

Hypoxia signalling in cancer and approaches to enforce tumour regression

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Tumour cells emerge as a result of genetic alteration of signal circuitries promoting cell growth and survival, whereas their expansion relies on nutrient supply. Oxygen limitation is central in controlling neovascularization, glucose metabolism, survival and tumour spread. This pleiotropic action is orchestrated by hypoxia-inducible factor (HIF), which is a master transcriptional factor in nutrient stress signalling. Understanding the role of HIF in intracellular pH (pH_i) regulation, metabolism, cell invasion, autophagy and cell death is crucial for developing novel anticancer therapies. There are new approaches to enforce necrotic cell death and tumour regression by targeting tumour metabolism and pH_i-control systems.

We have learned, over the past two decades, how mammalian cells perceive signals to induce cell-cycle progression, proliferation and survival. Two major pathways that are frequently mutated in human cancer, the Ras–extracellular signal-regulated kinase (ERK)^{1–3} and the phosphatidylinositol-3-OH kinase (PI(3)K)–AKT⁴ (see the review in this issue by Shaw and Cantley, page 424) signalling cascades, are activated by a vast array of growth factor polypeptides, hormones and extracellular matrix proteins⁵. Activation of these two pathways is sufficient to trigger multiple cycles of division and survival of normal cells under the ‘rich’ conditions of tissue culture. *In vivo*, however, growing cells must constantly instruct the microenvironment to maintain a supply of essential nutrients. It is remarkable that the Ras–ERK and PI(3)K–AKT pathways also control the expression of the ubiquitous vascular endothelial growth factor-A (VEGF-A), which is a key factor in vascularization/angiogenesis^{6,7}. During embryonic development or in the context of tumour expansion, growing cells rapidly outstrip the supply of nutrients. Although cells sense and respond to variations in concentrations of all nutrients, oxygen sensing has emerged as a central control mechanism of vasculogenesis^{8,9}.

At the heart of this regulatory system is HIF^{10,11}, which controls, among other gene products, the expression of two key angiogenic factors: VEGF-A¹² and angiopoietin-2 (Ang-2)¹³. This finding has placed the hypoxia-signalling pathway at the forefront of nutritional control — a notion reinforced by the fact that growth factors enhance HIF expression and converge with hypoxia in inducing maximal expression of VEGF-A.

HIF can induce a vast array of gene products controlling energy metabolism, neovascularization, survival, pH_i and cell migration, and has become recognized as a strong promoter of tumour growth¹⁴. This pro-oncogenic feature is only one facet of the dual action of HIF. Besides being a ‘guardian’ of oxygen homeostasis, HIF is capable of inducing pro-apoptotic genes¹⁴ leading to autophagy and cell death, which can be features of hypoxic tissues. In this regard, HIF can be likened to p53, which has dual roles as a guardian of genome integrity and a promoter of apoptosis.

In this review we highlight the most recently revealed features of hypoxia signalling, and the role of *hif* as a master gene controlling nutritional stress, angiogenesis, tumour metabolism, invasion and autophagy/cell death. Finally, we discuss potential new and exciting approaches to

enforce tumour regression by exploiting the emerging basic knowledge of tumour metabolism, autophagy and cell death.

Regulating angiogenesis with VEGF-A and angiopoietin-2

Growth factors and hypoxia converge in the regulation of key angiogenic genes. The cellular expansion of tumours progressively distances cells from the vasculature, and thus from oxygen and nutrients. Tumour cells, like growing embryonic cells, send out signals that initiate the formation of new blood vessels. This adaptive process, termed angiogenesis, is a general feature of every tissue; however, it is often exacerbated in solid tumours. Thus, new tumour vessels showing structural malformations, chaotic blood flow and local regions of hypoxia might nonetheless prevail. Although many molecules and receptors have been characterized in this biological process, at least two factors seem critical for initiating vessel sprouting. These are VEGF-A^{15,16} and Ang-2 (ref. 17), which are two receptor ligands expressed and secreted in response to hypoxia. VEGF-A is expressed in most cells, and attracts and guides sprouting neovessels into oxygen-depleted regions of the tumour mass^{18,19}. Endothelial cells situated at the tip of the sprouts sense and navigate through the environment using long filopodia that are rich in VEGF receptor-2 (VEGFR-2)¹⁹. Thus, migration of the tip cells is guided by a graded distribution of VEGF-A, particularly the long spliced forms that are retained in the extracellular matrix.

Although in hypoxia the binding of HIF to the *vegf* promoter is a key determinant in its expression, two other major transcriptional controls are mediated through the Ras–ERK and PI(3)K–AKT pathways^{6,20} (Fig. 1a). VEGF-A messenger RNA is upregulated by the ERK pathway through the phosphorylation of the transcription factor Sp1 and its recruitment to the proximal region of the *vegf* promoter²¹ (Fig. 1a). This regulation is independent of hypoxic stress and reflects the intensity of growth-factor stimulation or oncogenic signals. Transcriptional activation also occurs through ERK-induced phosphorylation of HIF-1 α ²² and the coactivator p300, which might improve the accessibility of RNA polymerase II to the *vegf* promoter. Other levels of regulation of VEGF-A occur, including the stabilization of the mRNA through the stress-activated kinase p38 (ref. 23), and translation by means of internal ribosome entry site (IRES) sequences present in 5′ non-coding regions of VEGF-A²⁴ and HIF-1 α mRNAs²⁵, which are two important attributes for translation of VEGF-A under nutrient deprivation. This is another

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point of convergence between growth factors and hypoxia signalling at the level of translation. As a 'survival' cytokine, VEGF-A is translated under conditions where the cell's general translational machinery is turned off.

The second molecule induced by hypoxia is Ang-2, a receptor ligand restricted to endothelial cells^{17,26} that allows vessel remodelling by antagonizing the related molecule Ang-1. As shown in Fig. 1b, Ang-1, through Tie-2 receptor tyrosine kinase signalling and platelet-derived growth factor-B (PDGF-B) action, induces pericyte recruitment²⁷ and

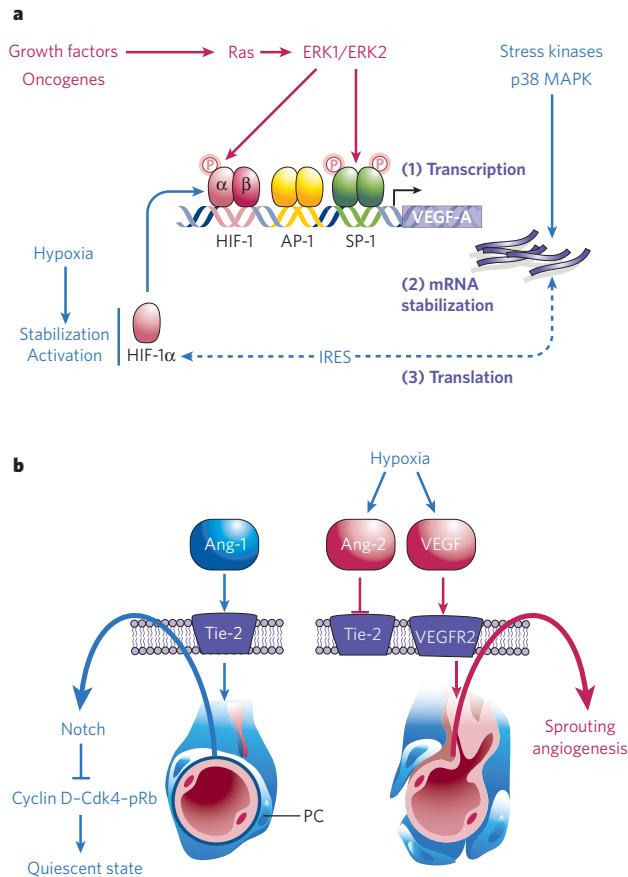


Figure 1 | VEGF-A and angiopoietin-2 are key angiogenic factors induced by hypoxia. **a**, Control of vascular endothelial growth factor-A (VEGF-A) expression. VEGF-A expression is controlled at three levels: transcription, messenger RNA stability and translation. The Ras–MEK–extracellular signal-regulated kinase (ERK) pathway stimulates transcription through phosphorylation of the transcription factors Sp1 and hypoxia-inducible factor-1α (HIF-1α) subunit, and their recruitment to the *vegf* promoter. The transcription factor activator protein-1 (AP-1) might also modulate *vegf* transcription. HIF-1 is a heterodimer of a hypoxia-stabilized and activated α -subunit and an oxygen-insensitive β -subunit. VEGF-A mRNA is stabilized through the stress-activated kinase p38, and the translation of VEGF-A is ensured under hypoxic and nutrient-depleted conditions by means of internal ribosome entry site (IRES) sequences. Under these energy-reduced conditions, classic cap-dependent translation is inhibited. **b**, VEGF-A and angiopoietin-2 (Ang-2) are two angiogenic factors induced by hypoxia. Blood capillaries are maintained in a mature and dormant state through the recruitment of pericytes (PC) through platelet-derived growth factor-B (PDGF-B) and the signalling of the endothelial receptor Tie-2 upon Ang-1 binding. In addition, activation of the Notch pathway through cyclin D/Cdk4 and retinoblastoma protein (pRb) phosphorylation contributes to the quiescence of endothelial cells. Ang-2 is an antagonist ligand for Tie-2 in endothelial cells and, like VEGF-A, is induced under low oxygen conditions through the HIF. The initiation of sprouting angiogenesis requires the destabilization of capillaries. This action is mediated by Ang-2, thereby blocking Tie-2 signalling and allowing VEGF-A-induced cell migration and division. MAPK, mitogen-activated protein kinase.

maturation of blood capillaries. These capillary endothelial cells are rendered quiescent through the activation of the Notch pathway²⁸, thus becoming unresponsive to VEGF-A action, unless Ang-2 is also secreted, leading to vessel destabilization. Ang-2 is a natural Ang-1 antagonist, which displaces Ang-1 from its receptor thus arresting Tie-2 signalling. Therefore Ang-2 secretion from Weibel–Palade bodies²⁹ is a critical, and perhaps limiting, step in angiogenesis permitting vessel remodeling upon VEGF-A action. It is remarkable that this angiogenic 'couple' — VEGF-A and Ang-2 — is expressed under hypoxic control when and where nutrients are needed. However, the precise mechanism of regulation of Ang-2 expression in hypoxia remains to be defined.

A master regulator of oxygen homeostasis

More than a decade ago, Semenza and colleagues showed that the nuclear factor HIF binds to the *epo* gene and induces its transcription in hypoxia¹⁰. HIF is now known to induce many genes involved in the response to hypoxia^{14,30}. HIF was shown *in vitro*, in a variety of cell-culture systems, to be activated at a cut-off point of about 5% oxygen (40 mmHg), and to progressively increase its activity with a decrease in oxygen gradient down to 0.2–0.1% oxygen (1.6–0.8 mmHg), close to anoxia. HIF belongs to the large family of basic-helix–loop–helix (bHLH) proteins and is a heterodimer of a constitutively expressed and stable HIF-1 β subunit, and one of three oxygen-regulated HIF- α subunits (HIF-1 α , HIF-2 α or HIF-3 α). HIF activation is a multi-step process involving HIF- α stabilization, nuclear translocation, heterodimerization, transcriptional activation and interaction with other proteins (see refs 31, 32 for reviews). In the nucleus, HIF binds to so-called hypoxia-response elements (HREs), with the minimal core sequence 5' -RCGTG-3', which are adjacent to auxiliary motifs specifying the responsive genes (about 100 genes have now been characterized). Yet identifying exactly how HIF becomes activated in hypoxia has been challenging.

HIF does not directly sense variations in oxygen tension (p_{O_2}). The key regulation is orchestrated by a class of 2-oxoglutarate-dependent and iron-dependent dioxygenases belonging to the largest known family of non-haem oxidizing enzymes (EC 1.14.11.2). Because the activity of these enzymes is strictly dependent on the cellular p_{O_2} , they are the true oxygen-sensing molecules controlling the hypoxic response³³. Two types of oxygen sensor control HIF action. The first are referred to as prolyl hydroxylase domain (PHD) proteins^{34–36}. PHDs hydroxylate two prolyl residues (P402 and/or P564) in the human HIF-1 α region referred to as the oxygen-dependent degradation domain (ODDD; Fig. 2). This HIF- α modification specifies rapid interaction with the tumour-suppressor protein von Hippel–Lindau (VHL), a component of an E3 ubiquitin ligase complex^{37,38}. Subsequently, HIF- α subunits become marked with polyubiquitin chains that drive them to destruction by the proteasomal system^{39,40}. HIF-1 α , in well-oxygenated cells (21% O_2), displays one of the shortest half-lives (<5 min) among cellular proteins⁴⁰. Of the three PHD isoforms in humans, PHD2 is the key limiting enzyme targeting HIF-1 α for degradation under normoxic conditions⁴¹, whereas the physiological roles of PHD1 and PHD3, which are active under chronic hypoxia, remain to be investigated (see ref. 33 for a review).

The second type of oxygen sensor controlling the hypoxic response is an asparaginyl hydroxylase, referred to as factor inhibiting HIF-1 (FIH)⁴². This enzyme hydroxylates an asparagine residue (N803) in the most carboxy-terminal transcriptional activation domain (C-TAD) of human HIF-1 α . This covalent modification abrogates C-TAD interaction with transcriptional co-activators, such as p300 and its paralogue CBP (Fig. 2). Thus, the two metabolic sensors, PHD2 and FIH, by controlling both the destruction and inactivation of HIF- α subunits, ensure full repression of the HIF pathway in well-oxygenated cells. An intriguing feature of HIF- α subunits is the occurrence of apparently 'bicephalous' transcriptional activation domains (amino (N)-TAD and C-TAD). Interestingly, only C-TAD, which interacts with p300/CBP, is subjected to hydroxylation and inhibition by FIH. We propose that N-TAD and C-TAD could discriminate between the induction of different hypoxic genes, as deletion of C-TAD in a naturally occurring spliced form of HIF-1 α retains one-third of the transcriptional activity⁴³.

In a model describing the microenvironment of a blood vessel (Fig. 3), we propose that cells exposed to a decreasing oxygen gradient will first express a subset of genes regulated by N-TAD, followed by a second subset of genes regulated by C-TAD at lower oxygen concentrations. This model integrates the interesting finding that PHDs have a much lower affinity for oxygen and 2-oxoglutarate than FIH⁴⁴. Therefore, if this notion applies *in vivo*, PHDs will be inactivated at oxygen values that still maintain FIH activity and therefore keep C-TAD under repression. This model is supported by the existence of at least two classes of HIF-dependent gene: those that are sensitive and non-sensitive to the activity of FIH⁴⁵. Thus, the two TADs, together with the oxygen-sensitive discriminator FIH, constitute a cellular device that allows fine-tuning of specific HIF gene expression along the hypoxic gradient⁴⁵. One particular HIF-induced gene that seems not to be repressed by the activity of FIH is the pro-apoptotic *bnip3*, a member of the BH3-only protein family of cell death factors⁴⁶. This finding came as a surprise, because we were expecting that a gene inducing cell death should be maintained under strict and tight control, and expressed only under severe hypoxic conditions. The current hypothesis is that BNIP3-induced cell death is revealed only by severe acidosis associated with deep O₂ depletion⁴⁷ (Fig. 3). BNIP3 'activation' by acidosis requires further investigation.

HIF meets the mTOR pathway

As well as activating angiogenesis, hypoxic stress also leads to attenuation of protein synthesis by means of emerging regulatory mechanisms implicating the mTOR (mammalian target of rapamycin) pathway.

mTOR is a conserved serine/threonine protein kinase that phosphorylates a series of substrates involved in protein translation, including the eukaryotic initiation factor 4E-binding protein-1 (4EBP1) and ribosomal p70 S6 kinase (S6K)^{48,49}. As a chief orchestrator of protein synthesis, the mTOR pathway integrates a variety of signals. mTOR is activated by Rheb-GTP, a small G protein, itself negatively regulated by the GTPase activity of the tumour suppressor complex TSC2-TSC1, which was first identified as being mutated in patients with tuberous sclerosis complex (TSC)^{50,51} (Fig. 4). The upstream activators of mTOR

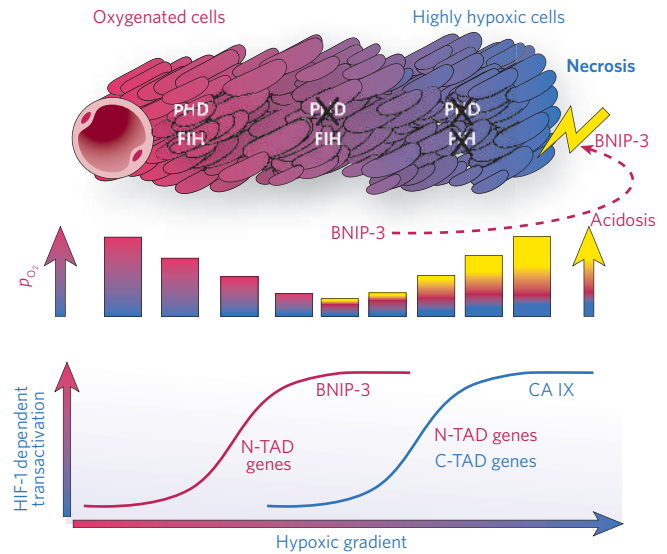


Figure 3 | Working model of two sets of HIF-1-regulated genes. In the tissue microenvironment, cells situated at various distances from blood capillaries will experience different oxygen tensions (p_{O_2}), as illustrated by a decreasing gradient. A parallel increase in the extracellular acidity due to the accumulation of lactate and CO₂ is noted as cells become more distant from capillaries. Hypoxia induces the expression of carbonic anhydrase IX (CA IX), which helps to retain a relatively neutral intracellular pH. The expression of the proapoptotic protein BNIP-3 is induced under moderately hypoxic conditions, but requires acidosis to promote cell death. Thus, under the extreme conditions of low p_{O_2} and acidosis, necrotic areas are often visible. A decreasing p_{O_2} gradient from the blood vessel to the tumour core will also determine the activity of the prolyl hydroxylase domain (PHD) proteins and factor inhibiting HIF-1 (FIH). The Michaelis constant (K_m) of the PHD proteins and FIH predict that the former has a lower affinity for oxygen and is therefore more rapidly inhibited than the latter. So, at a moderate p_{O_2} , HIF-1 α (hypoxia-inducible factor-1 α) will be stable because the PHD proteins are inhibited, but genes dependent on carboxy-terminal transcription activation domain (C-TAD) activity will not be induced because C-TAD inhibition is maintained by FIH activity. However, genes requiring only the amino-terminal transcription activation domain (N-TAD) will be induced. As the p_{O_2} decreases further, the inhibition of C-TAD will be released and HIF-1 α will attain full transcriptional activity. In this way the 'bicephalous' transcriptional nature of HIF-1 α will, in an FIH-dependent or FIH-independent manner, differentially regulate two sets of genes.

are myriad growth factors, hormones and extracellular matrix components known to promote cell growth and survival through activation of the PI(3)K-AKT and Ras-ERK cardinal pathways. All these activators converge at the level of the TSC2-TSC1 integrator complex. Direct phosphorylation of TSC2, by either AKT (see the review in this issue by Shaw and Cantley, page 424) or ERKs⁵², inhibits its intrinsic GTPase activity leading to mTOR activation. Interestingly, nutrients (amino acids and glucose) also inhibit the TSC2-TSC1 complex through a mechanism that has not been fully resolved, which results in activation of protein synthesis^{53,54}. Translation of HIF-1 α has been found to be particularly sensitive to growth factors that activate mTOR^{55,56}.

In contrast to these multiple mTOR-activation inputs, mTOR, and therefore protein synthesis, is shut down under stress conditions generated by energy depletion or hypoxia (Fig. 4). In response to an increase in the AMP:ATP ratio, the upstream kinase LKB1 phosphorylates and activates AMP-activated protein kinase (AMPK)⁵⁷. Once activated, AMPK phosphorylates several downstream substrates, resulting in a decrease in energy demand by switching off ATP-consuming pathways⁵⁸. Direct phosphorylation of TSC2 by AMPK leads to activation of the TSC2-TSC1 complex and subsequent mTOR inhibition. HIF/hypoxia negatively regulates mTOR in two ways:

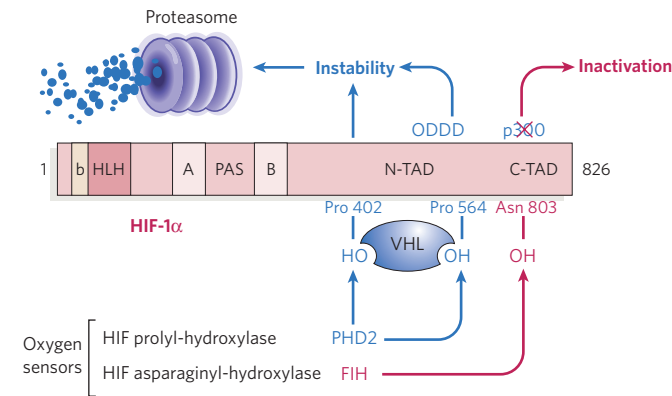


Figure 2 | Oxygen sensors contribute to the destruction and inactivation of HIF-1 α . The transcription factor HIF (hypoxia-inducible factor) is a member of the basic-helix-loop-helix PerArntSim (bHLH-PAS) family of proteins with two PAS domains, A and B. HIF is a heterodimer of an oxygen-sensitive α -subunit and an oxygen-insensitive β -subunit. Two oxygen sensors termed prolyl-hydroxylase domain (PHD) protein and factor inhibiting HIF-1 (FIH) determine, respectively, the stability and activity of HIF-1 α . The PHDs, by hydroxylating two proline residues (402 and 564) in a region called the oxygen-dependent degradation domain (ODDD), initiate the binding of a component of an E3 ubiquitin ligase, the von Hippel-Lindau (VHL) protein, which marks HIF-1 α for destruction by the proteasome. FIH, by hydroxylating an asparagine residue in the carboxy-terminal transcriptional activation domain (C-TAD) of HIF-1 α , inhibits the binding of cofactors, such as p300, that are required for the transcription of certain HIF-dependent genes. A second transcriptional activation domain, N-TAD, which overlaps the ODDD, is FIH independent and might be implicated in distinct gene expression.

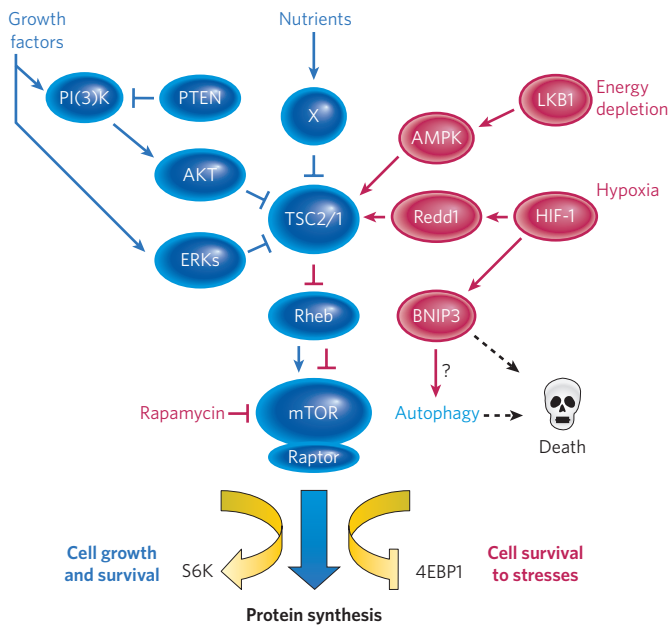


Figure 4 | Hypoxia meets the mTOR pathway. The blue arrows in the left and central parts of this diagram denote the converging pathways activating mTOR (mammalian target of rapamycin) at the level of the tumour suppressor complex (TSC2–TSC1). mTOR, which is sensitive to rapamycin, controls protein synthesis through the phosphorylation of 4E-binding protein-1 (4EBP1) and p70 S6 kinase (S6K). Growth factors, through AKT-dependent and extracellular signal-regulated kinase (ERK)-dependent phosphorylation, suppress the GTPase activity of the TSC complex, leading to full activation of GTP-Rheb, which is the activator of the mTOR–raptor complex. Nutrients and growth factors cooperate in the optimal activation of this pathway, which is essential for relaying growth and survival signals. By contrast, depletion of nutrients or energy (amino acids, ATP or oxygen) inhibits mTOR through independent activation of the TSC complex (red arrows). Suppression of mTOR in response to hypoxia requires gene induction (Rebb1), whereas a decrease in ATP rapidly shuts down mTOR by activation of the AMP kinase (AMPK), thereby directly phosphorylating TSC2. The hypoxia-mediated inhibition of mTOR favours the concomitant induction of pro-apoptotic BNIP3 and macro-autophagy, which are two processes that are often associated with necrotic cell death in tumours. HIF-1, hypoxia-inducing factor-1; LKB1, serine/threonine kinase; PI(3)K, phosphatidylinositol-3-OH kinase; PTEN, phosphatase and tensin homologue.

first, hypoxia inhibits mTOR by an increase in AMP, leading to the activation of AMPK⁵⁹; and second, a more direct link between HIF and mTOR was established in *Drosophila*, in which the HIF-induced paralogue gene products, Scylla and Charybdis (REDD1/RTP801 in mammals), activate the TSC complex resulting in mTOR inhibition^{60,61}. Therefore, hypoxia, through two independent mechanisms — AMPK activation and HIF-induced REDD1 — suppresses mTOR activity (Fig. 4). These restricted nutrient conditions associated with low mTOR activity favour rapid activation of macro-autophagy, which is the ultimate survival process before cell death⁶². We believe that the rapid induction of BNIP3 in a hypoxic microenvironment contributes to cell survival via autophagy up to a point of no return, in which severe acidic areas induce necrotic cell death by means of a BNIP3-dependent action^{47,63}. Human tumour cells often silence, by promoter methylation, the expression of BNIP3 (ref. 64). Whether this BNIP3 ablation allows tumour cells to invade, metastasize and resist this nutrition-deprived and hypoxia-induced cell death requires further investigation⁶⁵.

From hypoxia to tumour invasion

Hypoxia, or genetic alterations of the hypoxia signalling cascade^{66,67} leading to the constitutive expression of HIF, could promote intense and chaotic neovascularization that facilitates tumour spread.

It has now been firmly established that HIF has important roles in tumour progression. Several immunohistochemical analyses have indicated that HIF-1 α and HIF-2 α are overexpressed in primary and metastatic human cancers, and that the level of expression, either as a result of tumour hypoxia or genetic alterations, is correlated with tumour angiogenesis and patient mortality^{14,67}. Tumour progression and invasion are often associated with the increased capacity of the cells to promote extracellular matrix remodelling, increased migration and digestion of the basement membrane. Are these key features of cancer cells regulated by HIF? From the myriad genes induced by HIF, only a limited set of gene products possesses this potential. This group includes vimentin, fibronectin, keratins 14, 18 and 19, matrix metalloproteinase 2 (MMP2), cathepsin D and urokinase plasminogen activator receptor (uPAR)¹⁴. Other factors promoting migration can be added to this list, such as the autocrine motility factor (AMF)⁶⁸, the receptor tyrosine kinase c-Met⁶⁹ and the cytokine receptor CXCR4 (ref. 70; Fig. 5).

Another landmark of invasion and a crucial feature of epithelium–mesenchyme transition (EMT) is the loss of E-cadherin expression⁷¹. Interestingly, hypoxia and genetic lesions (*vhl* deletion in renal cell carcinoma) leading to HIF activation are associated with a concomitant loss of E-cadherin. After its proposal in 1999 (ref. 72), the first evidence linking HIF to decreased expression of E-cadherin was demonstrated in ovarian carcinoma: immunolocalization of nuclear HIF-1 α showed a strong topological correlation with loss of E-cadherin⁷³. Another immunological study in early genetic lesions in kidney showed concomitant expression of carbonic anhydrase IX (CA IX), an HIF-dependent gene product, with loss of E-cadherin⁷⁴. The intriguing question is how hypoxia represses E-cadherin. E-cadherin is controlled at the transcriptional level by the labile nuclear factor family, Snail/Slug⁷¹, so that any situation leading to Snail/Slug activation will repress E-cadherin. An unexpected link has just emerged in a class of secreted enzymes, the lysyl oxidase family (LOX), which is known to modify extracellular matrix components⁷⁵ but also to have some intracellular function, such as activation of Snail. Two key lysine residues, K98 and K137, of Snail have been found to be essential in a LOXL2-induced conformational change that renders Snail partly immune to glycogen synthase kinase- β 3 (GSK- β 3)-induced degradation⁷⁶. Interestingly, high expression of LOX and LOXL2 was previously reported only in breast cancer cells with a highly invasive metastatic phenotype⁷⁷. In a separate study, stable ablation of LOX by short-hairpin RNA (shRNA) in the MDA-231 breast cancer line reduced lung and liver metastasis in mice bearing orthotopic tumours⁷⁸. Perhaps the most exciting and relevant finding for this discussion is that LOX is highly induced by HIF-1 (refs 30, 79), therefore establishing the missing link in this new molecular cascade of hypoxia signalling and metastasis: HIF–LOX–Snail–E-cadherin (Fig. 5). Besides this predicted cascade, an alternative mechanism in renal cell carcinoma has been proposed in which HIF-1 could alter the mRNA levels of the E-cadherin repressors TCF3, ZFH1A and ZFH1B⁸⁰.

Approaches to enforcing tumour regression

A strong correlation between HIF-1 α expression and increased patient mortality is well documented for many types of human cancer. In tumour-xenograft and orthotopic mouse models, manipulation of the levels of either HIF-1 α or HIF-2 α has demonstrated a causal link between HIF- α expression and tumour progression¹⁴. Perhaps the best example concerns the renal cell carcinoma line 786-0, lacking VHL, in which it was elegantly demonstrated that HIF-2 α expression was indeed causal for tumour growth⁸¹. Along this line, it is interesting to note the role of HIF-2 α in promoting cancer stem cells by specific octamer-binding transcription factor 4 (OCT-4) induction⁸². Collectively, these findings suggest that HIF, a promoter of early invasive lesions, is a promising therapeutic target in cancer. A lot of effort has been put into identifying molecules that successfully inhibit HIF-1 α expression and/or activity⁸³. However, the pleiotropic action of HIF could represent a major concern with such an approach. It might be more appropriate to identify and target the key gene products specifying HIF-dependent invasion and tumour metabolism. The first good example of targeting an HIF-

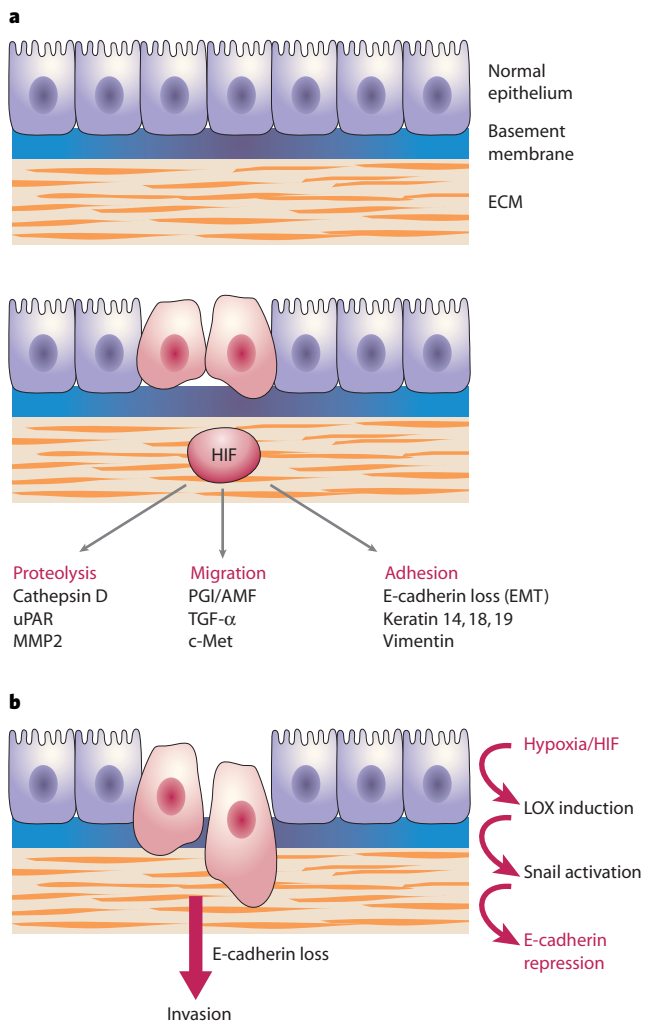


Figure 5 | Hypoxia-induced loss of E-cadherin through the lysyl oxidase-Snail activation pathway. Cell migration from the primary tumour and invasion into adjacent connective tissue are two steps leading to metastasis in carcinomas. Invasion requires a proteolytic modification of the extracellular matrix (ECM), migration and loss of cell-cell adhesion. **a**, Hypoxia-inducible factor (HIF) induces markers that activate proteolysis, including cathepsin D, urokinase-type plasminogen-activator receptor (uPAR) and matrix metalloproteinase-2 (MMP2), and factors stimulating migration such as phosphoglucose isomerase/autocrine-motility factor (PGI/AMF), transforming growth factor- α (TGF- α) and the spreading factor c-Met. Epithelial-mesenchymal transition is also considered to be a trait of tumour cell invasion and is associated with a downregulation of epithelial cadherin (E-cadherin), which is essential for maintaining cell-cell adhesion. Normal epithelial cells lie on a basement membrane that makes contact with the ECM. **b**, Under low-oxygen conditions (hypoxia), HIF is active and induces the expression of lysyl oxidase (LOX). A member of the same family, LOXL2, was found to activate the transcription factor Snail, which is a strong repressor of E-cadherin. This pathway might account for the invasion and metastatic process induced by hypoxia.

dependent gene product is the anti-VEGF-A therapeutic strategy⁸⁴. Major advances have been reported recently in the treatment of metastatic colon and renal cancers with this anti-angiogenic approach, and others, such as targeting Ang-2 (ref. 85), are likely to follow.

However, every success story has its drawbacks. The concern about anti-angiogenic therapy is the inherent selection of cancer cells that adapt to more hypoxic conditions and, in particular, to tumour acidosis, which is a hallmark of anaerobic glycolysis^{86,87}. What new approaches can we propose to anticipate and counteract this drawback? In solid tumours, immunohistochemistry often shows large fronts of HIF-1 α nuclear expression delineating areas of necrosis. How can we magnify and

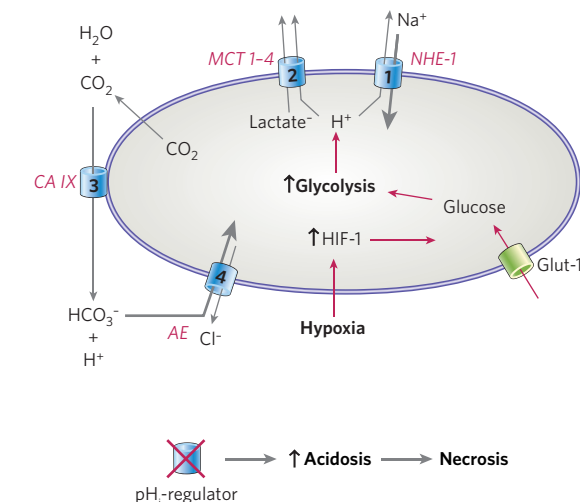


Figure 6 | Intracellular-pH-regulating systems as potential anti-cancer targets. The intracellular pH (pH_i) of tumours can become highly acidic as a result of the overproduction of lactic and carbonic acids. To survive and proliferate, cells must extrude these acids and maintain a balance between the extracellular and pH_i through the activity of several pumps, exchangers and transporters. Intracellular H⁺ ions are primarily extruded through the growth-factor-activatable and amiloride-sensitive Na⁺/H⁺ exchanger (NHE-1; target 1). Lactic acid is excreted from cells by members of the H⁺/lactate co-transporter family (monocarboxylate transporters (MCTs) 1-4; target 2). Metabolically generated CO₂ diffuses rapidly across the plasma membrane and meets the membrane-bound ectoenzyme carbonic anhydrase IX (CA IX) or CA XII, which converts it into carbonic acid (target 3). Uptake of the weak base HCO₃⁻ via a member of the Na⁺-dependent and Na⁺-independent Cl⁻/HCO₃⁻ exchangers contributes to intracellular alkalinization (target 4). Inhibiting target 1 profoundly reduces tumour incidence in the context of glycolytic tumour cells. It is expected that combined pharmacological actions on targets 1-4 will accelerate tumour necrotic cell death by intracellular acidosis. Glut-1, glucose transporter-1; HIF-1, hypoxia-inducible factor-1.

accelerate necrotic cell death in tumours? Although tumour cells have selected many mechanisms to escape and resist programmed cell death, they cannot avoid ATP depletion-induced necrosis. Several steps must be combined to enforce necrotic cell death in tumours: the first is to magnify acidosis by increased anaerobic glycolysis, the second is to antagonize pH_i regulation and the third might be to stop macro-autophagy.

The first of these steps can be conveyed through anti-angiogenic treatment, which is the best way to enforce tumour hypoxia and HIF-1 expression, and to stimulate the production of lactic acid and CO₂ in the tumour.

For the second step, we need to identify the key pH_i-regulating systems to be antagonized (representative examples are outlined in Fig. 6). Hypoxic tumour cells in particular 'learn' how to counteract local acidosis, which is a major constraint in protein synthesis and cell growth. They induce, through HIF-1 α membrane-bound ectoenzymes, CA IX or CA XII, which catalyse the hydration of CO₂ to bicarbonate⁸⁸. The rapidly formed bicarbonate is pumped in by a member of the ubiquitous bicarbonate/Cl⁻ exchanger family⁸⁹, leading to cellular alkalinization and subsequent acidification of the extracellular milieu, to the benefit of the cell. Two additional key transporters, extruding H⁺ from the cell, are the lactate/H⁺ symporters, in particular monocarboxylate transporter-4 (MCT-4) induced by HIF-1 (refs 90, 91), and the growth factor and amiloride-sensitive Na⁺/H⁺ exchanger (NHE1)^{92,93}. Preliminary experiments conducted with Ras-transformed fibroblasts deleted in *nhe1*, in glycolysis (*pgi*) or in both, have established proof of principle for tumour regression by manipulating tumour acidosis⁹⁴. Tumour cells lacking *nhe1* showed a drastic reduction in tumour incidence unless the production of lactic acid was genetically suppressed⁹⁴. An ideal

translation of this concept to tumour therapy would be to associate specific inhibitors for NHE1 (cariporide), bicarbonate/Cl⁻ exchangers (S3705)⁹⁵, MCT-1 lactate/H⁺ symporter⁹⁶ and/or blockers of carbonic anhydrases (acetazolamide).

The third step to accelerate tumour necrosis might be to suppress, in combination with other treatments, macro-autophagy⁹⁷, which is a process commonly induced by hypoxia and representing the 'ultimate' nutritional source for tumour cells to survive low-nutrient conditions⁹⁸. We expect that this 'pH_i-targeted' therapy, combined with anti-angiogenesis to increase hypoxia-mediated acidosis, will synergistically induce the collapse and massive shrinkage of solid tumours.

Preclinical studies exploiting the inducible expression of shRNA together with the above-mentioned drugs in tumour mouse models should lead to the following: the identification of the best molecular HIF-dependent targets to suppress; the recognition of the best associations of drugs targeting tumour metabolism; and the definition of the most appropriate therapeutic windows.

Concluding remarks and perspectives

The HIF signalling cascade has emerged as an important nutrient-driven genomic response allowing tumour cells to survive, expand and invade. As a result, tumour hypoxia or HIF expression is strongly associated with a diminished therapeutic response and malignant progression⁹⁹. We should therefore consider systematically assessing by immunohistochemistry both HIF-1 α /2 α and E-cadherin expression in biopsies of patients presenting with early tumour lesions. Detecting an early marker of potential malignancy and identifying patients at risk would be a step forward for clinicians, but cannot be compared with a cure. Drugs targeting HIF-1 will be available soon and there is no doubt that they will have important clinical applications^{14,83}. However, in inflammatory cells, HIF-1 serves a key function in energy metabolism and its blockade results in severe immunodeficiency¹⁰⁰. Thus, as discussed for nuclear factor- κ B (NF- κ B; see the review in this issue by Karin, page 431), prolonged and prominent inhibition of HIF-1 is unlikely to be proposed in cancer prevention. Alternatively, as emphasized in this review, it seems more appropriate to target the most relevant HIF-induced gene products/functions of the tumour microenvironment. We strongly believe that the singularities of tumour metabolism will serve as a source of inspiration for developing novel cancer therapeutic approaches.

Although the biology of hypoxia signalling has progressed rapidly, and many of the HIF-induced gene products have been characterized, their exact functions within the tumour microenvironment remain to be determined. New exciting areas in HIF signalling that intercept with the NF- κ B response and inflammation¹⁰¹, cell differentiation, Notch signalling¹⁰² and stem cell auto-renewal⁸² are blowing fresh oxygen into this area of cancer biology. ■

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