1, in het eerste hoofdstuk. VHL en HIF-1α kunnen alleen maar aan elkaar binden als er voldoende zuurstof aanwezig is. Daardoor blijft onder die omstandigheden de hoeveelheid HIF-1α eiwit in de cel laag, en kan het verder geen andere genen activeren. Zodra de concentratie van zuurstof afneemt, kan VHL niet meer binden aan HIF-1α. Hierdoor neemt de hoeveelheid HIF-1α in de cel toe en wordt het naar de celkern getransporteerd, waar het bepaalde genen activeert (de 'HIF-1α target genes'). VHL kan zowel de hoeveelheid HIF-1α, als die van HIF-2α reguleren. Het is echter ook een gen dat bij inactivatie kanker veroorzaakt; het Von Hippel Lindau syndroom. Mensen met mutaties in het VHL gen, hebben een verhoogde kans op de ontwikkeling van tumoren in bepaalde organen waaronder in de hersenen en nieren. Deze tumoren zijn vaak zeer intensief doorbloed. Naast

een grote hoeveelheid HIF-1α en HIF-2α, worden ook een verhoogde hoeveelheid eiwitten van de 'HIF target genes' aangetroffen in deze tumoren.

Na de ontdekking van HIF-1α, bleek dat het in veel verschillende tumoren in verhoogde hoeveelheden wordt gemaakt. Net als voor hypoxie, geldt voor de meeste soorten tumoren dat veel HIF-1α gecorreleerd is met een minder goede prognose, terwijl weinig of geen HIF-1α gecorreleerd is met een betere prognose. Deze correlatie staat aangegeven in het eerste hoofdstuk, tabel 1. HIF-1α is in staat is om direkt de expressie van genen te reguleren die een belangrijke rol spelen bij bijvoorbeeld metastase of de angiogenese. Een eiwit dat een belanrijke rol speelt bij dit laatste proces is de 'Vascular Endothelial Growth Factor' of VEGF; dit is een van de 'HIF-1α target genes'.

Een bepaald type kanker waarbij een grote hoeveelheid van HIF-1α en VEGF correleert met de moeilijkheid van behandeling en het agressieve karakter, zijn de hersentumoren van het type Glioblastoma Multiforme. Echter, het is niet precies duidelijk welke rol deze factoren spelen bij de ontwikkeling van deze tumor. In hoofdstuk 2 staan de resultaten beschreven van de proeven die zijn uitgevoerd om hier meer duidelijkheid over te scheppen. Dit is gedaan door de Glioblastoma in een muis te reproduceren, maar op zo'n manier dat de tumorcellen het eiwit HIF-1α niet meer kunnen maken. De cellen waarin dit is gedaan zijn de astrocyten, dit is hetzelfde celtype waaruit deze tumor bij de mens ontstaat. De astrocyten zijn verkregen van speciale muizen, en tijdens het kweken genetisch veranderd. Na die verandering zijn de cellen die geen HIF-1α meer kunnen maken HIF KO genoemd. De controlecellen zijn HIF WT genoemd; zij zijn nog wel in staat HIF-1α te produceren. Die cellen zijn vervolgens in muizen geïmplanteerd waarvan het immuunsysteem is uitgeschakeld. Hierdoor kunnen ze deze cellen niet meer afstoten, waardoor die uitgroeien tot een tumor. Dit is een veel gebruikte methode om de functie van een bepaald gen bij de ontwikkeling van kanker te onderzoeken, of om een nieuwe therapie tegen kanker te testen. Meestal worden de cellen in de flank van de muis gebracht en als de tumor groot genoeg is, kan deze eruit worden gehaald en onderzocht worden op bepaalde veranderingen. Dit hebben wij ook gedaan, maar om de Glioblastoma Multiforme te kunnen reproduceren, zijn deze cellen eveneens in de hersenen van deze muizen geïmplanteerd. Uit de tumorgroei in de flank van de muis bleek dat HIF-1α nodig is om te kunnen groeien; de HIF KO tumor was kleiner in vergelijking tot de HIF WT. De verwachting was vervolgens dat dit resultaat gereproduceerd zou kunnen worden als dezelfde cellen in de hersenen zouden groeien. In de hersenen vormden de HIF KO echter geen kleinere tumoren in vergelijking tot de controle groep. Daarnaast bleek dat de HIF KO astrocyten in kleine groepjes rond de bestaande bloedvaten van de hersenen groeiden, en zich door de hersenen hadden verspreid. Verdere analyse van deze tumoren liet zien dat in zowel de flank als in de hersenen, de HIF KO tumoren niet in staat waren geweest om nieuwe bloedvaten te kunnen maken. Toch leidde dit alleen tot een verminderde tumorgroei in de flank. Een van de grote verschillen tussen de hersenen en de flank van de muis is dat de hersenen zeer intensief doorbloed zijn, terwijl de flank in vergelijking minder goed doorbloed

is. Dat de HIF KO cellen niet hun eigen bloedvaten konden maken, had daardoor alleen een groei-remmend effect wanneer ze in de flank groeiden. In de hersenen konden ze profiteren van het al aanwezige netwerk van bloedvaten, en konden ze om de vaten heen groeien; het niet kunnen uitvoeren van de angiogenese was hier geen belemmerende factor.

Zoals beschreven kunnen mensen die lijden aan het VHL syndroom tumoren krijgen waarin grote hoeveelheden HIF-1α, HIF-2α en de eiwitten van de verschillende 'HIF target genes' zoals VEGF worden aangetroffen. Het is onduidelijk in welke mate deze factoren tot de groei van de tumor bijdragen. Om dit te onderzoeken is dezelfde strategie toegepast als hierboven beschreven, waarbij astrocyten van muizen zondanig genetisch veranderd zijn dat ze geen VHL (VHL KO), of geen HIF-1α en VHL (HIF-1α/VHL KO), of geen VEGF en VHL (VEGF/VHL KO) meer kunnen maken. Die cellen zijn in de muis geïmplanteerd, in zowel de flank als de hersenen. De resultaten hiervan staan beschreven in hoofdstuk 3. Uit de analyse van de tumoren die in de flank waren geïmplanteerd, bleek dat HIF-1α niet bijdraagt aan de groei van de VHL KO tumoren; de VHL KO en HIF-1α/VHL KO tumoren waren even groot. Echter, de VEGF/VHL KO tumoren waren kleiner, wat erop duidt dat deze factor belangrijk is voor de tumorgroei van de VHL KO astrocyten op deze locatie in de muis. Vervolgens werden de cellen in de hersenen geïmplanteerd. Ook in deze locatie droeg HIF-1α niet bij aan de tumorgroei van de VHL KO astrocyten. Echter, verlies van VEGF expressie in de VHL KO astrocyten had evenmin een effect op tumorgroei; de muizen met de VEGF/VHL KO astrocyten in de hersenen hadden dezelfde overlevingstijd als de muizen met de VHL KO cellen. Deze resultaten zouden verklaard kunnen worden door de compensatie van de aanwezige andere groeifactoren of door HIF-2α, die alleen een voordeel gaven als de HIF-1α/VHL KO, VEGF/VHL KO en VHL KO cellen in de hersenen groeiden. Om dit laatste te onderzoeken, zouden de proeven herhaald kunnen worden met astrocyten die zowel HIF-2α als VHL niet meer kunnen maken (HIF-2α/VHL KO).

Een andere aanpassing van een cel aan hypoxie is de verandering van energiebron. Bij voldoende zuurstof wordt energie gehaald uit een biochemisch proces welke zuurstof vereist. Als daar een gebrek aan komt, dan kan de cel energie halen uit de glycolyse waar geen zuurstof voor nodig is. Het is mogelijk om de glycolyse te remmen met behulp van een bepaalde stof: 2-deoxy-D-glucose (2DG). In het geval dat een cel volledig afhankelijk is van de glycolyse als energiebron en vervolgens wordt blootgesteld aan 2DG, dan sterft deze uiteindelijk af. Dit verband is een aantal jaren geleden aangetoond: cellen die blootgesteld staan aan hypoxie, zijn gevoeliger voor 2DG dan wanneer ze onder normale zuurstof concentratie groeien. Omdat HIF-1α essentieel is voor de glycolyse, hebben we getest of cellen die veel HIF-1α maken, gevoeliger zijn voor 2DG. Dit zou kunnen betekenen dat 2DG de tumorvorming in VHL patiënten tegen kan gaan, zonder dat dit tot grote bijwerkingen zou leiden. Daartoe hebben we de VHL KO astrocyten blootgesteld aan 2DG. De resultaten van deze proeven staan beschreven in hoofdstuk 4. Tijdens het kweken van deze cellen in een schaaltje, bleek dat 2DG effectiever de groei kon remmen van VHL KO cellen in vergelijking tot de VHL WT cel-

len. Echter, wanneer deze cellen als tumor in de flank van een muis groeiden, had 2DG geen groter remmend effect op de groei van de VHL KO tumoren. Het kan zijn dat in deze situatie, de groeifactoren die in die VHL KO cellen worden gemaakt zoals VEGF, compenseerden voor de remming van de glycolyse. Dit valt af te leiden uit de resultaten die zijn beschreven in hoofdstuk 3, waaruit blijkt dat het gebrek aan VEGF in de VHL KO cellen tot een verminderde tumorgroei in de flank leidt.

Het remmen van HIF-1α lijkt een aantrekkelijke methode om de tumorgroei te reduceren, omdat hiermee de processen worden aangepakt die ten grondslag liggen aan de ontwikkeling van een tumor naar een meer agressief karakter. Er zijn dan ook farmacologische stoffen ontdekt die de activiteit van HIF-1α kunnen remmen. Deze stoffen verminderden de tumorgroei in de flank van een muis, net als in het geval van de HIF KO astrocyten (hoofdstuk 2). Ook is het reduceren van de productie of activiteit van VEGF onderzocht als therapie voor mensen die lijden aan het VHL syndroom. Echter, deze therapie leidde niet tot een afname van kanker in deze patiënten. De resultaten die in hoofdstuk 3 staan beschreven, duiden eveneens op een beperkt effect van VEGF inactivatie op het verminderen van tumorcellen die VHL kunnen maken, al werd dit pas duidelijk door dit te bestuderen in de omgeving waar deze cellen normaal gesproken de tumor vormen. Zoals beschreven in dit proefschrift, kan het moduleren van de factoren HIF-1α of VEGF soms leiden tot onverwachte resultaten, afhankelijk van de locatie waar deze cellen groeien. Het is dus belangrijk om bij de ontwikkeling van therapiën die erop gericht zijn om deze eiwitten te remmen, ook het effect ervan te evalueren tijdens de groei van een tumor in het oorspronkelijke orgaan waar deze is ontstaan.

Publications

Barbara Blouw, Hanqui Song, Tarik Tihan, Jenel Bosze, Napoleone Ferrara, Hans-Peter Gerber, Randall S. Jonhnson and Gabriele Bergers.
The hypoxic response of tumors is dependent on their microenvironment.
Cancer Cell 4:133-146 (2003)

Barbara Blouw, Volker H. Haase, Hanqiu Song, Gabriele Bergers and Randall S. Johnson.
Loss of vascular endothelial growth factor expression reduces vascularization, but not growth, of tumors lacking the Von Hippel-Lindau tumor suppressor gene.
Oncogene in press

Rob Helton, Jiankun Cui, John R. Scheel, Julie A. Ellison, Chris Ames, Claire Gibson, **Barbara Blouw**, Ling Ouyang, Ioannis Dragatsis, Scott Zeitlin, Randall S. Johnson, Stuart A. Lipton and Carrolee Barlow.
Brain-Specific Knock-Out of Hypoxia-Inducible Factor –1α Reduces Rather Than Increases Hypoxic-Ischemic Damage.
The Journal of Neuroscience 16: 4099-4107 (2005)

Curriculum Vitae

Barbara Blouw werd geboren op 5 April 1972. Na het doorlopen van de MAVO en HAVO, behaalde zij in 1994 het VWO diploma op het Saenredam College in Zaandijk. In 1995 begon zij aan de studie Medische Biologie aan de Vrije Universiteit in Amsterdam. Zij liep stage bij de vakgroep Medische Oncologie bij de groep van Dr. Epie Boven. Van october 1998 tot september 1999 verbleef zij in verband met een door het Nederlandse Kanker Bestrijding/Koningin Wilhelmina fonds (NKB/KWF) gefinancierde buitenlandse stage in de groep van Dr. Gerrit Los binnen de School of Medicine, Deparment of Medical Pharmacology aan de University of California te San Diego, USA. In maart 2000 behaalde zij haar doctoraal examen. Een maand later begon zij als onderzoeker in opleiding bij de afdeling Moleculaire Carcinogenese op het Nederlands Kanker Instituut in de groep van Dr. Hein te Riele. San Diego bleef trekken, en na enkele maanden besloot zij om terug te gaan. In januari 2001 begon zij haar promotie onderzoek als 'graduate student' onder leiding van Dr. Randall S. Johnson binnen de Division of Biology, Section of Molecular Biology aan de University of California, San Diego. Sinds oktober 2006 is zij werkzaam als post-doctoral fellow in de groep van Dr. Sara A. Courtneidge op het Burnham Institute for Medical Research te La Jolla, USA.

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of course it is great that you are also a little bit Dutch, but I really enjoyed our discussions about science and the politics in Europe or the US. Adam, besides talking about your mice with (too many?) interesting phenotypes, it was also fun to take care of your cats during the holidays. Christian, it was a pleasure to make you enthusiastic about immunohistochemistry, I hope you still like it. But as you know all too well by now, antibodies can also be really tricky sometimes. The only girl in the 'guys office', Courtney, thanks for always getting in the orders, no matter how great the rush was, and tracking them down if they had not come in. Good luck getting into Medical School.

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