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Targeting the molecular basis for tumour hypoxia

Veronica A. Carroll and Margaret Ashcroft

Tumour hypoxia stems from impaired oxygen delivery as a result of a disorganised tumour vasculature and inadequate blood supply. Hypoxic tumours are highly resistant to chemotherapy and radiation therapy and correlate with a poor patient prognosis. Hypoxia is a powerful stimulus for the expression of genes involved in cell survival and angiogenesis. A key factor in this process is hypoxia-inducible factor (HIF), which regulates transcription of hypoxia-activated genes. Efforts are currently under way to develop targeted cancer therapeutics to hypoxia-activated pathways, and in particular to the transcription factor HIF.

All tissues depend on oxygen delivery for normal metabolic and physiological functions. In higher organisms, simple diffusion of oxygen is no longer adequate to provide metabolising cells with the oxygen and nutrients they require. In order to overcome this limitation, specialised respiratory and circulatory systems have evolved in mammals to ensure effective oxygen delivery. In ischaemia and other vascular diseases, regions of hypoxia (lowered oxygen concentration) arise when oxygen delivery is ineffective or blocked. Cells restore oxygen homeostasis and attempt to meet their metabolic demands by switching on a broad range of genes involved in increased oxygen transport (erythropoietin), glucose uptake (GLUT-1), glycolysis (lactate dehydrogenase A), pH regulation (CA9 and CA12) and angiogenesis [vascular endothelial growth factor (VEGF), endothelin 1] (Refs 1, 2).

In tumours, rapid expansion of proliferating cells means that they often outgrow their vascular supply, resulting in impaired oxygen delivery and regions of tumour hypoxia. In addition, new blood vessels arising from tumour angiogenesis are disorganised and have poor blood flow, contributing to hypoxic areas within the tumour (Fig. 1). Although severe hypoxia will lead to cell death, many tumour cells have undergone genetic alterations that confer resistance to apoptosis. These cells are commonly deficient in the tumour

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suppressor p53: loss of p53 function – through mutation, deletion of the gene, or deregulation of the pathway – occurs in the majority of cancers (Ref. 3). p53-null tumours also display a reduced response to anti-angiogenic therapy, indicating a decreased dependency of these tumours on vascular supply (Ref. 4). As well as resistance to apoptosis, hypoxia promotes an aggressive tumour phenotype by initiating tumour cell survival mechanisms (Ref. 5), increased cell proliferation, migration (Ref. 6) and invasion (Ref. 7). Tumour cells activate these pathways by a variety of hypoxia-inducible transcription factors, including nuclear factor κB (NF-κB), activator protein 1 (AP-1) and p53. However, the major hypoxia-regulated transcription factor is hypoxia-inducible factor (HIF). Techniques such as serial analysis of gene expression (SAGE) and cDNA gene expression microarray have contributed to the identification of a large number of hypoxia-inducible genes (Refs 8, 9), many of which are regulated by HIF. At least 70 genes have been identified so far as targets of HIF, implicating this transcription factor as the predominant regulator of oxygen homeostasis in cells (for a comprehensive list of HIF target genes, see Ref. 9).

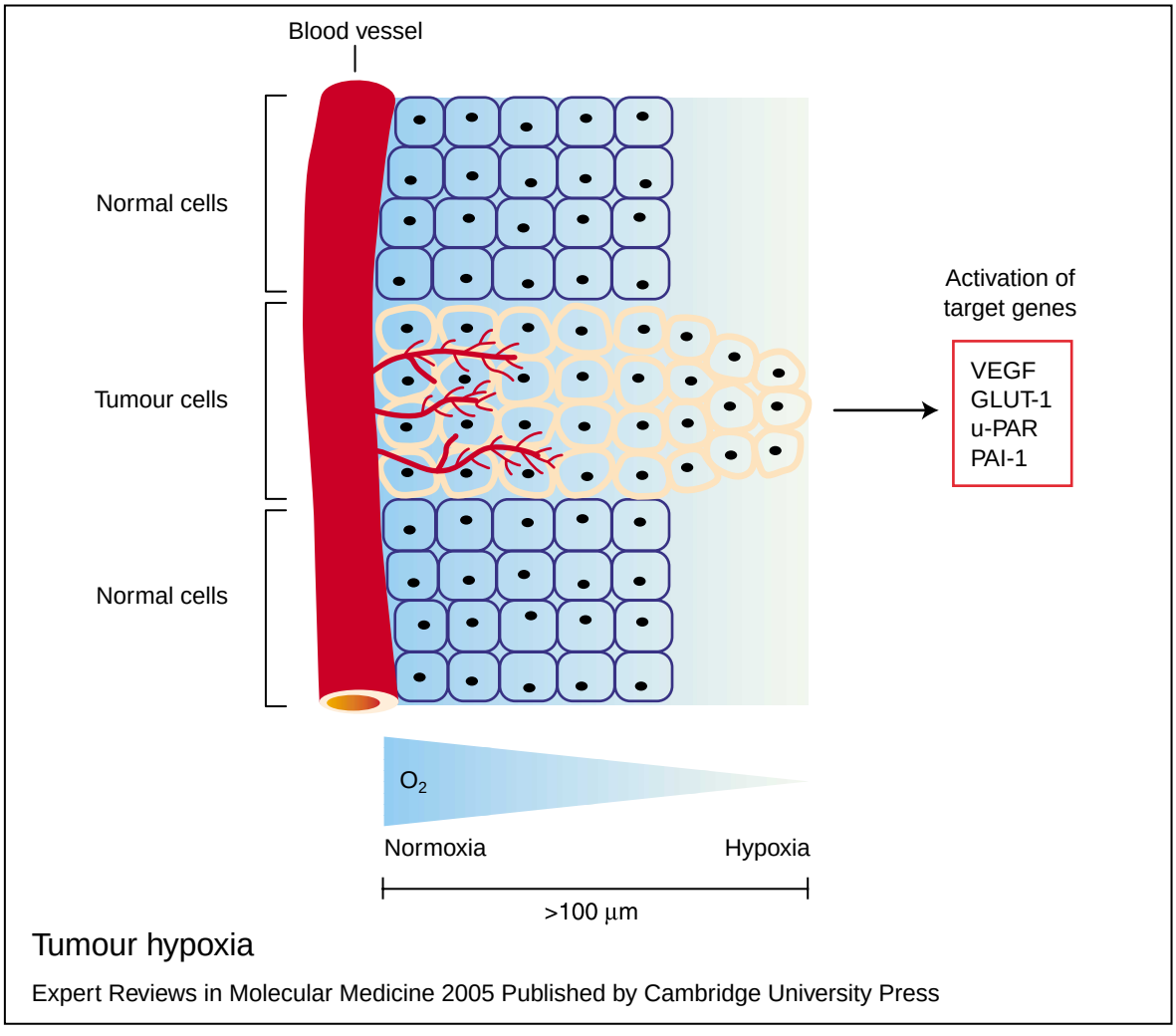

Figure 1. Tumour hypoxia. When a small, localised tumour outgrows its vascular supply (distances >100 μm) tumour hypoxia arises in regions with impaired oxygen delivery. Consequently, hypoxic cells switch on target genes involved in angiogenesis [vascular endothelial growth factor (VEGF)], glucose transport [glucose transporter 1 (GLUT-1)] and cell migration [urokinase-type plasminogen activator receptor (u-PAR) and plasminogen activator inhibitor 1 (PAI-1)]. Increased vascular supply to the tumour via the induction of new blood vessel formation (angiogenesis) encourages tumour growth and facilitates metastasis to distant sites.

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The central importance of HIF to hypoxic regulation has led to its recognition as a potential therapeutic target for treating hypoxic tumours (Refs 10, 11, 12). HIF-1α, the regulatory subunit of HIF-1, is widely expressed in a number of cancers and can lead to an aggressive malignant phenotype and tumour angiogenesis. HIF is therefore an attractive target for therapy. This review discusses the current strategies for targeting tumour hypoxia, with a particular focus on HIF as a potential therapeutic target. By way of introduction, the molecular mechanisms of HIF regulation are reviewed in detail.

HIF protein domains

HIF-1 is a heterodimer consisting of a HIF-1α subunit and a HIF-1β subunit [the latter is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT)] (Ref. 13). HIF-1 was first described as a hypoxia-inducible DNA-binding factor that mediates transcriptional activation of the human erythropoietin gene enhancer (Refs 14, 15), and was subsequently found to mediate hypoxic induction of genes involved in glycolysis (Refs 13, 16). HIF-1 heterodimers recognise a conserved DNA consensus sequence, known as the hypoxia-response element (HRE), located in the promoters of target genes. For full transcriptional activity, HIF-1 recruits the transcriptional co-activator p300/CREB-binding protein (CBP) (Ref. 17).

HIF subunits contain one basic–helix–loop–helix (bHLH) domain and two PER–ARNT–SIM (PAS) domains in their N-terminal regions (Fig. 2); it is these domains that are responsible for DNA binding (Refs 18, 19). HIF-1α and HIF-1β form heterodimers through protein–protein interactions via their PAS domains to form the HIF-1 complex. Recent data have shown that HIF-1α can also interact with Myc via its N-terminal region, indicating a more complex regulatory role for the N-terminal domain (Ref. 20). In addition, HIF-1α contains two transcriptional activation domains – the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD) – which are linked by an inhibitory domain (Refs 21, 22). The NTAD overlaps with a region known as the oxygen-dependent degradation (ODD) domain, and post-translational modification of this domain regulates the stability of the α-subunit (see below) (Ref. 23). HIF-1β also contains a transactivation domain (TAD) within its C-terminus; however, this does not appear to be required for HIF-1 activity, although it does have other functions (Ref. 24).

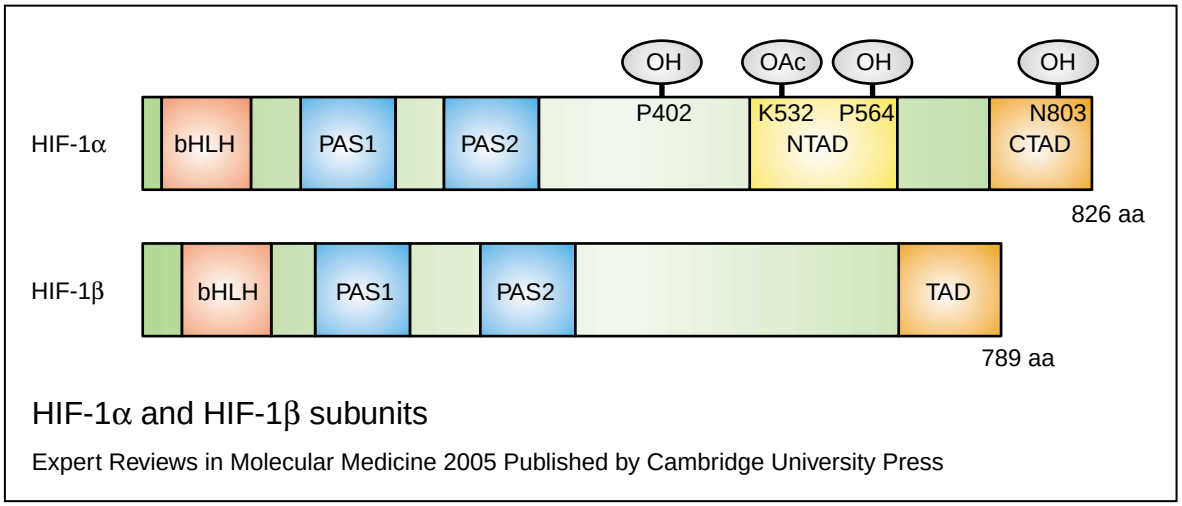

Figure 2. HIF-1α and HIF-1β subunits. Hypoxia-inducible factor (HIF)-1α and HIF-1β contain one basic–helix–loop–helix (bHLH) domain and two PER–ARNT–SIM (PAS1 and PAS2) domains in their N-terminal regions. The positions of post-translational hydroxylation (OH) and acetylation (OAc) of HIF-1α are indicated. Hydroxylation of two proline residues (at P402 and P564) and acetylation of lysine (at K532) within the oxygen-dependent degradation (ODD) domain (residues 401–603) and close to the N-terminal transactivation domain (NTAD) confers recognition by pVHL (the product of the von Hippel–Lindau tumour suppressor gene), leading to degradation of the α-subunit. Hydroxylation at N803 in the C-terminal transactivation domain (CTAD) of HIF-1α inhibits recruitment of coactivators required for HIF-1α transcriptional activity. HIF-1β contains one transactivation domain (TAD) in its C-terminus. Abbreviation: aa, amino acids.

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In addition to HIF-1α, two other α-subunits have been identified: HIF-2α and HIF-3α. HIF-2α, otherwise known as endothelial PAS domain protein (EPAS) (Ref. 25) and hypoxia-like factor (HLF) (Ref. 26), has 48% sequence identity with HIF-1α and is regulated in a similar manner (Ref. 27). By contrast, HIF-3α, also called inhibitory PAS (IPAS) domain protein, has a high degree of similarity in the bHLH and PAS domains with HIF-1α and -2α, but lacks the CTAD (Ref. 28). Although HIF-1α and HIF-2α mRNAs are not upregulated by hypoxia in most circumstances, HIF-3α is regulated at the mRNA level in response to hypoxia (Ref. 29) and exists as several splice variants (Ref. 30). HIF-3α is thought to negatively regulate the HIF system (Refs 31, 32).

Regulation of HIF

HIF-α is predominantly regulated by post-translational hydroxylation, acetylation and phosphorylation, although other mechanisms of regulation do exist (Ref. 9). HIF-1α and HIF-1β subunits are constitutively expressed in cells at the mRNA level. Both HIF-1α and HIF-2α protein stability is tightly regulated by changes in cellular oxygen levels. In normoxia (normal oxygen concentrations), HIF-1α protein is rapidly degraded, but protein levels are stabilised at low oxygen concentrations. Rapid degradation of the α-subunit under normoxia occurs by hydroxylation of two proline residues (Pro402 and Pro564) within the ODD domain (Refs 33, 34, 35, 36) (Fig. 3). Hydroxylated HIF-1α is subsequently recognised by the product of the von Hippel–Lindau tumour suppressor gene (pVHL). pVHL, as part of a multisubunit ubiquitin ligase complex, tags the α-subunit with polyubiquitin, which allows recognition by the proteasome and subsequent degradation (Refs 37, 38, 39, 40). Three mammalian prolyl hydroxylases have been identified that hydroxylate HIF-1α – namely PHD1, 2 and 3 – in a reaction requiring oxygen, ferrous ions (Fe^{2+}) and 2-oxoglutarate (Refs 41, 42). Gene silencing with small interfering RNA (siRNA) has revealed a predominant role for PHD2 in hydroxylation of HIF-1α (Ref. 43). In hypoxia, proline hydroxylation is inhibited because oxygen is limiting. Lack of HIF-1α hydroxylation prevents pVHL from recognising HIF-1α, which leads to its accumulation and dimerisation with HIF-1β. In addition to hydroxylation, acetylation of HIF-1α at Lys532 by the acetyl transferase ARD1 also promotes pVHL binding and proteasomal degradation (Ref. 44).

In normoxia, additional regulation by hydroxylation of HIF-1α occurs at Asn803 in the CTAD. This is mediated by an asparagine hydroxylase, also known as factor inhibiting HIF (FIH). In this case, hydroxylation of Asn803 prevents activation of HIF target genes by blocking recruitment of transcriptional co-activators that compete for the same binding site (Refs 45, 46, 47, 48).

Another level of control of HIF-1α is achieved by negative regulation of HIF-1α mRNA by a natural antisense HIF (aHIF) in tumour cells and in response to prolonged hypoxia (Refs 49, 50).

As well as oxygen-dependent regulation, HIF-1α can be regulated by oxygen-independent mechanisms. HIF-1α expression and transactivation of target genes are induced by growth factors [such as insulin-like growth factor 1 (IGF-1), epidermal growth factor and heregulin], inflammatory mediators (tumour necrosis factor α and interleukin 1β), hormones (insulin) and oncogenes (Ras V12, v-Src) (for review, see Ref. 51). It is thought that the majority of these factors converge on two common cellular kinase pathways: the mitogen-activated protein kinase and the phosphoinositide 3-kinase (PI3K)/Akt signalling pathways. Activation of HIF-1 and its transcriptional targets mediated by these pathways can promote tumour growth and angiogenesis in addition to that induced by hypoxic signalling. Therefore, therapeutic targeting of HIF-1α might also be of benefit in tumours where growth-factor and oncogenic signalling contribute to tumour development.

HIF and tumour-suppressor function

Increased understanding of the pathways regulating HIF has shed light on the effects of loss of certain tumour suppressor genes. As indicated above, pVHL is important for proteasomal degradation of HIF-1α in normoxia. Loss of, or mutations in, the gene encoding pVHL give rise to von Hippel–Lindau (VHL) disease, a cancer syndrome with a predisposition to highly angiogenic renal cell carcinomas and haemangioblastomas (Ref. 52). Gene array studies in pVHL-negative renal carcinoma cells have revealed several upregulated pVHL target genes (Refs 53, 54, 55, 56), some of which are hypoxia-inducible and are known targets of HIF-1. It has been demonstrated that HIF-1α induction is an early event in the kidneys of patients with VHL disease (Ref. 57) and it has been suggested

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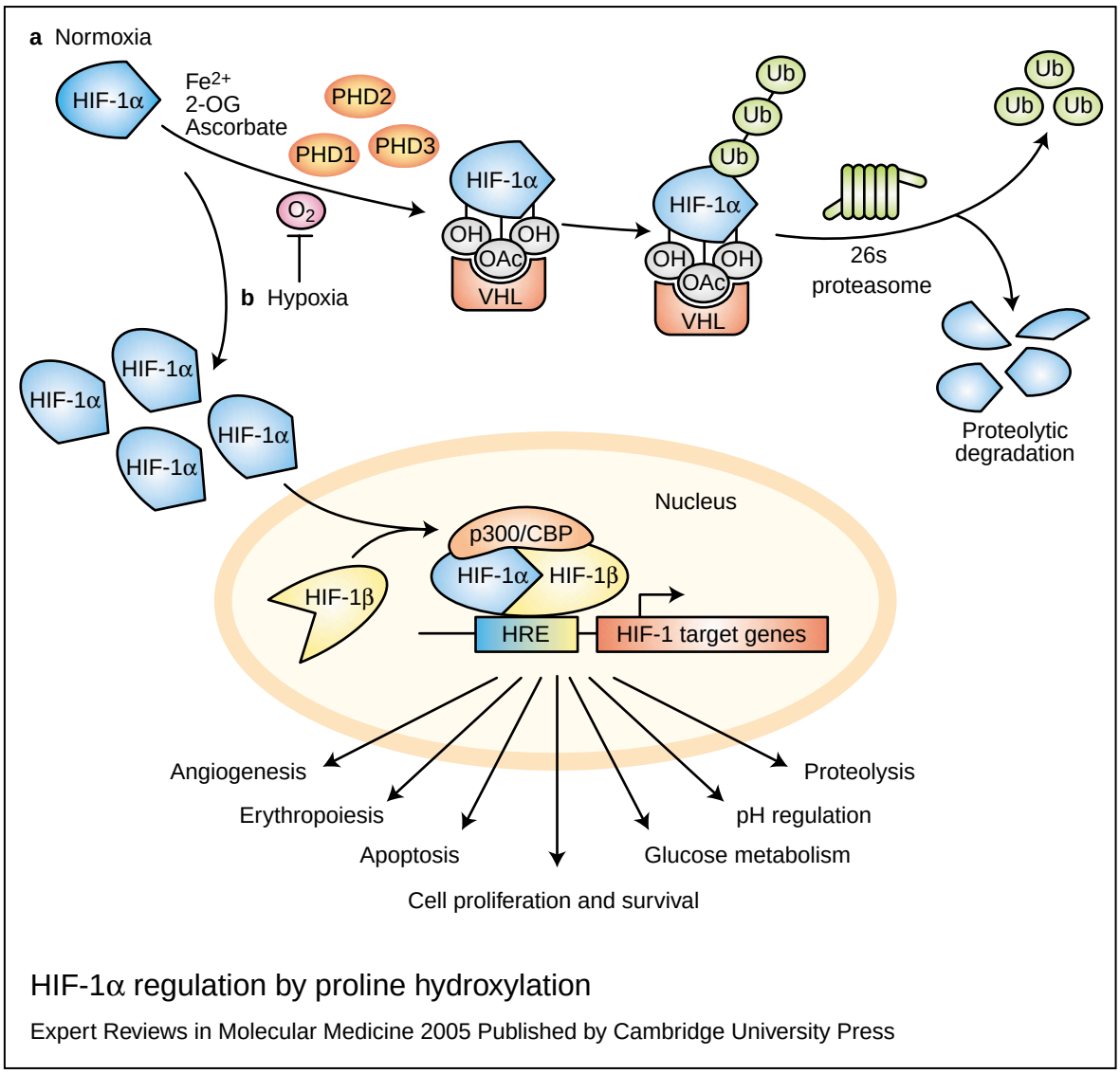

HIF-1α regulation by proline hydroxylation

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Figure 3. HIF-1α regulation by proline hydroxylation. (a) In normoxia, hypoxia-inducible factor (HIF)-1α is hydroxylated by proline hydroxylases (PHD1, 2 and 3) in the presence of O_2, Fe^{2+}, 2-oxoglutarate (2-OG) and ascorbate. Hydroxylated HIF-1α (OH) is recognised by pVHL (the product of the von Hippel–Lindau tumour suppressor gene), which, together with a multisubunit ubiquitin ligase complex, tags HIF-1α with polyubiquitin; this allows recognition by the proteasome and subsequent degradation. Acetylation of HIF-1α (OAc) also promotes pVHL binding. (b) In response to hypoxia, proline hydroxylation is inhibited. VHL is no longer able to bind and target HIF-1α for proteasomal degradation, which leads to HIF-1α accumulation and translocation to the nucleus. There, HIF-1α dimerises with HIF-1β, binds to hypoxia-response elements (HREs) within the promoters of target genes and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity. A range of cell functions are regulated by the target genes, as indicated. Abbreviation: CBP, CREB-binding protein; Ub, ubiquitin.

that deregulation of HIF-1 activity contributes to the angiogenic phenotype of VHL disease by upregulating genes such as VEGF. In addition, structural analysis of a hydroxylated HIF-1α peptide and the pVHL complex revealed that HIF-1α inserts into a highly conserved binding pocket within pVHL, a site that is commonly mutated in VHL disease (Refs 58, 59), indicating that overexpression of HIF-1α might be a causative event for the tumours observed that are associated with this disease. In support of this, data from mouse models show that binding of

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pVHL to HIF-2α is necessary for inhibition of the tumourigenic phenotype of VHL disease (Refs 60, 61). Surprisingly though, targeted inactivation of pVHL in embryonic stem (ES) cells leads to HIF deregulation but not to increased tumour growth (Ref. 62). It may be that additional mutations are required for tumourigenesis. Nevertheless strong evidence exists to support the notion that renal cell carcinoma would be a valid tumour type for HIF-targeted therapy.

Other tumour suppressors such as PTEN and p53 can regulate HIF-1α, although the mechanisms involved are not as clearly defined as those of pVHL. The *PTEN* gene encodes a phosphatase that negatively regulates the PI3K / Akt pathway. Loss of PTEN function results in hyperphosphorylation of Akt and upregulation of genes involved in cell proliferation and survival (Ref. 63). Loss of this tumour suppressor is significantly associated with glioblastoma formation (Ref. 64). In PTEN-null cells, HIF-1α is stabilised and target genes are activated following hypoxia (Ref. 65). After reintroduction of functional PTEN, however, HIF-1α stabilisation is lost, suggesting that there are additional mechanisms required for its stabilisation and activation. It is not clear whether PTEN loss of function would make tumours more resistant to HIF-based therapies.

Loss of p53 function in tumour cells results in resistance to hypoxia-mediated cell death via apoptosis. HIF-1 function might be deregulated in these cells because it has been shown that p53 negatively regulates HIF-1 transactivation (Refs 66, 67), and p53 interacts with HIF-1α during hypoxia (Ref. 68). One study, however, has shown the interaction between p53 and HIF-1α is indirect and that it is mediated by the oncoprotein HDM2, a target of p53 (Ref. 69). In addition, HDM2 can increase HIF-1α protein levels independently of p53 (Ref. 70). Although the precise mechanism of how HDM2, HIF-1α and p53 interact remains unclear, the p53 status of a tumour might be important for HIF-1α regulation in hypoxia, and the effectiveness of HIF-based therapies.

HIF-α subunit function and tumour growth

Zhong et al. (Ref. 71) were the first to show that HIF-1α was highly expressed in a wide variety of cancers including colon, breast and prostate carcinomas. Virtually all normal tissues were negative for HIF-1α staining, indicating the potential importance of HIF-1α for tumour progression. These results have since been confirmed by many other groups (see below). Both HIF-1α and HIF-2α are upregulated in clear-cell renal carcinoma (Ref. 72). Of significance is the observation that HIF-2α is present in a subset of infiltrating macrophages, indicating the importance of HIF activation in stromal cell populations of tumours (Ref. 73).

Mouse models have demonstrated that overexpression of HIF-1α or inhibition of HIF-1α degradation increases VEGF levels and angiogenesis (Ref. 74). However, tumour-targeted ablation of HIF-1 activity does not always lead to decreased tumour growth and tumour-associated angiogenesis. Xenografts with mouse hepatoma cells deficient in HIF-1β had lower tumour volumes and reduced angiogenesis (Ref. 75), indicating a requirement for the β-subunit for tumourigenesis, and indirectly a role for HIF-1α. However, the effects of HIF-1α on tumour growth from gene inactivation studies in mice are equivocal. In one study, xenografts grown from HIF-1α$^{-/-}$ knockout ES cells in nude mice did not decrease in size, most likely because of upregulated stress-induced proliferation and decreased apoptosis (Ref. 76). Hypoxia-induced VEGF, however, was decreased in these tumours, as was the number of large vessels, although the number of capillaries remained unchanged between wild-type and HIF-1α$^{-/-}$ tumours. In two subsequent studies, tumour growth of both HIF-1α$^{-/-}$ ES cells (Ref. 77) and HIF-1α-null fibrosarcomas was substantially retarded (Ref. 78). However, only the first of these studies, by Ryan et al. (Ref. 73), demonstrated a decrease in tumour vascularisation. No difference in vascular density was observed between wild-type and HIF-1α-null fibrosarcomas (Ref. 78). Furthermore, another study investigating HIF-1α$^{-/-}$ tumours showed that VEGF protein levels did not change between wild-type and HIF-1α$^{-/-}$ tumours (Ref. 79). It might be that tumour VEGF is derived from infiltrating macrophages and stromal cells in these models, or that VEGF is regulated in these tumours by other transcription factors, such as NF-κB, AP-1 or HIF-2. Of interest is the evidence that HIF-2 might regulate VEGF to a greater extent than observed for HIF-1 (Ref. 27). VEGF levels correlate strongly with HIF-2α induction (Ref. 80), and in some cell lines hypoxia and IGF-1 induction of VEGF is mediated by HIF-2 (Refs 81, 82). Furthermore, gene expression patterns in the developing mouse embryo show that while HIF-1α is ubiquitously expressed,

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HIF-2α is more highly expressed in the developing vasculature (Refs 25, 83, 84), indicating a more restricted role for HIF-2α in angiogenesis. Therefore, specific targeting of HIF-2, or combined inhibition with HIF-1 inhibitors, might be of benefit in certain angiogenic tumours. Recent studies have confirmed selectivity between HIF-1α and HIF-2α in the regulation of hypoxia-inducible genes. Genetic analysis studies utilising siRNA demonstrated that in cells that expressed both HIF-1α and HIF-2α, HIF-1α predominantly regulated genes such as carbonic anhydrase IX (CAIX), glucose transporter 1 (GLUT-1) and Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3), but HIF-2α was important in cells that expressed HIF-2α only (Ref. 85).

Although recent data have shown that hypoxia can stimulate both HIF-1α-mediated cell migration and invasion in in vitro assays, in vivo growth and migration of HIF-1-deficient cells in mice is highly dependent on the tumour microenvironment. Growth of HIF-1α-null astrocytoma cells varied depending on the site of tumour implantation. Tumour growth was slower in subcutaneously grown tumours as compared with wild-type controls. However, when HIF-1α-null astrocytoma cells were grown in the brain parenchyma, a more physiological tumour microenvironment for these cells, surprisingly, they exhibited an increased proliferation rate as compared with HIF-1α-expressing cells and were highly invasive (Ref. 86). It might be that HIF-1 target genes are activated differentially in different tumour microenvironments, and that in this particular study downregulation of apoptotic genes in HIF-1α-null cells allowed for enhanced tumour cell survival. Because of the large number of target genes that are regulated by the HIF pathway, it will be important to identify whether HIF target genes are differentially activated in different tumour microenvironments and the consequences for tumour progression.

Clinical implications

HIF-α subunit status in tumour prognosis and therapy

HIF-1α and HIF-2α expression is deregulated in many tumour types and might be important for tumour progression. Accordingly, a number of groups have analysed the impact of HIF-1α and HIF-2α expression in tumours for patient prognosis and have attempted to correlate HIF status with efficacy of chemotherapy and/or radiotherapy. In cervical cancer, HIF-1α expression was associated with decreased survival and reduced response to radiation therapy (Refs 87, 88), but was of no prognostic value for patients with ovarian tumours and did not affect response to platinum-based chemotherapy (Ref. 89). In a study of endometrial carcinoma, HIF-1α was associated with shorter patient survival (Ref. 90). Surprisingly, in non-small-cell lung cancer, HIF-1α- and HIF-1β-positive carcinomas were associated with longer patient survival times (Ref. 91). In a second study of this tumour type, HIF-2α, but not HIF-1α, was significantly associated with poor outcome (Ref. 92).

HIF-1α was highly expressed in over 90% of squamous cell carcinomas of the oropharynx as compared with the surrounding normal tissue, and was a strong independent risk factor for decreased survival in these patients (Ref. 93). In another study of squamous cell head and neck cancer, HIF-1α and HIF-2α were associated with greater risk of mortality by univariate analysis, of which only HIF-2α was significant following multivariate analysis (Ref. 94). In these patients, expression of either HIF-1α or HIF-2α was associated with poor response to carboplatin (Ref. 94). By contrast, however, HIF-1α expression was associated with improved disease-free survival in some patients with head and neck cancer (Ref. 95).

Bos et al. have shown that HIF-1α expression increases with histological stage of breast cancer (Ref. 96). In lymph-node-positive breast cancer, HIF-1α correlated significantly with poor outcome (Ref. 97) and had borderline significance in a study of lymph-node-negative cancer (Ref. 98). Patients with early oesophageal cancer have high HIF-1α-expressing tumours that have a reduced response to photodynamic therapy (Ref. 99), and in patients with oligodendrogliomas HIF-1α staining positively correlated with shorter survival (Ref. 100).

Taken together, these studies demonstrate that HIF-1α and/or HIF-2α are deregulated in a wide cross-section of tumours, but the predictive power of HIF for patient prognosis is not clear and varies considerably between different tumour types. This emphasises the critical need for suitable patient selection for future HIF-based therapies. Of interest are the data indicating that HIF-1α-negative cells might be more susceptible to chemotherapy and radiotherapy: HIF-1α$^{-/-}$ mouse embryo fibroblasts grown as experimental fibrosarcomas were more susceptible to carboplatin and etoposide (Ref. 101). This has important clinical implications, as

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combination therapies using different types of drugs – either HIF inhibitors or anti-angiogenics – in combination with chemotherapy or radiotherapy might achieve greater efficacy than traditional therapies alone. In addition, responses to radiation therapy are also dependent on the tumour microvasculature, with endothelial cell apoptosis being an important parameter for a favourable response (Ref. 102). Radiation induces tumours to secrete a variety of cytokines including VEGF, thereby decreasing the radiosensitivity of the tumour vasculature. Activation of a large selection of cytokines is mediated by HIF-1 (Refs 103, 104). For the above reasons, HIF-targeted therapy, perhaps in combination with conventional therapeutics, provides an exciting new strategy for the treatment of hypoxic tumours.

Therapeutic targeting of tumour hypoxia

A major problem in clinical oncology is the resistance of hypoxic tumours to radiation therapy and chemotherapy. Poor tumour blood flow results in impaired uptake and delivery of chemotherapeutic drugs, and reduced oxygen for the generation of toxic free radicals in radiation therapy. Historically, strategies to improve tumour oxygen status and blood flow include increased oxygen delivery to the tumour with carbogen gas (95% O_2; 5% CO_2) or with nicotinamide administration. These treatments may improve chemotherapy and radiotherapy efficacy.

However, another approach is to exploit the hypoxic environment itself, as an 'Achilles' heel'. For example, prodrugs can be designed that are activated to release a toxic product only in hypoxic areas. In addition, because one of the consequences of hypoxia is the upregulation of molecular pathways centred on HIF, the possibility of treating regions of tumour hypoxia with small-molecule inhibitors of HIF, and other HIF-based approaches, is an area of intense research. However, it is important to note that targeting tumour hypoxia is quite different to the specific targeting of HIF itself. The first aims to take advantage of the hypoxic environment in order to deliver a toxin or a therapeutic gene, whereas specific HIF-targeted drugs act by blocking the molecular pathways triggered by HIF. Since HIF regulates a broad range of genes involved in a variety of cellular responses within a tumour, inhibiting HIF might reduce cell migration, invasion and/or angiogenesis, as well as having effects on the hypoxic microenvironment. Paradoxically, inhibiting HIF might actually reduce blood flow to the tumour by a decrease in tumour angiogenesis, potentially reducing the value of chemotherapeutics. However, decreased tumour angiogenesis is overall favourable for patient prognosis, and studies have shown that HIF-1α-positive tumours generally show a reduced response to chemotherapy, not vice versa, supporting the argument for therapeutic targeting of HIF.

Hypoxia-activated prodrugs and gene therapy

Hypoxia-activated prodrugs are inactive under conditions of normal oxygen tension, but are activated in hypoxia (Refs 105, 106). Tirapazamine (TPZ) is one example of a bioreductive prodrug that is activated by tumour reductases (e.g. NADPH:cytochrome P450 reductase) under hypoxic conditions, thereby exhibiting a high selectivity and toxicity for hypoxic cells (Refs 107, 108, 109). TPZ shows promise in combination with chemotherapy or radiation therapy and is now in randomised clinical trials. Interestingly, a recent study has shown that TPZ activity can be greatly enhanced by adenoviral delivery of the P450 cDNA fused to an HRE sequence (Ref. 110). The HREs linked to therapeutic genes control gene expression in response to hypoxia and ensure that gene expression is limited to hypoxic cells (Refs 111, 112). This gene-directed enzyme–prodrug approach has proved promising, achieving a complete regression of tumour growth (Ref. 110), and highlights the potential for therapeutic intervention in this way. Other therapeutic approaches have used HIF antisense in combination with immunotherapy (Ref. 113) or with *VHL* overexpression (Ref. 114), and both showed improved efficacy. In addition, hypoxia- and HIF-dependent adenovirus-based therapies have been developed that specifically lyse HIF-expressing cells (Refs 115, 116, 117).

Small-molecule HIF inhibitors

There are several sites in the HIF pathway that are potential intervention points for inhibition by small-molecule inhibitors. These include inhibition of HIF protein synthesis, interference of HIF-dependent interactions, such as with HIF-1β, Hsp90 or p300, or inhibition of HIF transactivation. A number of small-molecule inhibitors of HIF have been described that act at one or more of these points, although their exact mechanism of

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action remains to be understood. The microtubule-depolymerising agent 2-methoxyestradiol (2ME2) efficiently blocks HIF-1α transactivation and has antitumour and anti-angiogenic activity (Ref. 118). The mechanism is unclear, but might be dependent on pVHL (Ref. 119). Other promising agents include inhibitors of the redox protein thioredoxin 1 (Trx-1) (Ref. 120), which positively regulates HIF-1α protein levels and angiogenesis (Ref. 121), and the HIF-1α inhibitors YC-1 (Ref. 122) and PX-478 (Ref. 123).

Some inhibitors are known to target specific HIF-dependent interactions. The molecular chaperone Hsp90 stabilises HIF-1α in a pVHL-independent fashion. Antagonists of Hsp90 such as geldanamycin and its analogue 17-allyl-aminogeldanamycin (17-AAG) are effective at inhibiting HIF-1α expression levels (Refs 124, 125). There is currently a high degree of interest in developing Hsp90 inhibitors for clinical use: a range of agents are under development, and 17-AAG is entering Phase II clinical trials (Ref. 126). Of particular interest is the fact that 17-AAG shows enhanced activity against the sugar–chaperone complexes that appear to predominate in cancer cells, potentially induced by a combination of oncoprotein expression and environmental pressures, such as tumour hypoxia (Refs 127, 128). The HIF-1α–p300 interaction has also been targeted. A gene therapy approach to deliver polypeptides to inhibit this interaction was successfully used to inhibit tumour growth in mouse models (Ref. 129). In addition, the small-molecule inhibitor chemotin, recently identified in a target-based high-throughput biochemical screen, blocked HIF-1α and HIF-2α binding to p300 and was shown to inhibit hypoxia-induced transcription and tumour growth (Ref. 130).

Cell-based high-throughput screens are also being used to identify novel small-molecule inhibitors of HIF. These systems generally utilise cells transfected with multiple HREs linked to a specific reporter gene construct. Cells express the reporter (for example, luciferase or β-galactosidase) in a HIF- and hypoxia-dependent manner. This allows efficient screening of large libraries of compounds for HIF-inhibitory activity. In this way, topoisomerase I inhibitors were also identified as having HIF-1-inhibitory activity (Refs 131, 132, 133).

Imaging tumour hypoxia

Rapid advances in imaging techniques such as magnetic resonance spectroscopy (MRS) and positron emission tomography (PET), and the development of novel probes and radiotracers, are allowing the noninvasive identification and quantification of hypoxia in tumours. Imaging techniques are important for selecting patients most likely to benefit from hypoxia-based treatments and for monitoring the efficiency of delivery and efficacy of hypoxia- and HIF-targeted drugs in vivo. Hypoxia probes such as the fluorinated nitroimidazole SR-4554 can be effectively used to detect areas of tumour hypoxia by ^{19}F MRS in preclinical studies (Refs 134, 135). A clinical Phase I study has also demonstrated the feasibility of SR-4554 detection in tumours by MRS (Ref. 136). In addition, targeting of hypoxic areas with HIF-specific reporter gene constructs has been shown recently using PET (Refs 137, 138). Interestingly, imaging of HIF-specific reporter gene expression revealed that induction of angiogenesis in the C6 tumours used in this study was not hypoxia dependent, but might have been due to constitutive oncogenic signalling in the tumours (Ref. 137). PET has also been used to correlate hypoxic tumours with levels of VEGF. In tumours expressing two- to fourfold differences in VEGF, a higher hypoxic fraction was observed in the low-VEGF-expressing tumours by using the novel PET radiotracer [^{18}F]fluoroetanidazole (Ref. 139). VEGF has also been imaged in vivo with PET by using ^{124}I-labelled VEGF antibody (Ref. 140). Apart from specific molecular imaging, PET can also be used to obtain functional data such as blood flow when monitoring efficacy of hypoxia-targeted drugs. In addition, the success of any HIF-targeted therapy will partly reside in the ability to equate changes in tumour growth with molecular endpoints, such as HIF status within the tumour, and effects on target genes such as VEGF.

Research in progress and outstanding research questions

Although rapid progress has been made in elucidating the oxygen-sensing mechanism of HIF-1 activation, many questions with regard to HIF-1 regulation are still unanswered. Growth-factor- and cytokine-mediated activation of HIF-1 occurs during normoxia, but quite how growth-factor-induced HIF-1α evades normoxic degradation is still unclear. HIF-1α is highly phosphorylated (Ref. 141), and although phosphorylation is thought to regulate HIF-1 transcriptional activity, how this occurs remains to be determined.

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A large body of evidence from mouse models and patient studies implicate HIF-1 in tumour progression. However, HIF-1 can potentially switch on genes that inhibit, as well as genes that enhance, tumour development. For example, inhibiting HIF-1 might prevent the upregulation of apoptotic genes such as BNIP3 and Nip3-like protein X (NIX) within the tumour (Refs 142, 143, 144), thereby promoting tumour cell survival. Orthotopic tumour models, as opposed to xenografts, have already indicated that HIF-1α-null cells exhibit an increased proliferation rate and are more invasive in a relevant tumour microenvironment (Ref. 86). Although HIF-1α has been shown to have a predominant role in mediating the transcriptional response to hypoxia in both endothelial and breast cancer cells (Ref. 85), HIF-2α clearly has distinct effects in tumour cells that have lost HIF-1α (Ref. 85). The extent to which HIF-1 and HIF-2 responses in tumour cells overlap, co-operate or are distinct is not yet fully understood, and will need consideration when developing strategies to target the hypoxia–HIF pathway. Clearly, a better understanding of the regulation of HIF target genes in different tumour microenvironments is required, as is the interplay between tumour cells, infiltrating macrophages and other stromal cells with respect to HIF activation and angiogenesis. The selection of the most appropriate patient populations will be important for achieving good clinical outcomes with HIF therapeutics, as will be assessment of the timing and duration of administration of HIF-targeted drugs. For this, it will be important to investigate the dynamics of HIF activation for tumour progression in order to predict when therapy might be most beneficial, ideally using noninvasive molecular imaging technologies. Detailed understanding of the molecular regulation of HIF, together with careful patient selection, should facilitate the development of an exciting new range of molecular therapeutics for targeted cancer treatment.

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Further reading, resources and contacts

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Online sources of general information on cancer and angiogenesis research:

http://www.cancer.gov (National Cancer Institute)
http://www.cancer.org.uk (Cancer Research UK)
http://www.angio.org (The Angiogenesis Foundation)
http://www.cancer.gov/clinicaltrials/developments/anti-angio-table (Angiogenesis inhibitors in trials)

Relevant virtual libraries and research databases:

http://www.ncbi.nlm.nih.gov (National Center for Biotechnology Information)
http://www.bioscience.org (Frontiers in Bioscience)
http://us.expasy.org (Proteomics server)
http://www.signaling-gateway.org (Cell signalling resource)

Features associated with this article

Figures

Figure 1. Tumour hypoxia.
Figure 2. HIF-1α and HIF-1β subunits.
Figure 3. HIF-1α regulation by proline hydroxylation.

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