Understanding hypoxia signalling in cells – a new therapeutic opportunity?

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ABSTRACT – The possibility that cells possess specific interfaces with molecular oxygen that have a prime function in biological control has long interested biologists. Specific 'oxygen-sensing' mechanisms have been defined in bacteria and yeast, but, until recently, have remained elusive in higher organisms. Studies of hypoxia pathways have now, however, revealed the existence of a series of non-haem Fe(II) and 2-oxoglutarate-dependent dioxygenases that catalyse oxygen-regulated hydroxylation of specific amino acids in a key transcription factor termed hypoxia-inducible factors (HIFs). These post-translational hydroxylations govern both the proteolytic stability and activity of HIF and therefore the transcription of many hundreds of human genes whose expression changes in accordance with cellular oxygen availability. This paper will review these developments and consider the biological and potential therapeutic implications.

Effective delivery of oxygen to metabolising tissues is a central physiological challenge for all large multicellular organisms. In man, the pulmonary, cardiac, vascular, and erythropoietic systems all contribute to the formidable task of ensuring appropriate delivery of oxygen to the body's approximately 10^{14} respiring cells, and very precise coordination of growth and physiological function is needed to avoid metabolic compromise or the risk of toxicity from excessive oxygenation.

The first major steps in the understanding of oxygen delivery by these systems can be traced to the times of Thomas Croone in the mid-17th century. The description of the blood circulation by William Harvey in *De motu cordis et sanguinis in animalibus* (1628) left an open question as to its purpose. Harvey's landmark deduction was based, in part, from the observation that rates of blood flow were much higher than previously supposed – and inconceivable without recirculation. Hence the focus of attention after this discovery was on the purpose of this rapid movement of the blood. Richard Lower (1631–91) working in Oxford with Robert Hooke (1635–1702) noted that while the blood leaving the heart for the lungs was blue, the blood returning from the lungs to the heart was red. By mixing blood with air in a glass vessel Lower noted the same colour change, concluding that 'nitrous spirit of the air, vital to life, is mixed with the blood during transit through the lungs.' It was to be another 100 years before the work of Priestley, Scheele and Lavoisier defined the essential 'spirit of the air' as oxygen and Lavoisier correctly described the chemistry of combustion, concluding that biological energy metabolism was essentially the same process. A further 100 years passed before the early environmental physiologists gained the first insights into the control mechanisms that respond to altered oxygen availability. In the late 19th century, a correlation between life at altitude and increased haemoglobin content of the blood was noted by Paul Bert,[1] but it was Mabel Fitzgerald (a colleague of JS Haldane on the expedition of 1911 to Pike's Peak, Colorado, to study breathing responses at altitude) who first clearly described the sensitivity of this response, illustrating that relatively minor reductions in barometric pressure at modest altitude were associated with a discernable elevation of haemocrit.[2] These observations were the first of many that ultimately defined the extremely sensitive control of red blood cell production in response to changes in blood oxygen availability. Although studies at this time also suggested the operation of a circulating factor in the regulation of red cell production,[3] hormonal control by erythropoietin was finally proved beyond doubt by Erslev's classic plasma transfer experiments in the early 1950s.[4]

Circulating levels of erythropoietin can be increased several 100-fold within hours of hypoxic stimulation. The response cannot be induced by metabolic poisoning with mitochondrial inhibitors, but it can be induced by transition metals, such as cobaltous ions, and iron chelators – distinctive properties that suggested the operation of a specific oxygen-sensing process and formed the point of entry for recent molecular analyses of oxygen sensitive signal pathways (reviewed in Jelkmann[5]). Two advances in the mid-1980s greatly facilitated this approach: molecular cloning of the erythropoietin

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gene and the development of tissue culture models for studying regulation by oxygen.

Unexpectedly, early studies of erythropoietin gene regulation revealed that the oxygen sensitive signal pathways that underlie erythropoietin regulation operate in essentially all mammalian cells irrespective of their relevance to erythropoietin production and that they regulate many other genes.[6] Central to the response is a series of closely related transcription factors, termed hypoxia-inducible factors (HIFs), that induce a very extensive transcriptional cascade – directly or indirectly controlling the expression of hundreds of genes in any given cell type. Hypoxia-inducible factor transcriptional targets are now recognised to play a key role in enhanced angiogenesis, as well as enhanced erythropoiesis, vasomotor regulation, matrix metabolism, cell proliferation and survival decisions, energy metabolism, and many other cellular and systemic responses to hypoxia (for review, see Semenza[7])(Fig 1). The recent elucidation of pathways that regulate HIF, as novel signalling systems mediated by post-translational protein hydroxylation, has provided some of the first molecular biochemical insights into the complex task of maintaining physiological oxygen homeostasis (for review see Schofield[8]). Given the prevalence of ischaemic and hypoxic pathology in human disease, these insights have also generated considerable interest as a potential basis for drug design. This article will outline the biological perspective of the new findings and consider the challenge of therapeutic translation.

The HIF hydroxylase system

Hypoxia-inducible factor is an α/β heterodimer of basic-helix-loop-helix proteins that binds DNA sequences within the hypoxia response elements (HRE) at the loci of target genes.[9] Both HIF-α and HIF-β subunits exist as a series of isoforms encoded by distinct genetic loci. The HIF-β subunits are constitutive nuclear proteins, whereas HIF-α subunits are inducible by hypoxia. The proteolytic stability of HIF-α and its transcriptional activity are regulated by distinct mechanisms that have the oxygen-dependent hydroxylation of specific amino acid residues in common. Hydroxylation at two prolyl residues (Pro 402 and Pro 564 in human HIF-1α) mediates interactions with the von Hippel-Lindau E3 ubiquitin ligase complex that targets HIF-α to the ubiquitin-proteasome pathway for proteolytic destruction.[10,11] These hydroxylations are catalysed by a series of three closely related HIF prolyl hydroxylases, termed prolyl hydroxylase domain (PHD) 1–3.[12] In a second hydroxylation-dependent control, b-hydroxylation of an asparaginyl residue in the C-terminal activation domain of HIF-α (Asn 803 in human HIF-1α) is catalysed by a HIF asparaginyl hydroxylase termed factor inhibiting HIF (FIH).[13–15] Hydroxylation at this site blocks interaction of the HIF-α C-terminal activation domain with the transcriptional coactivators p300/CBP (Fig 2). The HIF hydroxylases are all iron (II)- and 2-oxoglutarate-dependent dioxygenases that have an absolute requirement for molecular oxygen. In hypoxia, therefore, hydroxylation of both the prolyl residues and the asparaginyl residue is reduced, which allows HIF-α to escape von Hippel-Lindau (VHL) ubiquitin ligase complex-mediated proteolysis, to recruit coactivators, and to activate the transcription of hypoxia-inducible genes. The enzymatic process splits dioxygen, with one oxygen atom creating the hydroxylated amino acid and the other oxidising 2-oxoglutarate to succinate with the release of carbon dioxide (Fig 3). Iron (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 cobalt (II), with loss of catalytic activity, which accounts for the classic properties of activation of HIF, and induction of hypoxia-responsive genes such as erythropoietin, by cobaltous ions and iron chelators (for review, see Schofield[8] and Kaelin[16]).

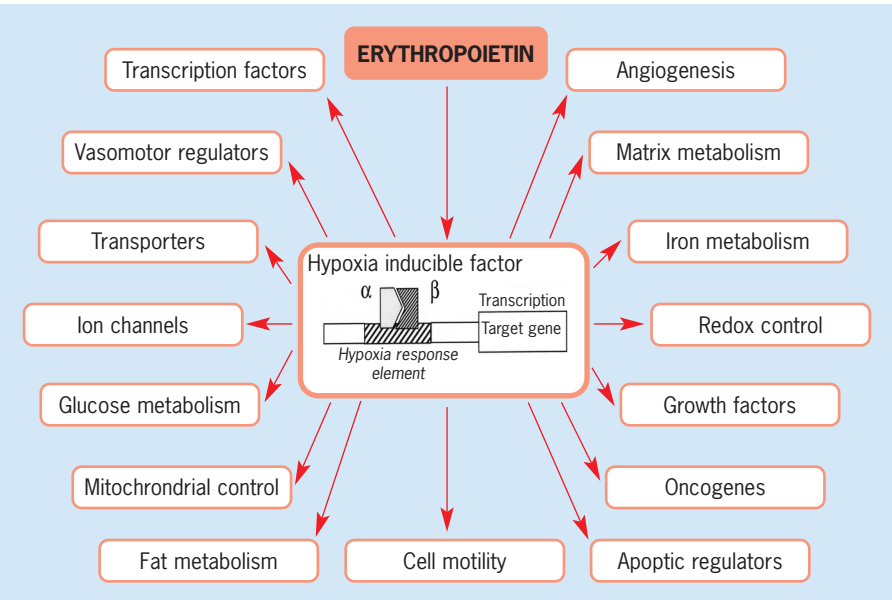

Fig 1. The hypoxia-inducible factor (HIF) transcriptional cascade directly regulates genes with key functions in a broad range of processes. The complex binds in a sequence-specific manner to control elements in DNA, termed hypoxia-response elements, at target gene loci.

The 2-oxoglutarate dioxygenase superfamily is widely represented across both prokaryotes and eukaryotes, but to date it is unclear whether the HIF hydroxylases have evolved unique catalytic features or are relatively ordinary 2-oxoglutarate-dependent oxygenases that simply use their absolute requirement for molecular oxygen in a signalling role. The evolutionary origin of the oxygen-sensing function of 2-oxoglutarate oxygenases in higher animals is also unclear. Members of the 2-oxoglutarate oxygenase family and related enzymes oxidise both small- and large-molecule substrates and are involved in diverse biological functions. None of these processes point clearly to an ancestral oxygen-sensing mechanism, however, and in lower organisms, such

as bacteria and yeast, other types of enzyme have been implicated in this role. Although strikingly conserved across nematode worms, insects, and vertebrates, both HIF and the HIF hydroxylases are apparently confined to higher eukaryotes – perhaps suggesting that the system developed in response to the challenge of oxygen homeostasis in multicellular animals.

Biological control of HIF hydroxylase activity

Given the very broad range of processes that manifest regulation by the HIF hydroxylase system and their operation in cells that operate at substantially different oxygen tension within the intact organism, an important challenge now is to understand how the biochemical process of oxygen-dependent protein hydroxylation can generate the flexibility necessary for a role in physiological oxygen homeostasis.

In vitro assays of enzyme kinetics indicate that the apparent K_m (concentration of substrate that gives half-maximal activity) for oxygen for the HIF hydroxylases is well above the physiological range, with reported values of 230–250 μM for the PHDs and about 100 μM for FIH.[12,17,18] Important caveats to these analyses exist, however, such as the use of relatively short peptides rather than native HIF-α polypeptides and the necessary use of unphysiological reaction conditions in the *in vitro* assays. Nevertheless, it seems likely that concentrations of oxygen in tissues, believed to be in the range of 10–30 μM, will essentially always be below the K_m for oxygen of the HIF hydroxylases and thus limiting for enzyme activity over the entire physiological range. For oxygen-sensitive operation of the system, it is also important that the overall cellular capacity for HIF hydroxylation is such that, within the physiological range, hydroxylation is rate limiting for HIF degradation or inactivation (Fig 4). Evidence that this is indeed the case is provided by observations that modest changes in enzyme activity achieved genetically by overexpression or small interfering RNA (siRNA)-mediated suppression of individual hydroxylase enzymes have clear effects on levels of HIF-α levels and HIF transcriptional target gene expression. In this respect, it is also interesting that the PHD enzymes exhibit marked inducible and cell-type specific patterns of expression. In particular, PHD2 and PHD3 are markedly inducible by hypoxia by mechanisms that include transcriptional activation by HIF itself. Increases in enzyme abundance will increase the rate of HIF hydroxylation at any given oxygen tension. Increased

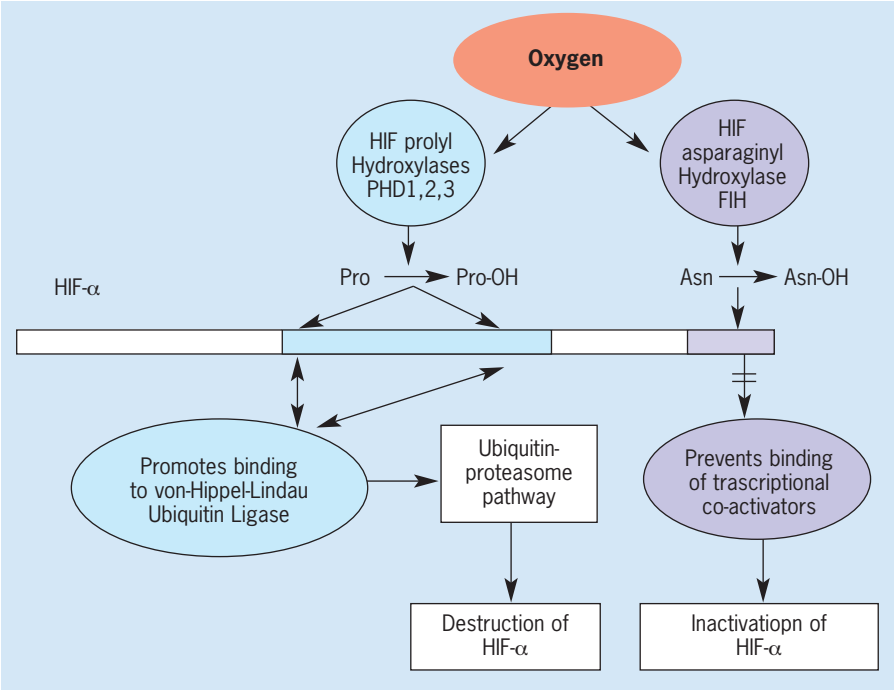

Fig 2. Dual regulation of hypoxia-inducible factor (HIF)-α subunits by oxygen-dependent prolyl (Pro) and asparaginyl (Asn) hydroxylation.

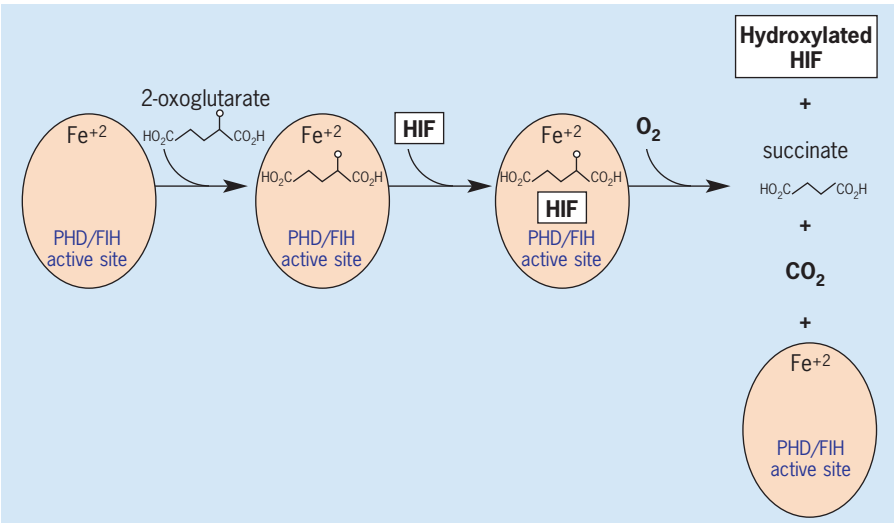

Fig 3. Reaction catalysed by the hypoxia-inducible factor (HIF) prolyl hydroxylase (PHD) enzymes and the HIF asparaginyl hydroxylase (FIH). On the basis of precedent for other enzymes of this type, molecular oxygen is postulated to bind the catalytic centre after ordered binding of the cosubstrate, 2-oxoglutarate, and the prime substrate, HIF. In a radical reaction at the catalytic iron centre, molecular oxygen is split, with one atom incorporated into the hydroxylated amino acid residue and the other into the oxidative decarboxylation of 2-oxoglutarate.

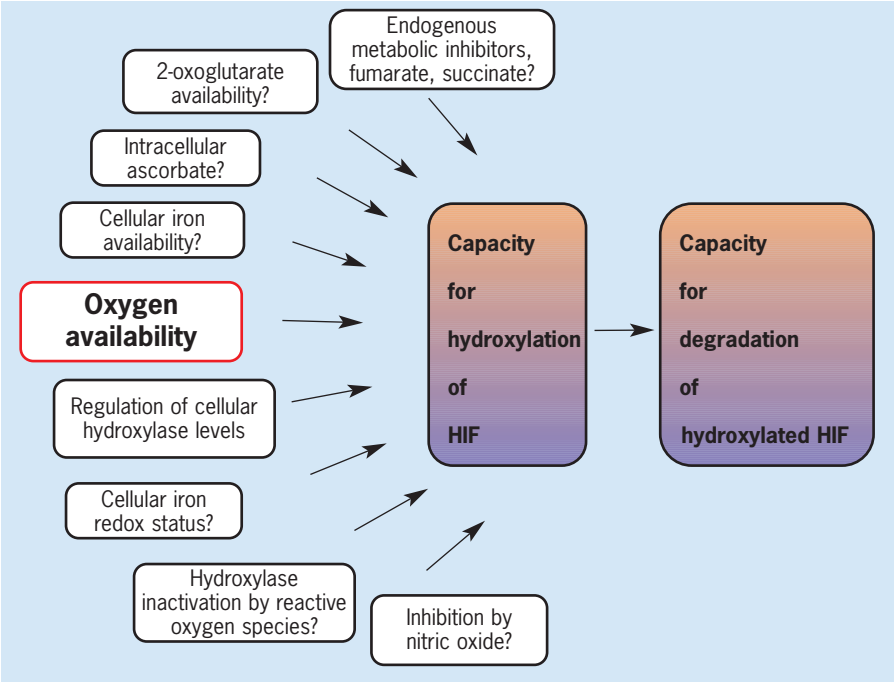

Fig 4. Actual and potential regulatory inputs to the hypoxia-inducible factor (HIF) hydroxylases. These processes are postulated to serve to enable flexible operation of the system in physiological oxygen homeostasis. Note that cellular capacity for hydroxylation of HIF must be in a similar range to cellular capacity for degradation of hydroxyated HIF for optimum oxygen sensitivity of the pathway.

levels of hydroxylase in hypoxic cells (or reduced levels in well-oxygenated cells) thus may serve a 'range-extending' function that matches hydroxylation capacity to that required for regulation of HIF over a wider range of oxygen concentrations.

Interestingly, emerging data suggest that HIF hydroxylase activity might also be controlled at a number of other levels in addition to the level of oxygen, potentially providing flexibility for directing physiological responses to hypoxia (Fig 4) (for review, see Schofield[8] and Kaelin[16]). In this respect, a number of properties of the HIF hydroxylases, including their relatively complex cosubstrate and cofactor requirements, are intriguing.

Use of the citric acid cycle intermediate 2-oxoglutarate as cosubstrate, and the action of other citric acid cycle intermediates such as fumarate and succinate as competitive inhibitors of 2-oxoglutarate binding, in *in vitro* assays of dioxygenase activity raises the interesting possibility of dual control by oxygen and energy metabolism. Whether and under what circumstances these metabolites reach critical levels for modulation of hydroxylase activity in cells, however, is unclear. Intriguingly, however, genetic defects in succinate dehydrogenase and fumarate hydratase (enzymes of the citric acid cycle) have been associated with tumours that manifest enhanced angiogenesis or activation of HIF, or both, possibly arising from suppression of hydroxylase activity by accumulation of succinate or fumarate.[19,20]

Another property that may contribute to control is dependence on iron (II) and ascorbate. As noted above, iron binding by the 2-histidine-1-carboxylate motif at the catalytic centre is relatively labile and the HIF hydroxylases are readily inhibited by iron chelators, which explains the activation of HIF transcription by such agents. Ascorbate is another cofactor that is needed for the full catalytic activity of many 2-oxoglutarate-dependent dioxygenases, including the HIF hydroxylases. The precise mechanism of action is unclear, although in the case of one group of enzymes – the procollagen prolyl hydroxylases – ascorbate seems to be used in reduction of the catalytic iron centre after uncoupled cycles that generate an inactive oxidised iron centre. Whether physiological changes in cellular iron (II) availability or ascorbate affect HIF hydroxylase activity *in vivo* is unclear. Addition of iron or ascorbate to tissue culture medium, however, readily suppresses the accumulation of unhydroxylated HIF-α that is frequently observed in rapidly growing, but apparently well-oxygenated, cultures of tumour cells. This indicates that, at least under these conditions, availability of these cofactors does indeed become limiting. It will now be of interest to determine whether similar mechanisms contribute to HIF activation and excessive angiogenesis observed in native cancer growth.

Other possibilities supported by evidence in cultured cells are that HIF hydroxylase activity may be inhibited by nitric oxide or inactivation of the catalytic iron centre by oxygen radicals, which potentially links the pathway to other signalling systems. Again, the challenge now is to determine to what extent such processes operate physiologically, particularly in the intact organism.

Therapeutic development

The role of the HIF transcriptional cascade in many adaptive and potentially protective physiological responses to hypoxia has suggested that pharmacological augmentation of HIF activation might be used in the treatment of hypoxic or ischaemic conditions. The enzymatic basis of HIF regulation, together with the requirement of the HIF hydroxylases for cosubstrates such as 2-oxoglutarate, provides a typical system for drug targeting through development of competitive 2-oxoglutarate analogues or more complex inhibitors. Indeed, such an approach has previously been taken in attempts to develop procollagen prolyl hydroxylase inhibitors that might limit tissue fibrosis. Some of the prolyl hydroxylase inhibitors developed in this way also inhibit HIF hydroxylases and clearly activate HIF target genes. Relative specificity for HIF versus procollagen prolyl hydroxylases is observed for certain compounds, however, which supports the feasibility of selective inhibition.

Analysis of the action of HIF hydroxylase inhibitors and other means of HIF activation in models of anaemia and ischaemic vascular diseases has suggested efficacy in a number of situations. Therapeutic development, however, still presents a number of challenges. Bioinformatic predictions made possible by genome-sequencing projects suggest the existence of an extensive family of 2-oxoglutarate oxygenases, with up to 40 or so predicted members encoded by the human genome.[21] Such insights provide the means to identify and limit potential unwanted 'off-target' effects from relatively unselective 2-oxoglutarate analogues. Nevertheless, to achieve and prove specificity for PHD enzymes against a range of 2-oxoglutarate oxygenases with known and unknown functions remains a challenging task. The pleotrophic nature of the HIF transcriptional response also creates both opportunity and challenge. This is well illustrated by considering the role of HIF in promoting two processes that might be of medical benefit – erythropoiesis and angiogenesis. In each case, activation of HIF can promote an effective response, and in each case efficacy is most probably based on the ability of HIF activation to regulate a range of targets in the relevant pathway. In angiogenesis, therefore, the aim of pharmacological HIF activation would be to augment the physiological activation of angiogenesis by hypoxia. The HIF pathway modulates the expression of not only a range of key angiogenic growth factors such as vascular endotheial growth factor (VEGF) but also growth factors receptors and molecules that play ancillary roles in the angiogenic process, such as matrix metalloproteinases (for review, see Pugh[22]). This coordinated response will likely induce more effective angiogenesis than treatment with any one factor. For instance, short-term exposure to the growth factor VEGF is associated with the growth of leaky vessels that may be unwanted in the treatment of ischaemic tissue. In contrast, transgenic expression of a stabilised HIF-1α gene in the skin of mice promotes the growth of new vessels that show little leakage.[23] Activation of HIF thus may offer advantages over treatment with recombinant VEGF.

In anaemia, the efficacy and safety of recombinant erythropoietin sets a high barrier for any new treatment. However, additional functions of the HIF system, such as induction of other haematopietic growth factors or receptors and alterations in iron metabolism that support efficient erythropoiesis, likely may enable treatment of conditions that are currently partly or completely refractory to erythropoietin. The pleotrophic effects of HIF activation thus may be of benefit in each of these conditions. Promotion of angiogenesis, however, is likely to be undesirable in a treatment aimed at promoting erythropoiesis and vice versa.

Current insights into the HIF hydroxylase system gained through biochemical analysis and observation in tissue culture system would suggest that separation of these effects might be difficult to achieve. Observations in intact organisms, however, provide a different perspective. Despite the pleotrophic effects of HIF activation in tissue culture, the well-studied effects of systemic hypoxia at altitude are largely confined to effects on erythropoiesis and respiration. Although hypoxic induction of angiogenesis is clearly observed in injured and neoplastic tissue, the normal circulation in the intact organism seems much less responsive. The reasons for this paradox remain unclear. It seems likely that additional levels of control serve to limit the expression and action of HIF target gene products in the cells of the intact organism, but the mechanisms are not well understood. Further insights into these processes are important but can most likely be obtained only in the intact organism.

Thus, just as the switch of experimental effort into tissue culture systems provided the impetus for molecular analysis of the cellular response to hypoxia, it is now clear that effective therapeutic translation will require a refocus on studies in the intact organism and, wherever possible, man. Further molecular analysis will be important in guiding these studies and in revealing potential risks of unwanted effects. Nevertheless, as with other potential drug targets, it is important that insights into the massive complexity of physiological pathways that are now possible through molecular and genomic analyses are used to assist, rather than outface, safe therapeutic development.

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