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Regulation of angiogenesis by hypoxia: role of the HIF system

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The regulation of angiogenesis by hypoxia is an important component of homeostatic mechanisms that link vascular oxygen supply to metabolic demand. Molecular characterization of angiogenic pathways, identification of hypoxia-inducible factor (HIF) as a key transcriptional regulator of these molecules, and the definition of the HIF hydoxylases as a family of dioxygenases that regulate HIF in accordance with oxygen availability have provided new insights into this process. Here we review these findings, and the role of HIF in developmental, adaptive and neoplastic angiogenesis. We also discuss the implications of oncogenic activation of extensive, physiologically interconnected hypoxia pathways for the tumor phenotype.

Historical perspective

Beyond a certain size, simple diffusion of oxygen to metabolizing tissues becomes inadequate, and specialized systems of increasing complexity have evolved to meet the demands of oxygen delivery in higher animals. In mammals, the lungs, heart, vascular, and red blood cell systems all contribute to this important task, and precise coordination is required to avoid metabolic compromise or the risk of toxicity from excessive oxygenation. The first experimental indication that angiogenesis must be subject to some form of metabolic regulation was provided by the striking correlation between muscle capillary density and the metabolic rate of different species revealed in Krogh's classical morphometric studies, performed more than 80 years ago[1]. This concept was strongly supported by intervention studies that aimed to alter metabolic demand and observe effects on capillary density. Thus, immobilization can reduce muscle capillary density, whereas continuous neural stimulation can increase muscle capillary density[2,3].

Further insights into angiogenesis came from pathophysiological studies of vascular and neoplastic disease. In premature infants exposed to high concentrations of oxygen, development of the retinal circulation is attenuated. Pathological new vessel growth, causing blindness from retrolental fibroplasia, can be precipitated by hypoxia arising from a return to normal inspired oxygen concentrations[4]. Tumor development presents a different challenge, arising from the increasing metabolic demands of the growing mass of cells. In keeping with this, early work established that many tumors develop a severely hypoxic microenvironment[5] and secrete angiogenesis-stimulating factors[6]. In wounding, capillary injury generates a hypoxic environment, and altered oxygenation of experimental wounds alters the restorative angiogenic response[7]. Arguing from the importance of macrophages in wound repair, Knighton and colleagues showed that hypoxic culture induced the secretion of an angiogenic factor by bone marrow–derived macrophages[8]. Following the molecular characterization of several angiogenic growth factors, landmark studies in the early 1990s showed that hypoxia could induce expression of platelet-derived growth factor mRNA[9] and vascular endothelial growth factor (VEGF) mRNA in tissue culture[10]. In tumors, VEGF mRNA expression is substantially enhanced in zones surrounding necrotic foci, suggesting a mechanism by which a hypoxic microenvironment might stimulate tumor angiogenesis[10,11]. Taken together, these studies clearly indicated that oxygen availability was an important regulator of angiogenesis, but did not show whether oxygen itself or some consequence of hypoxia on energy metabolism was the trigger[12].

In part, difficulties in understanding the nature of the oxygen-sensitive signal arose from the relatively slow time course of angiogenic responses in vivo. This position contrasts with hormonal regulation of vascular oxygen content by the haematopoietic growth factor erythropoetin. Circulating erythropoietin increases several hundred fold within hours of hypoxic stimulation (reviewed in ref. 13). This highly dynamic characteristic enabled greater physiological characterization, including recognition that the response could not be induced by metabolic poisoning with mitochondrial inhibitors[14], but could be induced by transition metals such as cobaltous ions[15]—distinctive properties that suggested the operation of a specific oxygen-sensing process. Subsequent recognition that transcriptional regulation of erythropoietin and key angiogenic growth factors are linked to a common control mediated by a transcriptional complex, HIF-1, refocused attention on the possibility that angiogenesis can be regulated specifically by oxygen availability[16–19], and provided a means to address the underlying mechanisms through studies of the regulation of HIF.

HIFs and regulation by protein hydroxylation

HIF-1 is an αβ-heterodimer that was first recognized as a DNA-binding factor that mediates hypoxia-inducible activity of the erythropoietin 3′ enhancer[20,21]. Following early studies of the oxygen-dependent activity of the erythropoietin 3′ enhancer in a wide variety of non-

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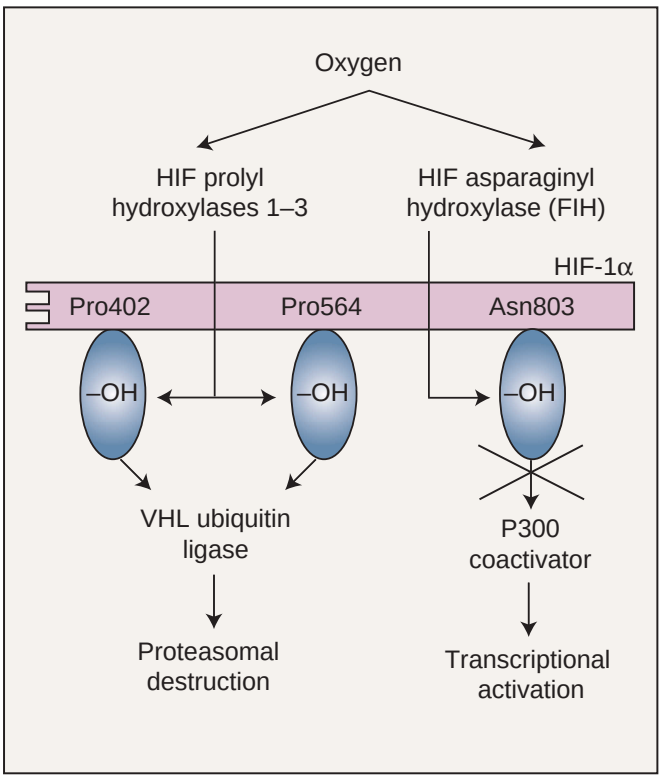

Figure 1 Dual regulation of HIF-α subunits by prolyl and asparaginyl hydroxylation. Hydroxylation sites are indicated for the human HIF-1α polypeptide.

erythropoietin-producing cells[22], it rapidly became clear that the HIF system is a key regulator of many other processes. These include angiogenesis among a broad range of cellular and systemic responses to hypoxia (reviewed in refs. 23,24).

Both the HIF-α and HIF-β subunits exist as a series of isoforms encoded by distinct genetic loci. HIF-1β subunits are constitutive nuclear proteins, whereas HIF-α subunits are inducible by hypoxia. Among three HIF-α isoforms, HIF-1α and HIF-2α appear closely related and are each able to interact with hypoxia response elements (HREs) to induce transcriptional activity[25,26]. In contrast, HIF-3α appears to be involved in negative regulation of the response, through an alternately spliced transcript termed inhibitory PAS domain protein[27]. HIF-α subunits are regulated by a multistep process involving changes in activity, abundance, mRNA splicing and subcellular localization (reviewed in ref. 24). Recent analysis of post-translational modifications that mediate these processes has revealed an unexpectedly direct interface with the availability of oxygen, through a series of non-heme, iron-dependent oxygenases that hydroxylate specific HIF-α residues in an oxygen-dependent manner[28–36].

Hydroxylation at two prolyl residues (Pro402 and Pro564 in human HIF-1α) mediates interactions with the von Hippel–Lindau (VHL) E3 ubiquitin ligase complex that targets HIF-α for proteasomal destruction[28–30,34]. Each site can interact independently with VHL E3, potentially contributing to the extremely rapid proteolysis of HIF-α that is observed in oxygenated cells[34]. These sites contain a conserved LxxLAP motif and are targeted by a newly defined prolyl hydroxylase activity, that in mammalian cells is provided by three isoforms termed PHD (prolyl hydroxylase domain) 1–3 (refs. 31,32). Determining the relative importance of different PHD isoforms in the regulation of HIF-α and other potential hydroxylation targets is the subject of active research. In a second hydroxylation-dependent control, β-hydroxylation of an asparaginyl residue in the C-terminal activation domain of HIF-α (Asn803 in human HIF-1α)[35] is regulated by a HIF asparaginyl hydroxylase[33,36] termed FIH (factor inhibiting HIF)[37]. Hydroxylation at this site blocks interaction of the HIF-α C-terminal activation domain with the transcriptional coactivator p300.

In oxygenated cells, these hydroxylation reactions provide a dual mechanism of HIF inactivation that involves proteolytic destruction and inhibition of transcriptional activity (**Fig. 1**). The HIF hydroxylases are all Fe(II)- and 2-oxoglutarate-dependent dioxygenases that have an absolute requirement for molecular oxygen, allowing HIF to escape inactivation in hypoxia. The enzymatic process splits dioxygen, with one oxygen atom creating the hydroxylated amino acid, and the other oxidizing 2-oxoglutarate to succinate with the release of CO_2 (reviewed in ref. 38). Fe(II) at the catalytic centre is loosely bound by a 2-histidine-1-carboxylate coordination motif, and may be displaced or substituted by other metals, such as cobaltous ions, with loss of catalytic activity. These findings therefore couple angiogenic regulation to metabolic demand through minimal pathways linking molecular oxygen availablity, HIF hydroxylase activity, HIF-dependent transcription and angiogenic growth factor expression (**Fig. 2**). They also account for the upregulation of angiogenic growth factors by cobaltous ions and iron chelators and indicate a route to therapeutic manipulation of angiogenesis.

Characterization of the HIF pathway has therefore provided a clear focus for investigation of the role of hypoxia in angiogenesis associated with development, ischemic and hypoxic disease, and neoplasia. Nevertheless, hypoxia influences many other transcriptional pathways, such as those mediated by fos and jun[39], NF-kB[40] and p53 (refs. 41,42). How these pathways interface with HIF, and whether they involve different targets for currently defined and other predicted protein hydroxylases, is the subject of active investigation. Recent data implicating prolyl hydroxylation in the VHL-dependent ubiquitylation of subunit 1 of RNA polymerase II indicates that regulatory prolyl hydroxylation extends beyond the HIF system[43].

Effects of hypoxia on molecular determinants of angiogenesis

Angiogenesis is a complex process, involving multiple gene products expressed by different cell types, all contributing to an integrated sequence of events[44]. Consistent with a major role for hypoxia in the overall process, a large number of genes involved in different steps of angiogenesis are independently responsive to hypoxia in tissue culture. Examples include nitric oxide synthases involved in governing vascular tone, growth factors such as VEGF, angiopoietins, fibroblast growth factors and their various receptors, and genes involved in matrix metabolism, including matrix metalloproteinases, plasminogen activator receptors and inhibitors, and collagen prolyl hydroxylase[16–19,45–61] (**Table 1**). Similarly, many of the individual phenotypic processes in angiogenesis such as cell migration or endothelial tube formation can be induced by hypoxic tissue culture[62–64]. These analyses in isolated systems have sometimes apparently shown both positive and negative effects of hypoxia, for instance on endothelial proliferation[65–67]. It is not yet clear whether such phenomena reflect fundamentally different responses based on severity of hypoxia, or counter-regulatory controls that are normally integrated in a productive way.

Experiments on cells bearing inactivating mutations in the HIF pathway have emphasized the importance of HIF (particularly HIF-1α) on the regulation of genes involved in angiogenesis[64,68–70]. For a substantial number of these genes, promoter analysis has also clearly identified HREs that interact directly with HIF. The expression of other genes may be affected indirectly by secondary cascades of gene regulation. Even with respect to a single molecule, there may be many interfaces with

hypoxia and hypoxia pathways (**Fig. 3**). This is well-illustrated for VEGF. In hypoxia, VEGF transcription is upregulated by HIF[18,19], mRNA stability is increased by binding of proteins to specific sequences in the 3′ UTR[71], and an internal ribosomal entry site allows preserved translation in the face of normal cellular hypoxic shutdown[72]. The biological activity of secreted VEGF is further influenced by hypoxia-inducible expression of the Flt-1 receptor[49], post-transcriptional regulation of the Kdr receptor[50] and VEGF-induced effects on soluble Flt-1 (ref. 73), which inhibits VEGF action (**Fig. 3**).

Essential role of HIF in developmental angiogenesis

In keeping with the central role of HIF in responses to hypoxia observed in tissue culture, targeted inactivation of either *Hif1a* (refs. 68,70,74) or *Arnt* (encoding HIF-1β)[75] in the mouse results in abnormal vascular development and embryonic lethality. In *Hif1a*$^{-/-}$ mice, defects in angiogenesis have been observed in both the yolk sac and in the developing embryonic tissues themselves, and are associated with severe hypoxia in the abnormal tissue. Despite marked reduction in VEGF expression by *Hif1a*$^{-/-}$ embryonic stem cells in hypoxic tissue culture, analysis of the developing *Hif1a*$^{-/-}$ embryos detected a surprising increase in *VEGF* mRNA expression. This suggests that compensatory stimuli may operate to induce VEGF, and that reduced vascular development may be mediated by other pathways such as the enhanced mesenchymal cell death that is observed in *Hif1a*$^{-/-}$ embryos[74].

Targeted inactivation of HIF-2α results in quite different and variable phenotypes. Though one study showed a defect in vascular remodeling, with abnormally fenestrated capillaries resulting in local hemorrhage[76], differing phenotypes have been observed in two other studies. These have shown instead either a defect in fetal catecholamine production[77] or a defect in lung maturation involving surfactant deficiency in the subset of *Hif2a*$^{-/-}$ offspring that survived to term[78]. The reason for variability is unclear, although it appears more likely to reflect differences in genetic background rather than incompletely inactivated *Hif2a* alleles. Overall, the results suggest that despite similar activity on HRE-linked reporter genes, HIF-1α and HIF-2α have important nonoverlapping, nonredundant functions in the regulation of endogenous gene expression in development. The unexpected and variable phenotypes presumably reflect the complexity of these responses.

Though studies with hypoxia-sensitive cell markers have shown regions of hypoxia during normal development[79], it is not yet clear whether, and to what extent, developmental abnormalities associated with genetic activation of HIF-α reflect lack of basal or hypoxia-induced HIF-α expression. More clearly related to hypoxia are the effects of partial inactivation of the *Hif2a* gene in a mouse model of the retinopathy of prematurity[80]. Affected mice showed striking reduction in the neovascularization response that normally follows return from hyperoxia to normoxia. Surprisingly, this was not associated with changes in expression of a range of angiogenic growth factors, but rather with blunted hypoxia-inducible erythropoietin expression. Given the protective effect of erythropoietin against light-induced retinal degeneration[81], the results raise the possibility that erythropoietin has a local function in retinal oxygen homeostasis. Overall, these studies emphasize the importance of HIF in developmental angiogenesis, although they have again revealed unexpected complexity.

Ischemic hypoxic disease

Because HIF-α subunits are destroyed by proteolysis in the presence of

Table 1 Summary of the action of hypoxia on some molecules involved in different steps in angiogenesis

Steps in angiogenesis	Stimulatory factors	Inhibitory factors
Vasodilation	Nitric oxide synthases[45]	
Increased vascular permeability	VEGF[16,18,19]	Angiopoietin-1 (ref. 46); Tie-2 (ref. 47)
	Flt-1 (refs. 48,49)	
	Kdr[18,50]	
Extravasation of plasma proteins	VEGF[16,18,19]	Angiopoietin-1 (ref. 46); Tie-2 (ref. 47)
Endothelial sprouting	Angiopoietin-2 (refs. 51,52);	
	Tie-2 (ref. 47)	
Degradation of extracellular matrix	Balance between MMPs (MMP-2; ref. 53) and TIMPs (TIMP-1; ref. 54)	
	Collagen prolyl-4-hydroxylase[55]	PAI-1 (ref. 56)
Liberation of growth factors	uPA receptor[57]	Thrombospondin-1 (ref. 60)
(including VEGF, IGF-1and bFGF)		PAI-1 (ref. 56)
Endothelial cell proliferation & migration	Interplay between VEGFs[16,18,19], angiopoietins[46–52] and FGFs[59]	
	MCP-1 (ref. 60)	
	PDGF[17,59]	
Pericyte and smooth muscle recruitment	PDGF[17,59]	
Endothelial assembly & lumen acquisition	$VEGF_{121/165}$ (refs. 16,18,19)	$VEGF_{189}$ (refs. 16,18,19)
	Angiopoietin-1 (ref. 46); Tie-2 (ref. 47)	
	Integrins[61]	Thrombospondin-1 (ref. 58)
Stabilization of nascent vessels	PAI-1 (ref. 12)	
Maintenance, differentiation and remodeling	Angiopoietin-1 (ref. 46); Tie-2 (ref. 47)	Angiopoietin-2 (refs. 51,52); Tie-2 (ref. 47)

Red, direct transcriptional targets of HIF identified by functional studies of HREs in promoter elements; light red, hypoxia-responsive genes in which HIF or related pathways are indirectly implicated by responses to cobalt, iron chelators or VHL inactivation; gray, responses not so far known to be connected to the HIF pathway. IGF, insulin-like growth factor; bFGF, basic fibroblast growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase plasminogen activator; MCP, monocyte chemoattractant protein; PDGF, platelet-derived growth factor; PAI, plasminogen activator inhibitor.

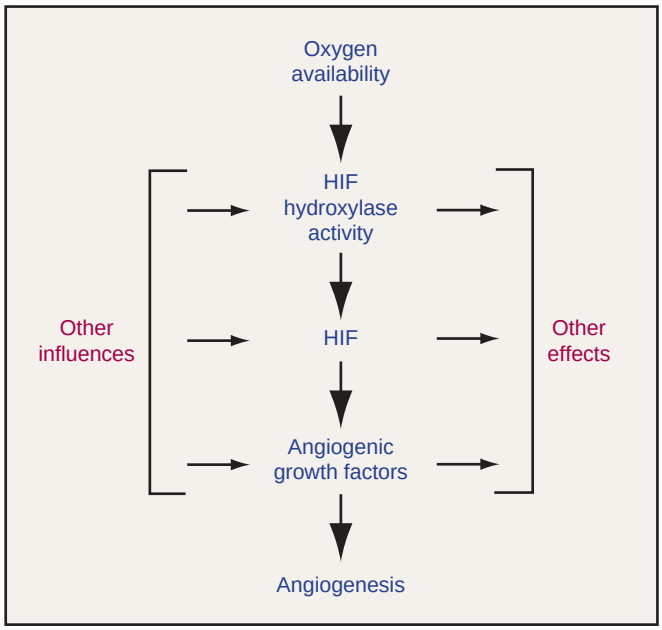

Figure 2 Pathways linking angiogenesis to oxygen availability through the regulation of HIF. The minimal links are indicated in bold. Each step is also influenced by other inputs that shape the final response to hypoxia.

oxygen, immunohistochemical staining of tissues for levels of HIF-α provides a guide to the activity of the system. Such studies have shown that HIF-α levels are generally low in normoxic rodent tissues, and may be undetectable even in physiologically hypoxic regions such as the renal medulla[82]. Levels are substantially increased after systemic hypoxia or tissue ischemia, although the extent and time course of induction varies between tissues[83–85]. HIF-α induction is often cell-type-specific within a particular region, suggesting that cellular thresholds for activation of the response may differ[82,84,85]. Other studies have shown induction of HIF-1α in the pre-eclamptic placenta[86], macrophages from inflamed joints[87], the retina after hypoxic preconditioning[81,88], and wounded skin[89], indicating that the system is activated in a broad range of ischemic, hypoxic and inflammatory conditions.

These studies raise important questions as to the role of HIF activation in determining the outcome of ischemic injury and whether manipulation of HIF could improve this. Therapeutic activation of HIF might in theory be applied before the ischemic stress as a form of preconditioning stimulus, or used in the peri-ischemic period to augment the natural response.

Several strategies have been used successfully for experimental activation of HIF. Deletion of the central oxygen-dependent degradation domain results in a stable and constitutively active HIF-1α molecule. Transgenic expression of such a molecule in skin using a keratin K14 promoter results in marked activation of HIF transcriptional targets and overgrowth of blood vessels. These vessels are not leaky and the enhanced skin vascularity was not associated with edema[90]. This contrasts with reports of proangiogenic therapy with VEGF where edema is often observed, at least early after VEGF application, and suggests that HIF activation might avoid this potential complication. An alternative approach has used the N-terminal DNA-binding and dimerization domain of HIF-1α fused to the transactivation domain of herpes simplex virus VP16 as gene therapy. In a rabbit hindlimb ischemia model, administration of this naked DNA resulted in an improvement, determined both in terms of the number of blood vessels and regional blood supply[91]. Similar results have recently been reported in a rat myocardial infarction model[92].

Other investigators have focused on the possibility of inhibiting degradation of native HIF-α. PR39, a macrophage-derived peptide that interacts with the proteasome and stabilizes HIF, has been shown to increase peri-infarct vascularization in mouse cardiac tissue[93]. In a potentially more specific approach, overexpression of peptides corresponding to either of the VHL-binding prolyl hydroxylation sites in human HIF-1α has been shown to block degradation of the native molecule, promote HIF transcriptional activity, and generate an angiogenic response[94].

Perhaps the most exciting possibility, however, is the use of small-molecule inhibitors of the HIF hydroxylases. Inhibition of the HIF hydroxylases by 2-oxoglutarate analogs will stabilize HIF and activate the transcriptional response[29]. Such an approach was first developed for the inhibition of procollagen prolyl hydroxylase as antifibrotic therapy. Data generated using procollagen prolyl hydroxylase inhibitors will now require re-evaluation, following the recent recognition that many of these compounds also inhibit the HIF hydroxylases. For instance, favorable responses to one such compound, FG0041, in a rat model of myocardial infarction were observed even in the face of little detectable fibrosis in control animals[95], and may reflect the action of this compound on HIF hydroxylases[96]. Given the new insights into the extent of the 2-oxoglutarate-dependent oxygenase family[97,98], it is likely that development of more specific inhibitors will be necessary. Nevertheless, distinctive features of the 2-oxoglutarate and HIF peptide-binding sites revealed by structural analysis of the asparaginyl hydroxylase FIH suggest that this should be achievable[98].

The role of HIF in tumor angiogenesis

As may be predicted from its physiological role, HIF is also important in tumor angiogenesis. Upregulation of the HIF system is observed in many common cancers and occurs by a multiplicity of genetic and environmental mechanisms.

In addition to activation by hypoxia, the HIF system is also induced or amplified by a wide range of growth-promoting stimuli and oncogenic pathways such as the insulin, insulin-like growth factor-1, epider-

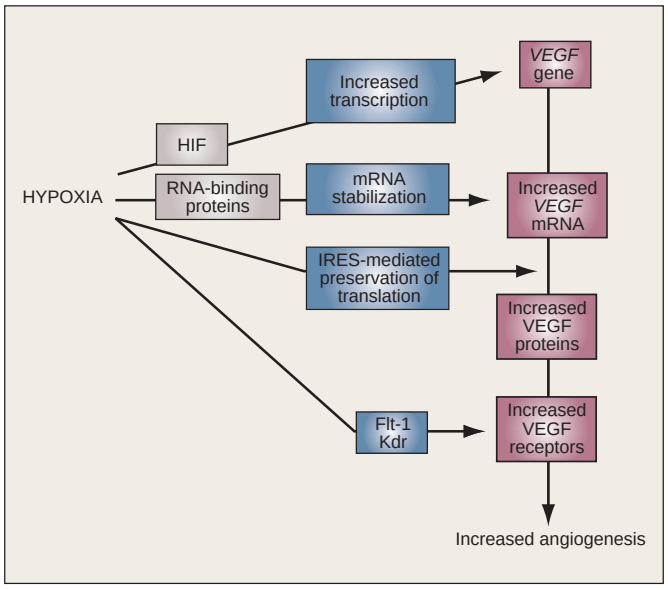

Figure 3 Multiple interfaces of hypoxia pathways with the angiogenic growth factor VEGF. IRES, internal ribosomal entry site.

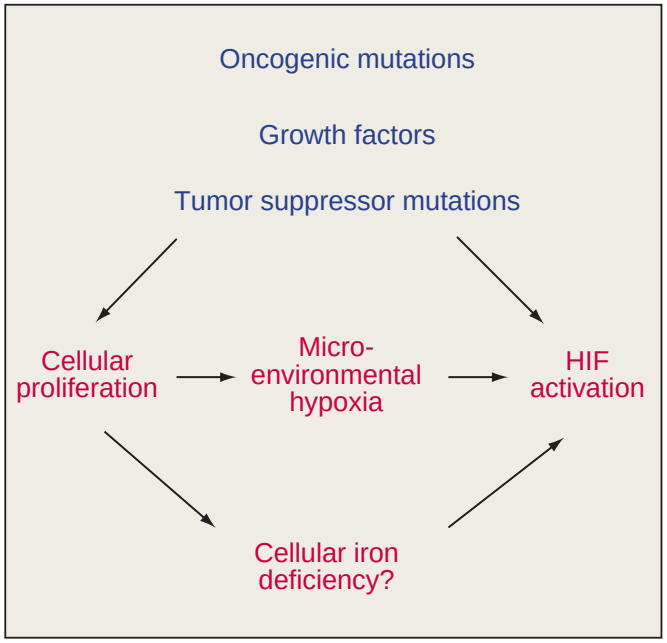

Figure 4 Links between growth-promoting pathways and HIF activation that upregulate HIF in cancer.

mal growth factor and mutant Ras and Src kinase pathways. Tumor suppressor mutations, including PTEN, p53, p14ARF, and most strikingly pVHL, also activate the HIF system (reviewed in refs. 24,99,100). As outlined above, pVHL is a key component of the oxygen-sensitive pathway, and inactivation results in constitutive stabilization of HIF-α subunits and upregulation of the transcriptional response. Other mechanisms of oncogenic and growth factor stimulation of the HIF pathway are less clearly defined, although induction of HIF1α mRNA levels and enhanced translation, stability and activity of HIF-α protein have all been described. In keeping with the known transduction pathways mediating responses to these stimuli, both the MEK–MAP kinase[101] and the phosphatidylinositol-3 kinase–Akt[102] systems have been implicated in these processes.

Microenvironmental activation of HIF in cancer occurs at the simplest level by physiological activation of the oxygen-sensitive pathways by hypoxia within the growing mass of cells. Surprisingly, however, studies using hydroxylation-specific antibodies have indicated that prolyl hydroxylation of HIF is often incomplete in tumor cells, even in fully oxygenated tissue culture[103]. Under these conditions, HIF-α degradation can be enhanced by supplementation with iron or ascorbate, most probably by promotion of HIF hydroxylase activity[104]. Precarious iron balance is a well-recognized characteristic of rapidly growing tumor cells[105]. Taken together, these findings suggest that reduction of cellular iron availability, as well as hypoxia, may limit HIF hydroxylase activity in cancer cells and contribute to the general activation of HIF in tumors (**Fig. 4**).

Given the multiplicity of mechanisms activating the HIF system in cancer, and the many HIF target genes involved in the angiogenic process, it is tempting to propose a simple system whereby activation of HIF in cancer promotes angiogenesis and hence tumor growth. If true, such a model would strongly support the HIF system as an anticancer target.

Several studies have sought to test this model genetically in studies of experimental tumor formation by cells that are defective in particular components of the HIF pathway. The first of these used a series of mouse hepatoma cells that are functionally defective for HIF-1β and cannot form an active HIF complex. When grown as subcutaneous tumors, the mutant cells manifest a near-total loss of the peri-necrotic enhancement of *VEGF* gene expression that is apparent in wild-type cells. This was associated with marked reductions in tumor angiogenesis and growth rates[106]. Similar reductions in growth and vascular density in tumors grown from human colon and breast carcinoma cell lines have been observed after expression of a peptide that antagonizes HIF transcription by abrogating the interaction of HIF-α and p300 (ref. 107).

Effects of targeted inactivation of mouse HIF-1α on the behavior of embryonic stem cell–derived teratomas have been variable, with neither vascularity nor growth being positively correlated with an intact HIF system in every study[69,70,108]. Positive effects of HIF-1α on tumor growth without discernable effects on tumor vascularity have been observed, not only in teratomas, but also in fibrosarcomas derived from SV40- and H-Ras-transfected fibroblasts[108,109]. That these effects are not cell-autonomous is suggested by mixing experiments in which a small proportion of *Hif1a*$^{+/+}$ embryonic stem cells can rescue the

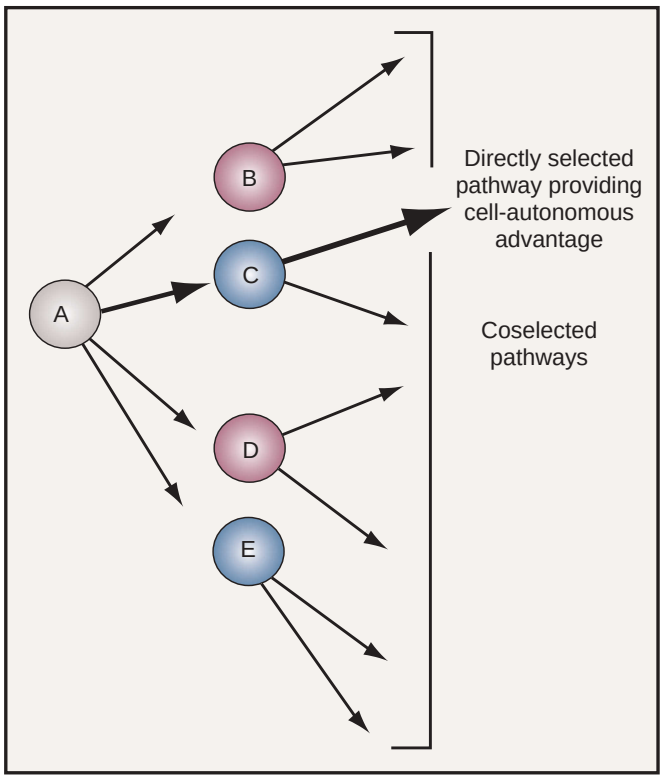

Figure 5 Schematic illustrating the coselection of pathways in cancer. A–E are molecules that normally operate in physiologically hardwired pathways, indicated by arrows. The broad red arrow represents an oncogenically activated pathway that promotes a cell-autonomous selective survival advantage. Other pathways are coselected because of pre-existing physiological connections and are upregulated in the developing tumor. They may coincidentally provide a growth advantage to the growing tissue mass (green), be neutral (blue), or even inhibit growth (black). Positive selective pressure operates on the red pathway, and tumor-associated activating mutations of both A and C are anticipated. Molecules B, D and E are activated by coselection after selection of cells bearing activating mutations of A. The coselected pathways commonly will not harbor activating mutations as they are not themselves under positive selective pressure. For the same reason, the results of genetic inactivation or therapeutic intervention at points B, D and E in these coselected pathways will be unpredictable, despite the strong association of upregulation with cancer.

impaired growth of *Hif1a*$^{-/-}$ teratomas without themselves becoming over-represented in the tumor mass[108]. Overall, these results indicate that HIF has important non-cell-autonomous growth-promoting effects that extend beyond its role in angiogenesis.

Discrepancies between studies may reflect the balance of multiple effects in different cell backgrounds, with effects of HIF status on the differentiation of embryonic stem cells making this a particularly complex system for analysis of bulk growth. Given this evidence for the importance of cell background, other studies have focused on the functional consequences of HIF upregulation in the specific tumor types that are associated with VHL inactivation.

VHL disease is characterized by a highly tissue-restricted cancer predisposition involving renal cell carcinoma, hemangioblastoma, and pheochromocytoma, but rarely other tumors. Furthermore, the disease manifests a clear genotype-phenotype correlation, with functional studies of different mutations being consistent with a role of HIF upregulation in the predisposition to renal cell carcinoma (RCC) and hemangioblastoma but not pheochromocytoma (reviewed in ref. 110). Several groups have studied the tumorigenic potential of HIF in the RCC cell background. Transfection of the VHL-defective RCC line 786-0 with a wild-type VHL gene downregulates HIF activity[111], and suppresses growth as tumor xenografts in mice[112]. To address the existence of a causal link between these effects, 786-0 wild-type VHL transfectants have been further transfected with HIF-α polypeptides that are mutated at the C-terminal VHL hydroxyproline recognition site so as to allow accumulation even in the presence of VHL. In assays of tumor suppression by VHL, one study found that additional expression of stable HIF-2α blocked the tumor suppressor action[113], whereas a different group using similar methods found that stable HIF-1α expression did not block VHL tumor suppression, and in fact retarded growth still further[114]. In contrast, saturating overexpression of a peptide containing the site of prolyl hydroxylation recognized by VHL blocked tumor suppression[114]. Overall, these results suggest the VHL tumor suppressor effect is dependent on its ability to capture hydroxylated substrates that include, but are not necessarily confined to, HIF-2α.

The effect of VHL inactivation has also been studied in other cell types. Substantial upregulation of the HIF system is observed even in cell backgrounds unrelated to VHL-associated cancer[115,116]. Teratomas grown from VHL-defective embryonic stem cells, however, manifest reduced growth rates despite increased vascularity[116].

Coselection of hypoxia pathways in human development

At first sight, many of the above findings appear paradoxical. HIF is frequently upregulated in cancer, and at least in isolated tissue culture systems, the pathway has multiple effects that may be predicted to promote angiogenesis. Nevertheless, genetic studies have not always supported a simple model in which upregulation of HIF promotes tumor angiogenesis, and hence tumor growth. Oddly, though HIF is frequently a target of oncogenic pathways, direct activating mutations in HIF itself have not so far been described. How can these findings be explained? A potential answer lies in the effect of oncogenic activation of pathways that have extensive hardwired physiological connections.

Genetic studies indicate that the development of cancer is a multistep process in which mutation and clonal selection result in progressive development of the cancer phenotype[117]. In such a model, selection must operate at the level of the cell; the cancer phenotype would be predicted to represent the accrual of properties that had been selected through a cell-autonomous survival advantage. Many aspects of tumor biology are clearly at odds with this prediction. In the case of the angiogenic phenotype, it is difficult to understand how selection can operate, as the advantage is equally available to a population of cells, and clonal selection requires that a cell gain predominance over its neighbors by an individual rather than a group advantage.

If a genetic mutation affects the function of an extensive physiological pathway, however, then clonal selection of a particular property will result in coselection of many properties that are linked through the pathway. Such properties could then be acquired irrespective of whether they contribute positively, negatively or not at all to the overall advantage driving selection of the emerging clone (**Fig. 5**). In respect of HIF and hypoxia pathways, the enormous scale of interconnection suggests that these effects may be particularly prevalent. Data outlined above indicate that cellular proliferation, HIF activation and angiogenesis are linked by pathways that operate physiologically to preserve oxygen homeostasis. Thus, it is possible that hypoxia and angiogenesis pathways are simply coselected in cancer by these pre-existing links to events during cancer progression that are selected through cell-autonomous survival advantage. Such a possibility would be consistent with the observation that HIF and angiogenic growth factors are not common sites for direct mutational activation in cancer, and could explain why, despite common upregulation in cancer, the effects of inactivation of the HIF pathway are somewhat inconsistent.

The operation of coselection would also predict that hypoxia pathways might often be activated in a disorganized way in cancer, as activity reflects links to stochastic events driving clonal selection rather than a properly coordinated physiological response. This could potentially explain the paradox that tumors often develop an angiogenic phenotype that is ineffective in the delivery of oxygen. These arguments do not disqualify HIF or other components of hypoxia pathways as therapeutic targets in cancer, but they do indicate a need for caution in predicting the antitumor effect of intervention at a given point in these complex pathways—as is manifest by the genetic studies of HIF.

COMPETING INTERESTS STATEMENT
The authors declare competing financial interests (see the *Nature Medicine* website for details).

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