

Mechanisms of hypoxia signalling: new implications for nephrology

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Key points

- Hypoxia-inducible factors (HIFs) transduce transcriptional responses to hypoxia that involve hundreds to thousands of target genes.
- The oxygen-sensitive signal regulating HIF activity is generated by 2-oxoglutarate-dependent dioxygenases that catalyze the hydroxylation of specific HIF prolyl and asparaginyl residues to inactivate HIF in the presence of oxygen.
- Inhibition of the HIF prolyl hydroxylases by 2-oxoglutarate analogues mimics hypoxia and activates many, but not all, components of the HIF transcriptional response.
- Erythropoietin production by cortical interstitial fibroblasts in the kidney is very sensitive to activation of the HIF pathway.
- In diseased kidneys, erythropoietin production is reduced, but can be increased by HIF prolyl hydroxylase inhibitors.
- Activation of HIF has the potential to generate many other renal and systemic effects that will require consideration when HIF prolyl hydroxylase inhibitors are used clinically.

Abstract

Studies of the regulation of erythropoietin (EPO) production by the liver and kidneys, one of the classical physiological responses to hypoxia, led to the discovery of human oxygen-sensing mechanisms, which are now being targeted therapeutically. The oxygen-sensitive signal is generated by 2-oxoglutarate-dependent dioxygenases that deploy molecular oxygen as a co-substrate to catalyze the post-translational hydroxylation of specific prolyl and asparaginyl residues in hypoxia-inducible factor (HIF), a key transcription factor that regulates transcriptional responses to hypoxia. Hydroxylation of HIF at different sites promotes both its degradation and inactivation. Under hypoxic conditions, these processes are suppressed, enabling HIF to escape destruction and form active transcriptional complexes at thousands of loci across the human genome. Accordingly, HIF prolyl hydroxylase inhibitors stabilize HIF and stimulate expression of HIF target genes, including the *EPO* gene. These molecules activate endogenous *EPO* gene expression in diseased kidneys and are being developed for, or are already in clinical use for, the treatment of renal anaemia. In this Review, we summarize information on the molecular circuitry of hypoxia signalling pathways underlying these new treatments and highlight some of the outstanding questions relevant to their clinical use.

[H1] Introduction

Most organisms use oxygen to fuel cellular respiration and for other vital functions and, therefore, require adaptive responses to defend oxygen homeostasis. In small primitive organisms, these include regulation of metabolism and motility controls (which are used to find a more oxygenated environment)^{1,2}. By contrast, in higher animals, whose large size and high rates of metabolism create a greater challenge to oxygen homeostasis, complex oxygen delivery systems such as the lungs, heart and vasculature have evolved that require precise dynamic control. In humans, the rate of breathing is controlled to bring oxygen to the alveoli at the required rate, where it diffuses into the blood, binds to haemoglobin, and is transported to all organs of the body via the circulatory system. From the blood vessels, oxygen diffuses to the respiring cells. Within many tissues, including regions of the kidneys, oxygen diffusion gradients are complex and oxygen levels are highly heterogeneous³⁻⁵. Hypoxia, which we define as a state of insufficient oxygen levels for maintenance of normal cellular function, does not equate to a specific oxygen concentration, as many tissues function physiologically at levels equivalent to an atmosphere of 5% oxygen, and some at levels as low as 1% oxygen⁴. Thus, mechanisms that maintain oxygen homeostasis must operate over a very wide range of oxygen concentrations and in response to temporal challenges that span from seconds (for example, in the dynamic control of respiration) to days, weeks or months (for example, in metabolic and developmental adaptation).

Although many of these oxygen homeostasis systems are still poorly understood, investigation of the control of gene expression by oxygen levels has provided the first detailed molecular understanding of an oxygen-sensing system in humans. This discovery has enabled new insights into disease mechanisms associated with hypoxia and has revealed new therapeutic opportunities. The first evidence for such an oxygen-sensing process came from the altitude physiologists of the late nineteenth and early twentieth centuries, who found that blood haematocrit increased as an adaptive change to reductions in the partial pressure of atmospheric oxygen^{6,7}. The work of Erslev⁸ and others in the mid-twentieth century established the hormonal control of haematocrit by the hormone erythropoietin (EPO). Subsequently, EPO, which is synthesized by the kidneys and, to a lesser extent, the liver, was shown to operate in a highly sensitive feedback loop to control red blood cell production in response to changes in blood oxygen availability⁹. The 'sensing' of oxygen levels was widely believed to occur specifically in the cells within the kidney and liver that produce EPO. However, work on the transcriptional regulation of the *EPO* gene led to the recognition that the system operates widely across cell types, irrespective

of whether they produce the hormone¹⁰. This oxygen-sensing pathway is now established to control numerous physiological outputs and to directly or indirectly regulate thousands of genes.

Hypoxia-inducible factor (HIF), a heterodimer comprising an inducible α subunit (HIF- α ; the main hypoxiasensitive component) and a constitutively expressed β subunit (HIF- β), was identified as the key transcription factor regulating these transcriptional responses^{11,12}. The oxygen-sensitive signal that controls HIF activity is generated by a series of regulatory enzymes that catalyze the hydroxylation of specific prolyl¹³⁻¹⁶ and asparaginy¹⁷⁻¹⁹ residues in the HIF- α subunits; these enzymes are dioxygenases that split molecular oxygen and incorporate the oxygen atoms into their substrates (**FIG. 1**). Thus, their activity is suppressed during hypoxia. The basic system, which is present in the earliest animals, comprises a single HIF- α , a single HIF- β and a single HIF prolyl hydroxylase. Gene duplication events early in vertebrate evolutionary radiation created multiple isoforms, some of which were subsequently lost during further evolution²⁰. For instance, the human genome encodes three HIF- α isoforms and three isoforms of the HIF prolyl hydroxylase, which control HIF- α stability. Most animals, including humans, possess a single HIF asparagine hydroxylase that controls HIF transcriptional activity.

These advances have opened the possibility of therapeutic interventions targeting human oxygen-sensing pathways. Small molecules that inhibit the activity of the HIF prolyl hydroxylases (PHD inhibitors) effectively activate the HIF pathway and are currently in late-phase clinical trials, or have been licensed for the treatment of renal anaemia. The development of these and other agents that target different components of the HIF system has been described in other reviews²¹⁻²⁴, and will not be detailed exhaustively herein. In this Review, we outline the background biology of hypoxia signalling, review new advances in the understanding of the HIF hydroxylase system, and consider outstanding questions regarding the therapeutic manipulation of these pathways in kidney disease.

[H1] HIF transcription factors

Human HIF-1 was discovered as a protein complex binding to a regulatory DNA sequence at the *EPO* locus¹¹. Purification by affinity chromatography and identification of the encoding cDNAs revealed that HIF-1 was a heterodimer of bHLH-PAS (basic-helix-loop-helix-Per-ARNT-Sim) proteins, HIF-1 α (encoded by *HIF1A*) and HIF-1 β (encoded by *ARNT*)²⁵. Whereas HIF-1 α was a novel protein, HIF-1 β had been previously identified as the aryl hydrocarbon nuclear translocator (ARNT), a dimerization partner for the aryl hydrocarbon receptor (AHR) in a different transcriptional pathway²⁶. HIF-1 α was shown to mediate the oxygen sensitivity of the HIF complex, and to interact with HIF-1 β through a shared Per-ARNT-Sim (PAS) domain²⁷. Both HIF-1 α and HIF-1 β contain an N-terminal basic-helix-loop-helix domain that mediates DNA-binding, and C-terminal transactivation domains that are necessary to induce gene expression. The oxygen sensitivity of HIF- α proteins is conferred by an internal oxygen-dependent degradation domain, which includes the target prolyl residues whose hydroxylation mediates interaction with the von Hippel-Lindau tumour suppressor (pVHL), and by the C-terminal transactivation domain, which contains the target asparaginyl residue (**FIG. 2**)²⁸⁻³⁰. Under conditions of hypoxia, stabilization of HIF-1 α enables formation of the HIF-1 heterodimer, which binds hypoxia-responsive elements (HREs) in gene promoters and their transcriptional enhancers that contain the core sequence (RCGTG).

Humans and most vertebrate species have two paralogues of HIF-1 α ; HIF-2 α (otherwise known as endothelial PAS domain containing protein 1 (EPAS1) and encoded by *EPAS1*, which we term *HIF2A* in the text for simplicity) and HIF-3 α (encoded by *HIF3A*), were subsequently identified. All three HIF- α proteins are members of the class 1 group of bHLH-PAS proteins, which are able to dimerize with either of two class 2 bHLH-PAS proteins (HIF-1 β /ARNT and ARNT2) to form DNA binding complexes³¹. In most cells and tissues, the most abundant class 2 dimerization partner is HIF-1 β and inactivation of this protein results in near complete loss of HIF activity. However, in certain tissues, including the central nervous system and the kidneys, ARNT2 is highly expressed and has been shown to form HIF complexes and contribute to neural and neuroendocrine responses to hypoxia³².

HIF-1 α , HIF-2 α and HIF-3 α complexes with HIF-1 β /ARNT are termed HIF-1, HIF-2 and HIF-3. As indicated above, HIF-2 α was initially termed EPAS1, as it was found to be most strongly expressed in endothelial cells³³. However, HIF-2 α is not, in fact, confined to the endothelium³⁴, but it is indeed more tissue restricted than HIF-1 α . In the kidney, HIF-2 α is strongly expressed in interstitial cells, endothelial cells and the glomeruli, but its expression is largely absent from the tubular cells, in which HIF-1 α is the predominant isoform³⁵. HIF-1 and HIF-2 both transduce positive transcriptional

responses to hypoxia, although their transcriptional targets, kinetics of activation and oxygen dependence differ. HIF-1 α is induced more strongly by severe hypoxia, and its activity peaks within the first 24 hours of hypoxia, whereas HIF-2 α is induced by more moderate hypoxia and remains active for longer^{34,36}. These differences might, in part, reflect the specific actions transcriptional feedback loops. For instance, HIF negatively regulates HIF-1 α expression via an antisense transcript³⁷.

By contrast, the HIF-3 α isoform is less well understood, in part because of its complex pattern of expression, involving multiple variant transcripts derived from cell-specific patterns of alternative RNA splicing³⁸. Some splice variants, including the first isoform to be identified, inhibitory PAS domain protein (IPAS), lack oxygen-dependent degradation and transactivation domains and inhibit HIF transcriptional responses by complexing with the other HIF isoforms³⁹. Transcriptionally inactive heterodimers of HIF-3 α with both HIF-1 α and HIF-1 β have been identified in different settings, each exerting a dominant negative action on HIF-1-mediated transcription^{38,39}. In contrast to IPAS, full-length HIF-3 α contains an oxygen-dependent degradation domain that interacts with pVHL⁴⁰, and a transactivation domain. This form of the protein is reported to interact with HIF-1 β and to positively regulate an extensive set of transcriptional targets⁴¹. At least some HIF-3 α transcripts are, themselves, regulated by HIF, representing another feedback loop (**FIG. 3**)⁴².

[H1] Enzymatic oxygen-sensing mechanisms

The oxygen regulated signals that govern the activity of HIF are generated by enzymatic oxygen sensors that catalyse the post-translational hydroxylation of target residues in HIF- α polypeptides.

[H2] HIF prolyl hydroxylases

Identification of HIF- α proteins as the oxygen-dependent subunits of the HIF complex focused subsequent efforts on defining the molecular mechanisms of oxygen sensing that lay upstream in the HIF signal transduction pathway. As a first step, domains within the HIF- α polypeptide were characterized that could confer oxygen-regulated instability on heterologous proteins^{28-30,43}. Surprisingly, these domains did not seem to be regulated by protein phosphorylation pathways, which had been widely predicted to transduce the oxygen-sensitive signal²⁸. In a different line of work, certain hypoxia-inducible mRNAs were found to be upregulated in cell lines derived from clear-cell renal cell carcinoma (ccRCC)^{44,45}, which harbours inactivating mutations in the *VHL* gene. However, the mechanism was again unclear and, initially, a role for pVHL in the regulation of mRNA stability was proposed. The connection to HIF was discovered with the observation that pVHL and

HIF- α subunits physically interact and that pVHL is absolutely required for the oxygen-dependent proteolysis of HIF- α ⁴⁶. Further analyses identified pVHL as the recognition component of an E3 ubiquitin ligase complex that targets HIF- α for proteasomal destruction by binding to the same regions of HIF- α that had been shown to confer oxygen-regulated instability⁴⁷⁻⁵⁰.

Investigation of this interaction between HIF and pVHL led to the discovery that the key oxygen-regulated event promoting their association is the post-translational hydroxylation of specific amino acid residues in HIF- α ^{13,14}. Oxygen-dependent trans-4-hydroxylation of prolyl residues (Pro402 and Pro564 in HIF-1 α ; Pro405 and Pro531 in HIF-2 α ; Pro492 in HIF-3 α)^{13,14,40,50,51} results in a >1000-fold increase in affinity for pVHL⁵²⁻⁵⁴. The prolyl hydroxylation reaction is catalyzed by an enzyme first identified in *Caenorhabditis elegans*, and encoded by the *egl-9* gene¹⁵. Three homologous genes exist in humans (*EGLN1*, *EGLN2* and *EGLN3*), which encode closely related PHD enzymes (PHD2, PHD1, and PHD3)^{15,16}. These enzymes belong to the 2-oxoglutarate (2-OG)-dependent dioxygenase (2-OG oxygenase) superfamily. In the catalytic cycle, oxidation of the prolyl residue in HIF- α is coupled to the oxidative decarboxylation of 2-oxoglutarate in a redox cycle that involves the creation of a ferryl (Fe^{IV}=O) intermediate at the catalytic centre (**FIG. 1**; for a review, see REFS.^{20,55}).

Although all of the PHDs contribute to the regulation of HIF, PHD2 is the most widely expressed HIF prolyl hydroxylase and, in most cell types, it is the main regulator of HIF activity, particularly HIF-1^{56,57}. Accordingly, genetic inactivation of *Egln1* (encoding Phd2) in mice leads to severe placental and developmental heart defects and is lethal in embryonic life⁵⁸. By contrast, the roles of PHD1 and PHD3 in HIF regulation seem to be more tissue specific. PHD3 is also strongly induced by hypoxia and is, therefore, important at lower oxygen concentrations^{15,59}. At least in some circumstances, PHD1 and PHD3 are more active in the regulation of HIF-2 than of HIF-1⁵⁷. In part this observation might reflect preferences of PHD enzymes for the hydroxylation of different prolyl residues within the HIF polypeptide substrates and the differential importance of these prolyl residues in the regulation of HIF-1 versus HIF-2, HIF-2 being more dependent on C-terminal prolyl hydroxylation. Both HIF-1 α and HIF-2 α have two target residues (Pro402 and Pro564 in human HIF-1 α ; Pro405 and Pro531 in HIF-2 α). Though all PHD enzymes show a preference for the C-terminal sequence over the N-terminal sequence⁶⁰, this preference for the C-terminal sequence is greater for PHD1 than PHD2 and particularly marked for PHD3^{15,61}. Selectivity among HIF substrates is mediated by sequences contained within a mobile loop in the PHD polypeptide⁶², which moves to enclose the bound HIF peptide at the catalytic site⁶³. Interestingly, certain naturally occurring mutations within this region of human PHD2 have marked effects on selectivity for the N-terminal versus C-terminal

sites of prolyl hydroxylation⁶³. Taken together, these findings suggest that the design of small-molecule PHD inhibitors that have at least some selectivity with respect to their action on different HIF- α isoforms should be possible, although current PHD inhibitors do not manifest such selectivity⁶⁴.

All PHD isoforms have a low affinity for molecular oxygen and their catalytic activity is greatly reduced under conditions of low oxygen concentration^{15,61}, such as those that prevail physiologically in many tissues, including the kidney. The molecular basis of this special sensitivity to oxygen is not entirely clear. Studies of recombinant PHD2 have revealed an unusually slow reaction with molecular oxygen^{65,66}. This observation might arise from the need for molecular oxygen to displace a water molecule in order to access the catalytic centre (**FIG. 1**), which, in human PHD2, is stabilized via hydrogen bonding to the iron-co-ordinating aspartate residue at the catalytic site⁶⁶. Interestingly, loss of a proton from this water molecule under acidic conditions is associated with a higher catalytic rate for PHD2, and the oxygen-sensing function of PHD2 has been suggested to be modulated by pH within the physiological range⁶⁷.

Potentially, the activity of the PHD enzymes can also be modulated by oxidant stresses. As with many other 2-OG oxygenases, the PHD enzymes require ascorbate for full catalytic activity, and the action of ascorbate has been proposed to reduce the catalytic iron centre following the oxidation that occurs during uncoupled catalytic cycles⁶⁸. Whether failure of this process is responsible for the observed reductions in PHD activity following exposure of cells to oxidant stress is not yet clear. Different mechanisms of enzyme inactivation have been reported. One study reported spectroscopic evidence of oxidation of the catalytic iron centre of human PHD2 in cells exposed to oxidant stress following inactivation of oxidant defense pathways⁶⁹. However, a subsequent study focused on the importance of intracellular cysteine levels and on the vulnerability of specific cysteine residues to oxidation when levels are low⁷⁰. However, how these processes contribute to the physiological regulation of the system in the intact organism is still unclear. For instance, although marked effects of ascorbate supplementation on *HIF* and HIF target gene expression are observed in cultured cells⁷¹, little effect of ascorbate supplementation was observed on EPO production in scorbutic rats⁷². Interestingly, although PHD enzymes are sensitive to iron, in vitro studies suggest that, compared with other 2-OG oxygenases, their binding affinity for iron is unusually strong⁷³. This characteristic is shared by related prolyl hydroxylases, which perform oxygen-sensing functions in the slime mould *Dictyostelium Discoideum*⁷⁴. This property could possibly make the PHD enzymes relatively resistant to oxidant attack on the catalytic centre and

could, therefore, protect their function as molecular oxygen sensors from confounding signals arising from the redox chemistry of cellular iron.

[H2] FIH

In a second oxygen-dependent control mechanism, another HIF hydroxylase, which was first identified simply as a factor inhibiting HIF (FIH)⁷⁵, targets an asparagine residue (Asn803 in HIF-1 α ; Asn851 in HIF-2 α) in the C-terminal domain of the HIF- α protein^{18,19}. This residue is located within a hydrophobic region formed when HIF- α forms a complex with members of the p300-CBP co-activator family, such as histone acetyltransferase p300 (p300) and CREB-binding protein (CBP), which possess histone acetyl transferase activity and are involved in HIF target gene activation^{76,77}. Hydroxylation of Asn803 in HIF-1 α introduces a polar group, preventing this interaction and, therefore, impeding full transcriptional activation of relevant HIF target genes. Despite these insights, the role of FIH in HIF-mediated transcription is not fully understood. RNA polymerase II (RNA Pol II) is bound at the promoters of most hypoxia-inducible genes, even in normoxic cells^{78,79}. The induction of HIF is associated with processing of RNA Pol II along the transcribed gene, a process known as promoter pause release^{78,79}. For many HIF target genes, promoter pause release is associated with recruitment of the mediator complex⁷⁸. Whether FIH is involved in this process is not yet known. Although both HIF-1 and HIF-2 isoforms are susceptible to FIH-mediated hydroxylation, functional studies suggest that FIH predominantly inhibits HIF-1⁸⁰. In the kidney, FIH is strongly expressed in the distal tubule and podocytes, but seems to have different actions at these sites, restricting HIF-1 α target gene expression in tubules but having potentially HIF-independent transcriptional effects on some target genes (*CXCR4* and *VEGFA*) in podocytes⁸¹. Interestingly, genetic manipulation of the gene encoding FIH results in dysregulation of only a subset of HIF-1 target genes⁸².

Of note, the HIF prolyl hydroxylase inhibitors under current clinical use to induce HIF activity do not inhibit FIH⁶⁴. Thus, these PHD inhibitors can only partially upregulate the HIF transcriptional response. Biochemical analyses of its oxygen dependence have revealed that FIH activity is inhibited at lower oxygen tensions than the PHDs^{83,84}. This finding suggests that, with decreasing oxygen levels, PHD activity is reduced first, whereas FIH is still operating on HIF to reduce transactivation of selected targets⁸⁵. Only very low oxygen levels lead to a complete inhibition of both the PHDs and FIH, permitting maximum activation of the HIF response (**FIG. 2**).

FIH differs from the PHDs in a number of other respects. Its substrate binding cleft is more open⁸⁶, and it is more sensitive to inactivation by oxidant stress⁸⁷. In addition to HIF, FIH has been

shown to hydroxylate ankyrin repeat domain (ARD)-containing proteins such as the NF- κ B p105 subunit, the NF- κ B inhibitory subunit I κ B α , neurogenic locus notch homolog protein 1 (NOTCH1), tankyrase-2, or rabankyrin-5^{88,89}. In most ARD-containing proteins, the target residue for FIH-mediated hydroxylation is asparagine; however, when the residue is positioned at the target site in the ankyrin fold, hydroxylation has also been observed on aspartyl and histidiny residues^{90,91}. In vitro kinetic studies of FIH have indicated that some ARD-containing proteins have a higher affinity for FIH than HIF⁹². Thus, ARD substrates compete for HIF and, therefore, their abundance and/or hydroxylation status within the cell has the potential to modulate the activity of HIF⁹³. What is less clear is the function of ARD hydroxylation itself. The modification has the potential to stabilize the ankyrin fold, but the physiological consequence of this for signalling by ARD-containing proteins is unknown⁹⁴. Nevertheless, knockout of the gene encoding FIH in mice is associated with metabolic dysregulation that is distinct from that associated with HIF activation, which strongly suggests that FIH has physiological functions other than the regulation of HIF^{95,96}.

[H2] Potential role of other 2-OG oxygenases in signalling hypoxia

The involvement of two distinct classes of 2-OG oxygenase in the regulation of HIF has raised further questions regarding the role of these enzymes in transducing responses to hypoxia. For instance, an important outstanding question is whether the PHD enzymes, like FIH, have substrates other than HIF that transduce physiological responses to hypoxia. Overall, >20 non-HIF substrates have been reported for different PHD enzymes⁹⁷. Such PHD substrates might transduce other physiological responses to hypoxia and generate off-target effects (that is clinically unintended) of PHD inhibitors. They might also be expected to modulate the HIF system through competition. However, the biochemical activity of the PHDs on these non-HIF substrates not been confirmed using recombinant enzymes⁹⁸.

Another possibility is that trans-4 prolyl hydroxylations of proteins other than HIFs, which are catalyzed by enzymes other than the PHDs, interact with the pVHL degradation pathway by competing with HIF for occupancy of the hydroxyproline binding site in pVHL. Pro-collagen prolyl hydroxylases catalyze the hydroxylation of prolyl residues in the triplet repeat sequences in pro-collagens, an action that is important for the structural stability of the collagen triple helix⁹⁹. Collagen molecules that are hydroxylated, but not incorporated into its mature triple helix, have the potential to bind pVHL^{100,101}. Whether this interaction occurs physiologically and whether dysregulation of collagen binding has a role in pVHL-associated oncogenesis is unknown. Transmembrane prolyl 4-hydroxylase (P4H-TM), another putative trans-4-prolyl hydroxylase that is

sometimes termed PHD4, has been shown to affect the hydroxylation and activity of HIF under some circumstances^{102,103}. This enzyme has a transmembrane domain that facilitates localization to the endoplasmic reticulum, and seems to be more closely related to the collagen hydroxylases than the PHDs. At present, its direct substrates have not been identified, and whether its action on HIF is direct or indirect has not been resolved. Nevertheless, P4H-TM is highly expressed in the kidney and its inactivation in mice is associated with abnormalities in renal development and dysregulation of EPO production^{103,104}.

The HIF prolyl and asparaginyl hydroxylases belong to a large family of 2-OG oxygenases. In human cells, reactions catalyzed by 2-OG oxygenases include demethylation of DNA, RNA and histones, in addition to protein hydroxylation^{20,105}. Interestingly, the 2-OG oxygenases most closely related to the HIF hydroxylases catalyze the hydroxylation of ribosomal subunits and other proteins involved in translational control¹⁰⁶⁻¹¹⁰. Thus, 2-OG oxygenases are potentially involved at multiple steps in gene expression, from transcription factor regulation and epigenetic control of chromatin structure to RNA stability and protein translation. Whether these enzymes are involved in generating oxygen-sensitive signals that impinge on the HIF transcriptional cascade is not yet clear. Interestingly, some genes encoding 2-OG oxygenases are transcriptional targets of HIF^{111,112}. Moreover, marked sensitivity of their catalytic activity to hypoxia has been demonstrated experimentally for several members, including DNA and histone modifying enzymes^{113,114}, and histone demethylases manifesting high sensitivity to hypoxia have been implicated in non-HIF dependent responses to hypoxia^{115,116}.

[H1] Other mechanisms of HIF modulation

In keeping with the complexity of oxygen homeostasis, in addition to the core oxygen-dependent control of HIF activity by protein hydroxylation, HIF is also modulated by a wide range of other mechanisms operating at multiple levels. These include the transcription, mRNA processing, translation, and post-translational modification of the different HIF-encoding genes and their protein products (**FIG. 2**). In this section, we outline some of these processes, particularly examples that might influence the role of HIF in renal cell biology or provide therapeutic entry points. Importantly, many of these processes are specific to one or more HIF isoforms and, therefore, have the potential to alter their relative levels of expression and, consequently, shape the HIF target gene repertoire. In general, these pathways do not transduce oxygen-sensitive signals, although they often reflect the activity of systems, such as inflammatory pathways, that are activated in hypoxic

tissues or are linked to proliferative stimuli that require a homeostatic response to offset increased oxygen consumption.

[H2] Transcriptional regulation of HIF- α isoforms

Many signalling pathways impinge on the HIF system through effects on *HIF1A* and *HIF2A* transcript levels. For example, nuclear factor- κ B (NF- κ B) has been shown to stimulate *HIF1A* transcription by binding to its promoter¹¹⁷. Other studies have identified *HIF1A*¹¹⁸ or mouse *Hif1a*¹¹⁹ promoter binding by the transcription factors signal transducer and activator of transcription 3 (STAT3), SP1 and SP3. Angiotensin II has been identified as a humoral factor that increases rat *Hif1a* gene transcription in vascular smooth muscle cells via protein kinase C (PKC) signalling¹²⁰. In glomerular mesangial cells, high levels of glucose were reported to activate *HIF1A* transcription by inducing binding of carbohydrate responsive element binding protein (ChREBP) to the *HIF1A* promoter¹²¹. Multiple other proinflammatory pathways induce *HIF1A* mRNA expression, including those activated by lipopolysaccharide (LPS), tumour necrosis factor (TNF), interleukin-6 (IL-6) and IL-18 (for a review, see REF.¹²²).

The levels of the *HIF2A* and mouse *Hif2a* transcripts are also regulated by a range of differentiation, inflammatory and stress signals¹²³⁻¹²⁵. Although the transcription factors binding to its regulatory sequences have not been extensively studied, the transcription factors SP1, SP3 and E2F1 have been implicated in different responses^{124,126}. Interestingly, the marked tissue specificity of HIF-2 α expression that is observed under normal physiological conditions in nonmalignant cells is lost in cancer cells, which often express HIF-2 α irrespective of their origin³⁴. This appears, at least in part, to be due to de-repression of the *HIF2A* gene, a process which has been clearly observed in malignant transformation of renal tubular cells. Renal tubular cells ordinarily only express HIF-1 α ¹²⁷, but in pVHL-defective kidney cancer the malignant tubular epithelium usually expresses high levels HIF-2 α ^{128,129}. This process has been attributed to reduced methylation of the *HIF2A* promoter, as a consequence of reduced expression of DNA methyltransferase 3A (DNMT3A) (**FIG. 2**)¹³⁰. Accordingly, the DNA methyltransferase inhibitor 5-azacytidine has also been reported to induce the expression of HIF-2 α in neuroblastoma cells¹³¹. In addition to control by transcription factors and epigenetic regulators, work has also revealed control of *HIF1A* and *HIF2A* transcript levels by a variety of noncoding RNAs (**FIG. 3**; for reviews, see REFs.¹³²⁻¹³⁴).

[H2] Translational regulation of HIF- α isoforms

Investigation of dysregulated pathways in cancer has revealed many signals that influence the translation of HIF- α proteins. Activation of the mechanistic target of rapamycin (mTOR) or the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway by growth factors promotes HIF- α translation via phosphorylation of ribosomal protein S6 kinase 1 (S6K1), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) or 4E-BP2¹³⁵⁻¹³⁷. The extent that these pathways differentially affect HIF- α isoform expression is unclear. However, mTOR complex 1 (mTORC1) and mTORC2 exert differential effects on HIF-1 α and HIF-2 α expression¹³⁸. Thus, inhibitors with differential activity on these complexes might have the potential to alter the relative expression of these genes, an action that might be useful in the treatment of RCC, in which HIF-2 expression seems important for oncogenesis^{128,139}.

Other controls of HIF- α translation are highly specific and illustrate both the importance of specific HIF isoforms in particular aspects of hypoxia biology and the potential for targeted therapeutic intervention. Iron regulatory protein 1 (IRP1) specifically binds to the 5' untranslated region (UTR) of the *HIF2A* mRNA transcript and is, therefore, a strong translational repressor of HIF-2 α , but not HIF-1 α . IRP1-deficient mice manifested high levels of *Hif2a* and *Epo* mRNA and develop polycythemia and pulmonary hypertension¹⁴⁰, whereas pharmacological activation of IRP1 binding ameliorated HIF-2 α -driven erythrocytosis in a model of polycythaemia¹⁴¹.

[H2] Post-translational modifications and interactions of HIF

The role of HIF in many biological processes is reflected in its multiple post-translational modifications. HIF-1 α and HIF-2 α are both extensively phosphorylated, and phosphorylation of HIF- α subunits is directly implicated in control of their stability, activity and nuclear localization¹⁴². Many kinase pathways are implicated in HIF- α subunit phosphorylation, including the RAC- α serine/threonine-protein kinase (AKT; also known as PKB), glycogen synthase kinase-3 β (GSK3 β), ataxia telangiectasia mutated (ATM), and MAPK/ERK pathways (for a review, see REF.¹⁴²). In some studies, specific sites of phosphorylation have been implicated in specific functional controls. For instance, phosphorylation of Thr796 on HIF-1 α or Thr844 on HIF-2 α regulates transcriptional activity by affecting FIH-catalyzed hydroxylation of HIF-1 α at Asn803 or HIF-2 α at Asn851^{143,144}. In other instances, the phosphorylation event is HIF- α isoform specific. For instance, phosphorylation of Thr324 on HIF-2 α is specific to that isoform and prevents an interaction with DNA repair pathway proteins that is observed with HIF-1 α ¹⁴⁵.

Other post-translational modifications of HIF include methylation¹⁴⁶, sumoylation¹⁴⁷, and acetylation¹⁴⁸. Work on the role of lysyl acetylation on HIF-2 α is of interest, since it suggests that it

is not the acetyl modification of lysine itself, but the process of cycling between acetylated and deacetylated forms that regulates transcriptional activation. Surprisingly, both the NAD-dependent protein deacetylase sirtuin-1 (SIRT1; a lysyl deacetylase)¹⁴⁸ and CBP (a lysyl acetyl transferase)¹⁴⁹ were observed to enhance the transcriptional activity of HIF-2 α on the *EPO* gene, leading to the hypothesis that cycling between acetylated and deacetylated states augments HIF-2A transcriptional activity by mechanisms which are not yet understood¹⁴⁹. Further work showed that downregulation or knockout of the gene encoding cytoplasmic acetyl-CoA synthetase (ACSS2), an enzyme responsible for production of the acetyl donor acetyl-CoA, reduced EPO production, whereas treatment with exogenous acetate augmented EPO production and enhanced the rate at which anaemia was corrected in mice¹⁵⁰.

[H2] Ligand binding to HIF proteins

Structural studies of HIF proteins have raised the possibility that HIF activity is also regulated by endogenous metabolites that bind its PAS domains. PAS domains are involved in environmental signalling across many prokaryotic and eukaryotic organisms and, in some species, they perform oxygen-sensing functions¹⁵¹. Although oxygen-sensitive protein hydroxylation clearly controls the activity of HIF independently of its PAS domains, the possibility that signals are also transduced by the binding of small-molecule ligands to pockets within the PAS domains of HIFs has attracted interest³¹.

These studies have revealed an unusually large pocket in HIF-2 α at its dimerisation interface with HIF-1 β ^{152,153}. Synthetic small molecules that bind to this pocket can specifically block the activity of HIF-2¹⁵⁴, at least in part by inhibiting dimerization^{155,156}. These compounds show anti-tumour activity in the treatment of *VHL*-defective RCC¹⁵⁷ and are also being evaluated in recurrent glioblastoma, advanced solid tumours and familial von Hippel-Lindau disease that is associated with RCC¹⁵⁸⁻¹⁶¹. The powerful action of these exogenous ligands raises the important question as to whether endogenous ligands also exist that have a physiological role in regulating the HIF response, and whether HIF agonists, as well as the antagonists described above, might be developed. Notably, one of the alternative dimerisation partners of HIF-1 β , AHR, is a paradigm for PAS-ligand-activated transcription¹⁶². Although the question as to whether this mechanism is a general characteristic of these basic-helix-loop-helix PAS domain transcription factors remains open, structural studies have defined pockets, albeit substantially smaller, within the PAS domains of other members of this family of transcription factors that might be druggable or represent points of interaction with endogenous ligands¹⁶³.

[H1] HIF target gene selectivity

High-throughput DNA sequencing methods have now enabled the HIF transcriptional cascade to be investigated across the entire genome. The majority of studies of hypoxia have either used chromatin immunoprecipitation sequencing (ChIP-seq) to assay the binding of HIFs to DNA directly, or RNA sequencing (RNA-seq) to measure changes in the abundance of transcripts that respond directly or indirectly to HIF. Depending on the statistical threshold applied, pan-genomic studies have identified HIF binding sites in the order of thousands across the genome^{112,164-167}. Many of these HIF binding sites lie at a distance of tens to hundreds of kilobases from the nearest promoter¹⁶⁵. Nevertheless, analyses of chromatin conformation suggest that, through looping of DNA, the majority of HIF binding sites are in direct contact with the promoters of one or more genes that are inducible by hypoxia¹⁶⁸. Therefore, in most cells, there are likely to be in excess of 1000 direct transcriptional targets of HIF. In addition to gene products that directly mediate adaptive responses to hypoxia these transcriptional targets encode other transcription factors, epigenetic regulators of chromatin, factors involved in RNA processing and regulatory noncoding RNA networks^{79,169,170}, that greatly extend the complexity of the direct transcriptional response. Interestingly, a clear statistical association between HIF binding at a given locus and the regulation of gene expression at that locus is only observed for genes that are positively regulated by HIF, suggesting that the downregulation of gene expression by HIF is largely an indirect response mediated by transcriptional repressors that are induced by HIF¹⁷¹.

Genes that are controlled directly or indirectly by HIF mediate a very broad range of biological outputs. At the cellular level, responses to HIF include effects on differentiation, migration, cytoprotection, apoptosis, cycle control, and the function of specific organelles such as mitochondria (for review, see REFs.¹⁷²⁻¹⁷⁴). At the level of the organ or organism co-ordinated responses to altered HIF activity include the regulation of erythropoiesis, angiogenesis, energy metabolism, iron metabolism, matrix metabolism, inflammation, and immune regulation (for review see REFs.¹⁷⁵⁻¹⁷⁹). The need, in most medical situations, for relatively specific manipulation of one or other of these outputs, rather than activation or inactivation of the entire pathway, has generated interest in understanding the mechanisms by which different HIF transcription factors are directed to specific targets across the genome.

[H2] HIF isoform specificity

To date, most studies of transcriptional responses to specific HIF isoforms have focused on HIF-1 and HIF-2. Despite binding to an identical DNA sequence at HREs, HIF-1 and HIF-2 direct largely

distinct transcriptional systems^{165,167,180,181}. For instance, many metabolic responses are specifically dependent on HIF-1, whereas cell differentiation, reparative pathways, and more complex adaptive responses to hypoxia, including induction of erythropoiesis, are dependent on HIF-2^{180,182,183}. Interestingly, HIF-1 and HIF-2 also have different patterns of genomic distribution in relation to their transcriptional targets. HIF-1 generally binds to DNA at sites close to target gene promoters, whereas HIF-2 frequently binds to transcriptional enhancers that lie at a distance from the target gene¹⁶⁵, a pattern that is maintained across cell types with quite different complements of HIF target genes¹⁶⁷. Although some HREs bind both HIF-1 and HIF-2, very little cross-competition is observed when one or more HIF- α isoform is depleted, indicating that the two isoforms have intrinsically distinct, although overlapping, binding patterns¹⁶⁷. This DNA binding selectivity is amplified by post-DNA binding mechanisms that mediate transcriptional selectivity^{168,181,184}. Thus, even when both HIF- α isoforms bind to a particular control sequence, it might be that only one is transcriptionally active. Together with cell-type specific expression of HIF- α isoforms, this DNA binding selectivity generates highly distinct functional outputs for HIF-1 and HIF-2 (**FIG. 4**).

To date, investigation of the human transcriptional targets and tissue/cell-type specificity of expression HIF-3 has been much less complete¹⁸⁵. The most extensive pan-genomic studies have focused on zebrafish, in which, despite the creation of more HIF isoforms by an additional round of gene duplication during evolution, DNA sequence and chromosomal synteny clearly identify a HIF-3 α orthologue⁴¹. At least when overexpressed, Hif-3 α directs an extensive positive transcriptional response. Furthermore, of >150 genes that were found to be upregulated by Hif-3 in zebrafish embryos, almost 100 were also Hif-1 targets. These experiments also suggested that Hif-3 preferentially targets specific hypoxia pathways, such as the Janus kinase (JAK)–STAT and NOD-like receptor (NLR) signaling in zebrafish, and that at least some of the targets of Hif-3 in zebrafish are also responsive to HIF-3 in human cells⁴¹.

[H2] Determinants of HIF binding

Although studies support the existence of large numbers of direct HIF transcriptional targets^{112,165}, the number of HIF binding sites is considerably smaller than the number of core-HRE-binding motifs encoded by the genome, implying the existence of processes that shape the HIF transcriptional response by directing HIF binding to select HREs (**FIG. 4**). As defined at the *EPO* locus, HIF binding to the core HRE is prevented by DNA methylation¹⁸⁶. However, comparison of data on HIF binding sites¹⁶⁵ with patterns of methylation across the genome¹⁸⁷ reveals that although HIF binding sites are unmethylated, the genome also contains large numbers of unmethylated HRE sequences that

do not bind HIF¹⁸⁷. Most HREs that do bind HIF are in regions of open chromatin that are characterized by hypersensitivity to deoxyribonuclease (DNase)-mediated digestion¹⁶⁵, indicating that most HIF binding sites are pre-allocated and do not need hypoxia or HIF-mediated mechanisms to open chromatin^{112,168}. In keeping with this hypothesis, assays of chromatin conformation at HIF binding enhancers have revealed that physical interactions of remote HIF binding sites with their target gene promoters are already pre-formed in normoxic cells^{168,188}, enabling rapid HIF binding and target gene activation in hypoxic cells. Across the genome, >50% of high-fidelity HIF-binding sites are distinct between any two cell lines¹⁶⁷. Thus, additional cell-specific mechanisms must be responsible for these patterns. The most likely possibility is that other transcription factors co-operate or compete with HIF to define actual binding patterns at available sites. Comparative analysis of HIF-1 versus HIF-2 binding sites revealed very distinct associations with binding motifs and binding sites for other transcription factors^{167,189}. Further investigation of these interactions and the mechanisms underlying the targeting of HIF-1, HIF-2 and HIF-3 to different sites within the genome should improve our understanding of how the HIF transcriptional cascade is shaped to meet the challenge of oxygen homeostasis in different tissues and physiological circumstances. In the meantime, for HIF-1 and HIF-2, the intrinsically distinct patterns of binding across the genome provide a rationale for the therapeutic development of HIF-isoform specific antagonists and agonists.

[H1] Erythropoietin in renal disease

Given that *EPO* was the original archetypal HIF target gene, HIF prolyl hydroxylase inhibitors (which activate HIF) can, perhaps unsurprisingly, induce the production of EPO. Nevertheless, this exciting clinical development highlights some as yet unresolved questions in the field of hypoxic cell biology of the kidney. In particular, given the highly complex pathways activated by HIF, whether and how relatively selective induction of EPO production and erythropoiesis might be achieved is not yet clear.

Although *EPO* is a specific HIF-2 target, current PHD inhibitors do not directly take advantage of this phenomenon, as they are largely unselective with respect to the HIF substrate⁶⁴. Some selectivity among HIF targets will arise from their lack of action on the HIF asparaginyl hydroxylase FIH¹⁹⁰. As FIH is less active on HIF-2 than HIF-1⁸⁰, relatively selective activation of HIF-2 by PHD inhibitors will be achieved indirectly. Concentration of the compounds in the kidneys and liver could largely restrict drug exposure to these EPO-producing organs. In addition, the intermittent dosing (for example, three times weekly) used in some clinical studies¹⁹¹⁻¹⁹³ could possibly achieve a

differential pharmacodynamic effect on blood haematocrit. Since the life-span of red blood cells is of the order of 100 days, an intermittent effect on red cell production has the potential to be integrated more effectively than other biological responses to HIF activation. However, given the possibility that the number cells with the potential for EPO production might actually be increased in certain types of renal disease (see below), another interesting possibility is that at least some diseased kidneys contain a larger potential for EPO production than previously assumed. In considering this possibility, two largely unanswered questions are brought into focus; the actual mechanism of EPO failure in renal disease and the exact mechanism by which these agents correct this failure.

[H2] Extinction and reactivation of erythropoietin production in renal disease

In health, EPO is produced at sufficient levels to maintain normal haematocrit by a small population of renal interstitial fibroblasts situated close to the cortico-medullary junction¹⁹⁴⁻¹⁹⁶. In anaemia, the number of these fibroblasts producing EPO increases in a systematic manner; as the severity of anaemia or hypoxia increases, more cells, situated progressively more superficially in the kidney cortex, induce *EPO* mRNA expression^{194,197}. This pattern has been proposed to reflect alterations in renal oxygen gradients, although a precise correlation has not been verified experimentally. In renal disease, this process fails (**FIG. 5**) and anaemia becomes progressively more severe as the disease progresses, without an associated rise in EPO production⁹. This failure of EPO production has long been appreciated to be not absolute; patients with kidney disease and animal models of kidney disease subjected to intercurrent hypoxia have the potential to increase EPO production in conditions of severe hypoxia (for review see REF.¹⁹⁸). In keeping with this notion, multiple clinical trials now attest to the efficacy of pharmacological activation of HIF using PHD inhibitors in stimulating EPO production and in increasing haemoglobin levels in patients with chronic kidney disease (CKD) (for a review, see REF.²¹).

A possible explanation for the reduced EPO production in CKD is that, as renal tubular reabsorption decreases, oxygen consumption falls and intra-renal hypoxia is relieved, altering the relationship between oxygen delivery and hypoxic stimulation of the EPO-producing cells¹⁹⁹. Some evidence in support of this hypothesis was provided by renal-tubular-specific inactivation of pVHL. In this mouse model, decreased renal tubular oxygen consumption, consequent upon pVHL-dependent changes in metabolism, was proposed to reduce EPO production by interstitial fibroblasts by increasing intra-renal oxygen levels²⁰⁰. However, increased intra-renal oxygenation is not a general finding in CKD. For instance, increased hypoxia has been detected in diabetic,

hypertensive and cystic animal models of CKD, and acutely following unilateral ureteral obstruction (UUO), using direct measurement of the hypoxia-activated marker pimonidazole or using blood-oxygen-level-dependent MRI (BOLD–MRI)^{201,202}. Hypoxia has also been confirmed in human CKD using BOLD-MRI²⁰³. Hypoxia might result from reduced blood flow through rarefied capillaries in the injured glomeruli and the tubulo-interstitial bed, and is considered to be an important driver of progressive renal injury in CKD^{201,204}. Thus, although intercurrent hypoxia stress has the potential to alter EPO production in diseased kidneys, reduced EPO production in CKD cannot be attributed to the relief of physiological hypoxia within the diseased kidneys, but rather, the opposite.

Interestingly, renal injury is followed by a change in the EPO-producing interstitial fibroblasts to a myofibroblastoid phenotype, characterized by the expression of markers such as α -smooth muscle actin (α -SMA) and altered responsiveness of the *EPO* gene to anaemic or hypoxic stimuli²⁰⁵. Several studies have sought to better define the renal EPO-producing cells (REPCs) and their responses in kidney disease using lineage-tracing technologies^{202,206,207}. These studies have indicated that REPCs are of neural crest origin and are contained within a lineage specified by expression of the transcription factor forkhead box protein D1 (FOXD1)²⁰⁷. In normal kidneys, most REPCs are positive for the cell surface markers platelet-derived growth factor receptor- β (PDGFR- β) and 5'-nucleotidase (CD73), lie closely adjacent to the peritubular capillaries, and manifest long cellular protrusions^{202,207,208}. Interestingly, it has been proposed that REPCs or other cells derived from FOXD1-expressing precursors, rather than cells derived by transdifferentiation of the renal epithelium, are the source of the fibroblasts responsible for the fibrosis that characterizes progressive CKD²⁰⁹. This hypothesis is supported by lineage-marking experiments in which strong induction of an *Epo* transgene was used to mark renal cells with a history of EPO-producing capability; such cells accounted for the large majority of the intra-renal myofibroblastoid population that is subsequently observed in a mouse model of unilateral ureteric obstruction²⁰². Importantly, although only expressed at a low level in the untreated obstructed kidney, *Epo* mRNA was induced strongly in this population of cells by genetic inactivation of the PHD enzymes, and exceeded that observed in the contralateral kidney²⁰². If this phenomenon occurs generally in CKD, then patients with an increased intra-renal population of myofibroblastoid cells might respond particularly well to PHD inhibitors (**FIG. 5**). To date, clear evidence of such a response has not been reported, although some studies report the need to reduce the dose or withhold PHD inhibitors in some patients owing to an excessive rise in haematocrit²¹⁰. In one study assessing the response to a single dose of a PHD inhibitor it was observed that nephric patients on dialysis manifested a larger EPO

response than either healthy volunteers or anephric patients on dialysis, consistent with a high potential for EPO production in the diseased kidneys²¹¹.

As further results from clinical trials evaluating PHD inhibitors emerge, understanding whether or not ‘hyper-responding’ patients indeed exist will be of great interest. These findings will also focus attention on other questions regarding the biology of the REPCs. For example, the exact mechanisms accounting for a failure to express the *EPO* gene in CKD, despite severe parenchymal hypoxia, are still unclear. In non-EPO-producing cells, increased DNA methylation has been observed at the *EPO* gene locus¹⁸⁶, which might possibly occur in the REPCs in CKD²⁰⁵. In support of this theory, one study reported that 5-azacytidine increases EPO production by immortalized REPCs in culture²⁰⁸. However, methylation-based silencing of the *EPO* gene is inconsistent with the known modes of action of PHD inhibitors, which can rapidly reactivate *EPO* expression, and was not supported by direct analysis of the *EPO* promoter²⁰⁸. Given that *EPO* is a specific HIF-2 target gene, a switch from HIF-2 α towards HIF-1 α expression in REPCs could theoretically have the potential to decrease EPO production, as would altered PHD expression or a reduction in oxygen consumption by these specific cells. Interestingly, all PHDs are expressed in the kidney and their expression pattern is altered in acute kidney injury (AKI)²¹². In addition, differential expression of PHD enzymes has been proposed to account for heterogeneity in the responses of REPCs to *Phd2* gene inactivation in mice²⁰⁷.

Overall, however, neither the reasons for loss of EPO production after myofibroblastoid differentiation, nor the means of reactivation of EPO production are currently understood. Given the potential involvement of REPCs in the fibrosis that contributes to the progressive decline in renal function in CKD, investigation of the effect of PHD inhibitors on these processes will be of great interest²¹³.

[H1] HIF modulation in kidney disease

In the treatment of the anaemia of renal disease most studies have aimed to use the lowest doses of PHD inhibitors that are effective in maintaining or increasing haemoglobin levels. This strategy aims to avoid excessive rises in EPO, and to limit as yet unknown clinical effects of systemic HIF activation outside EPO-producing organs. However, all the cells in the kidneys themselves must be exposed to the drug. At present the clinical effects of PHD inhibitors on the kidneys, beyond induction of EPO, are not clear. Nevertheless a number of pre-clinical studies have tested the effects of specific or non-specific PHD inhibitors, or examined the effects of genetic interventions that alter

the expression of the PHDs, HIFs or VHL in a wide range of models of kidney diseases. These studies potentially predict effects on the kidneys that might arise from clinical use of PHD inhibitors and are outlined below.

[H2] Acute kidney injury

The ability of HIF activation to induce endogenous defence mechanisms against hypoxia, including metabolic reprogramming, cytoprotection, angiogenesis, or even EPO production, has provided a strong rationale for studies of the renoprotective effects of HIF stabilization in AKI. Induction of endogenous HIF protein expression has been observed in AKI, in both a range of animal models and in human renal biopsy tissues²¹⁴⁻²¹⁸. Hypoxia-induced activation of HIF is associated with renoprotective effects in AKI²¹⁹. Protection against AKI has also been reported in rodent models treated with both nonspecific 2-OG oxygenase inhibitors — such as cobaltous ions (for example, cobalt(II)-chloride), L-mimosine, and dimethyloxalylglycine (DMOG) — as well as PHD inhibitors similar to those that are currently being developed for clinical use in renal anaemia²¹⁹⁻²²⁴. Studies involving genetic manipulation of HIF also support a protective effect of HIF in mouse models of AKI. In one study of bilateral renal ischaemia in mice, heterozygosity for either *Hif1a* or *Hif2a* was associated with a poor outcome, whereas treatment with DMOG or L-mimosine improved outcome²²¹. Genetic interventions also enable the effects of HIF stabilization to be studied in specific renal tubular segments; for instance, stabilization of HIF- α specifically in the thick ascending limb ameliorated ischaemic kidney injury in a mouse model²²⁵.

As HIF-1 α is the predominantly expressed isoform in tubular cells, most studies have attributed renoprotection to that isoform. However, in different cell types, activation of HIF-2 might be important. Indeed, specific genetic inactivation of HIF-2 α , but not HIF-1 α , in endothelial cells led to increased kidney damage following ischemia–reperfusion injury in mice²²⁶. In general, these protective effects have been observed with pharmacological pretreatment or genetic interventions that are applied before the ischaemic stress, and are not observed when interventions are applied in the post-ischaemic period²²⁷. This finding suggests that the clinical use of such interventions to prevent AKI, which is generally difficult to predict, might be difficult, restricting their use to planned interventions such as cardiovascular surgery or chemotherapy. However, the findings do suggest that the PHD inhibitors in clinical use in the setting of renal anaemia could have effects on kidney disease beyond enhancement of EPO production. This notion is also supported by their action in other settings. In an experimental renal transplant model, one study observed effects of PHD inhibitors not just on immediate graft function, but also on long-term function of the allograft²²⁸.

Furthermore, stabilization of HIF in tubular cells of human renal transplants has been observed both shortly after transplantation, and later, in the setting of acute rejection²¹⁶. Thus, in addition to a role in organ preservation, HIF activation might have effects on the immune response to the renal allograft. However, these results require confirmation both in models of renal transplantation, and in further studies in humans.

[H2] Chronic kidney disease

In keeping with the activation of HIF by diverse hypoxic and non-hypoxic stresses and the importance of intrarenal hypoxia in CKD, induction of different components of the HIF system has been described in many forms of CKD. In addition, constitutive activation of HIF is a core feature of *VHL*-defective kidney cancer⁴⁶. In an attempt to understand the role of HIF activation in these pathologies, many studies have investigated the effects of genetic and pharmacological modulation of different components of the HIF system in different renal cell types (**TABLE 1** and **FIG. 6**; for a review, see REF.²²⁹).

The induction of fibrosis is observed following activation of HIF- α in the renal tubular epithelium by genetic inactivation of pVHL²³⁰. Moreover, overexpression of HIF-2 α alone in the renal tubule is associated with induction of fibrosis in previously normal kidneys²³¹. Somewhat surprisingly, neither genetic inactivation of pVHL nor forced activation of stabilizing mutant forms of HIF- α have been associated with strong proliferative responses in renal tubules that might underpin a direct oncogenic role^{231,232}. However, overexpression or activation of HIF-2 α , but not HIF-1 α , is associated with the development of cyst changes^{231,232}. Other studies of pVHL inactivation in specific renal cell populations have revealed marked effects on differentiation. For instance, inactivation of the *Vhl* gene in the mouse using renin-1d-driven Cre recombinase resulted in aberrant expression of the *Epo* gene in cells that ordinarily produce renin²³³. In mouse podocytes, activation of HIF by Cre-mediated inactivation of *Vhl* leads to a rapidly progressive glomerulonephritis, an effect that was attributed, at least in part, to the increased expression of the HIF-target gene *Cxcr4*²³⁴. In this setting, blockade of CXC chemokine receptor 4 (CXCR4) signalling using antibodies improved renal function. In human renal biopsy tissues from patients with rapidly-progressive glomerulonephritis, HIF was also upregulated in association with enhanced expression of CXCR4, compared with individuals with non-crescentic glomerulopathy²³⁴. Based on genetic studies in mice, hypoxia and HIF activation have been proposed to modulate glomerular barrier function and susceptibility to nephritis or glomerulosclerosis²³⁵⁻²³⁷. However, the mechanisms could

well be different in the human disease setting, and causal relationships with HIF expression in human CKD are difficult to define.

The effects of genetic manipulation of the HIF pathway have also been directly tested in models of progressive renal injury. Renal-tubule-cell-specific genetic inactivation of HIF-1 α in the mouse was found to be associated with decreased fibrosis in a UUO model²³⁸. Consistent with this observation, stabilization of HIF in renal tubular cells increased fibrosis in a renal ablation mouse model of CKD²³⁰. However, other work has reported different outcomes, leading to the proposal that the effects of HIF activation are cell-type specific, a hypothesis that is supported by the beneficial effect of myeloid-cell-restricted activation of HIF in a UUO mouse model²³⁹.

Further complexity is generated by reports that the effects of interventions targeting HIF are time-dependent or have positive and negative effects on outcomes via different mechanisms²⁴⁰. For instance, in a mouse model of renal fibrosis induced by adenine, genetically driven overexpression of HIF-2 α in renal tubules aggravated fibrosis in early disease, whereas, at later time points, it improved vascularization and was associated with reduced hypoxia and reduced fibrosis²⁴⁰.

The results of experimental studies also suggest that clinical effects on human kidneys might be disease-specific. For instance, activation of HIF has been observed in cyst-lining cells in human autosomal dominant polycystic kidney disease (ADPKD) and in a range of genetic mouse models, and has been attributed to ischaemic hypoxia caused by compression of the renal tissue by expanding cysts^{241,242}. In line with observations in the normal kidney, cystic epithelial cells express HIF-1 α , whereas stromal cells of the cyst lining express HIF-2 α ²⁴¹. Interestingly, in mice, combined deletion of *Hif1a* and *Pkd1* (which encodes polycystin-1) in the renal tubules ameliorated polycystin-1-associated cyst development, whereas treatment with a PHD inhibitor (similar to those currently being evaluated in clinical trials) exacerbated cystic disease²⁴³. Thus, at least in this context, activation of HIF-1 α seems to drive cyst development. Again, however, the outcome might be specific to the type of cystic disease. As outlined above, in disease associated with *VHL* inactivation, HIF-2 seems to drive cyst development^{231,232}. Furthermore, the cystic pathology generated via renal-tubule-specific inactivation of the gene encoding the renal tumour suppressor fumarate hydratase is exacerbated on concomitant inactivation of the *Hif1a* gene, suggesting that, in this setting, HIF-1 α functions to restrain cyst development²⁴⁴.

Studies of pharmacological activation of HIF in other models of CKD have largely been restricted to the use of nonspecific 2-OG oxygenase inhibitors. In models of diabetic nephropathy, activation of HIFs by cobalt(II) chloride resulted in decreased proteinuria and tubulo-interstitial damage, as well as improved function^{245,246}. Cobalt(II) chloride also improved outcomes in models of Thy-1-mediated

nephritis and in renal ablation models²⁴⁷⁻²⁴⁹, although some studies have reported adverse outcomes. In a remnant kidney model, both beneficial and deleterious effects of the nonspecific 2-OG oxygenase inhibitor L-mimosine were observed to be dependent on the timing and duration of administration²⁵⁰. Moreover, cobalt(II) chloride has been reported to damage podocytes and induce proteinuria²⁵¹. Other experimental systems have been used to infer adverse effects of HIF activation. For instance, in the genetically obese diabetic (*db/db*) mouse model, inhibition of Rho–Rho kinase (ROCK) signalling by fasudil simultaneously prevented upregulation of HIF-1 α and its target genes and reduced glomerulosclerosis²⁵².

In addition to the aforementioned findings in the kidneys, multiple systemic actions of HIF in innate and adaptive immunity, cardiovascular and pulmonary biology, metabolism and cancer have been described and reviewed elsewhere^{175,176,178,253}. Taken together, these observations demonstrate the enormous complexity of the effects that might arise from activation of HIFs at different times and in different cell types in the setting of renal disease (**FIG. 6**). To date, surprisingly few long-term studies have been published of experimental renal disease using the PHD inhibitors that are under current evaluation in clinical trials. In terms of predicting the consequences of using PHD inhibitors in patients with renal disease, current knowledge and model systems, therefore, have major limitations. In studies reporting HIF activation in renal disease, it is difficult to understand causal relationships to the disease process. Genetic interventions are usually ‘all or none’ in any given cell type and do not mimic the graded pharmacological induction of HIF that is produced by catalytic inhibition of the PHD enzymes. In terms of cancer risk, the time course, contextual specificity and multiple additional mutations that characterize *VHL*-associated renal cancer is also very different from that predicted from pharmacological activation of HIF. However, the current knowledge does indicate that PHD inhibitors — if used at sufficient dosages — will probably have a range of effects on renal cell biology, which will require careful consideration when clinical trial results are analyzed.

[H1] Conclusions

In conclusion, the elucidation of mechanisms by which cells sense and respond to oxygen levels has opened new fields in biology and medicine. The apparent simplicity of the major components, a series of regulatory dioxygenase enzymes that generate the oxygen-sensitive signals, and a set of transcription factors that transduce those signals, belies the complexity of the HIF hydroxylase pathways. The enormous reach of the HIF transcriptional cascade and its role in a vast number of adaptive responses to hypoxia thus presents many opportunities and challenges, both for therapeutic modulation of HIF in renal disease and for the understanding of renal pathologies.

The field of hypoxia signaling, which began with studies of EPO production by the kidneys, has now come to focus again on the kidneys where several important questions have been brought into focus. Exactly why EPO production fails in renal disease, whether it will be possible to selectively activate EPO production in diseased kidneys using PHD inhibitors, what effects PHD inhibitors might have beyond the stimulation of erythropoiesis, and which patients might respond best, are all largely unanswered questions. Meantime clinical trials of PHD inhibitors in pre-dialysis and dialysis patients are showing promise and should shortly begin to answer some of these questions.

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Author contributions

Both authors researched the data, fomulated the content and wrote the manuscript.

Competing interests

P.J.R. is a scientific co-founder of ReOx Ltd., an Oxford University spin-out company that seeks to promote the therapeutic development of prolyl hydroxylase inhibitors. P.J.R. has served as a member of GlaxoSmithKline's Research Advisory Board and holds equity in the company. J.S. declares no competing interests.

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Therapeutic modulation of hypoxia-inducible factors, which transduce adaptive transcriptional responses to hypoxia, is an emerging theme in kidney disease. This Review summarizes the hypoxia signalling mechanisms underpinning these novel treatments and highlights key remaining questions relevant to their clinical use.

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Figure 1 | **General mechanism of oxidation catalyzed by 2-oxoglutarate-dependent dioxygenases.**

The catalytic iron (Fe^{II}) is co-ordinated by a 2-histidine-1-carboxylate triad of amino acid residues. In the HIF prolyl hydroxylases which are also termed prolyl hydroxylase domain (PHD) proteins, the carboxylate is an aspartate residue. Binding of the substrate (that is, the target HIF polypeptide; green) promotes dissociation of a water molecule from the active site, enabling interaction with molecular oxygen (red). The reaction proceeds via a redox process, involving a ferryl ($\text{Fe}^{\text{IV}}=\text{O}$) intermediate. Oxidation of the prime substrate is coupled to the oxidative decarboxylation of 2-oxoglutarate (blue) to succinate and carbon dioxide.

Figure 2 | **Regulation of HIF-1 α and HIF-2 α .**

The schematic illustrates different modes of regulation of hypoxia-inducible factor 1 α (HIF-1 α) and HIF-2 α , encoded by the *EPAS1* (*HIF-2A*) gene. At the transcriptional level, transcription factors (for example, signal transducer and activator of transcription 3 (STAT3) and nuclear factor- κ B (NF- κ B)) and epigenetic modifiers (for example, DNA methyl transferase 3A (DNMT3A)) influence transcription from the two gene loci, *HIF1A* and *EPAS1* (*HIF2A*), which respectively encode HIF-1 α and HIF-2 α . HIF-1 α expression is negatively regulated by an antisense transcript, HIF1A antisense RNA 2 (*HIF1A-AS2*). At the translational level, mechanistic target of rapamycin (mTOR) activation modulates the translation of HIF proteins. In addition, iron regulatory proteins (IRPs; also known as IRE-BPs) specifically interfere with the translation of the *EPAS1* (*HIF2A*) transcript by binding to an iron-responsive-element (IRE) in the 5' untranslated region (UTR) of the transcript. At the post-translational level, hydroxylation (OH) of HIF- α polypeptides by HIF prolyl hydroxylases, also termed prolyl hydroxylase domain (PHD) proteins, is responsible for their regulation by oxygen. Under conditions of normoxia, hydroxylation of prolyl residues promotes binding of HIF- α subunits to the von Hippel-Lindau tumour suppressor (pVHL), the recognition component of an ubiquitin E3-ligase complex; ubiquitylation (Ub) targets HIF for proteasomal degradation. Conditions of moderate hypoxia (or PHD inhibitor treatment) inhibit HIF prolyl hydroxylation by the PHD1–3 enzymes; consequently, HIF- α dimerizes with HIF-1 β and activates transcription of target genes, such as *EPO*. Under conditions of severe hypoxia, asparaginyl hydroxylation of HIF- α by factor inhibiting HIF (FIH) is also inhibited, permitting interaction with the acetyltransferases p300 and CREB-binding protein (CBP) and further increasing transcription of FIH-sensitive HIF target genes.

Figure 3 | Feedback mechanisms of hypoxic gene regulation.

Hypoxia-inducible factors (HIFs) activate a series of genes that operate through feedback loops to control the activity of the pathway (for additional review see REF. ¹³³). Modes of regulation include downregulation of HIF- α by microRNAs (miRNAs) and antisense transcripts, such as HIF1A antisense RNA 1 (*HIF1A-AS1*), transcriptional induction of HIF prolyl hydroxylases (PHD2 and PHD3), expression of inhibitory isoforms of HIF-3 α that compete for binding to HIF-1 β , and histone-modifying enzymes that can modulate the binding or activity of HIF at target genes in chromatin-bound DNA.

Figure 4 | HIF isoform expression profiles and target gene selectivity.

Following inhibition of hydroxylation, cell-type-specific and tissue-specific accumulation of hypoxia-inducible factor α (HIF- α) isoforms, as well as their DNA binding selectivity, generate highly distinct functional outputs for HIF-1 and HIF-2. **a** | HIF isoforms exhibit tissue-specific expression profiles. HIF-1 α is widely expressed across normal tissues and cell types, whereas HIF-2 α expression in normal tissues is restricted to the endothelium and selected cells in the kidney, gut, lung, liver and carotid body; ^{35,127,254}. **b** | A number of mechanisms mediate HIF target gene selectivity. HIF-1 preferentially targets promoter-proximal sites to activate gene transcription, whereas binding of HIF-2 to DNA frequently occurs at promoter-distal or enhancer sites.^{165,167} **c** | Epigenetic mechanisms interfere with HIF–DNA binding by DNA methylation or altered chromatin state to define the cell-specific transcriptional program in response to hypoxia^{112,165,186}. **d** | HIFs co-operate with other transcription factors to bind at specific sites and regulate gene expression^{189,255} **e** | Post-DNA binding mechanisms, including recruitment of co-activators such as the histone acetyltransferase p300, modulate HIF transcriptional activity²⁵⁶.

Figure 5 | Erythropoietin regulation in normal and diseased kidneys.

Erythropoietin (EPO) is produced in the kidneys by cortical interstitial fibroblasts, termed renal EPO-producing cells (REPCs)^{195,196}, that are derived from a lineage specified by expression of forkhead box protein D1 (FOXD1)²⁰⁷. In the schematic, REPCs cells are coloured pink when they are inactive (that is, not producing EPO) and red when they are active (that is, producing EPO). In response to increasingly severe anaemic or hypoxic stimulation, the number of active REPCs in normal kidneys progressively increases from the deeper to more superficial regions of the renal cortex (part **a**). Kidney injury results in phenotypic changes in the REPCs, which adopt myofibroblastoid features and reduce *EPO* gene expression²⁰⁵. EPO production by myofibroblastoid cells in diseased kidneys

can be reactivated pharmacologically by inactivation of the HIF prolyl hydroxylase enzymes using prolyl hydroxylase domain (PHD) inhibitors (which activate HIF)²¹. In renal disease, populations of myofibroblastoid cells might be increased, potentially generating a larger population of REPCs, in which *EPO* gene expression might be reactivated by PHD inhibitor treatment^{211,213}. This hypothesis would suggest that EPO responsiveness to PHD inhibitors might vary in different renal disease settings. Some patients with low numbers of REPCs (part **b**) might respond less well than those with large numbers of REPCs (part **c**), in whom responses might be greater than those in normal kidneys.

Figure 6 | **Actions of HIF in the kidney.**

The potential effects of hypoxia-inducible factor (HIF) activation in different cells (highlighted in red) within the kidney are illustrated. In different experimental settings (principally mouse models) and in response to different modes of HIF modulation, effects of HIF activation include those that are both potentially beneficial and potentially harmful. These effects on the kidney include **a** | De-differentiation; for instance chronic activation of HIF-2 impairs differentiation of renal progenitor cells²⁵⁷, and the HIF-pathway is activated in de-differentiated tubular cells in early lesions of VHL-defective renal cancer²⁵⁸. **b** | Cyst growth; in different setting activation of HIF-1 or HIF-2 either promotes or restricts cyst growth^{231,232,242,243}. **c** | Erythropoietin (EPO) regulation; EPO is induced in renal interstitial fibroblasts^{195,196} by activation of HIF-2²⁵⁹ **d** | Tubule protection; both genetic and pharmacological activation of HIF have been reported to protection renal tubules in models of acute kidney injury²¹⁹⁻²²⁴. **e** | Glomerulonephritis and **f** | Inflammation; both intra-renal (podocyte) and general activation of HIF-2 have been reported to induce nephritis^{234,236,260}. **g** | Fibrosis; in different setting activation of HIF has been reported to ameliorate or provoke fibrosis in the kidneys^{224,230,231,237-239,246,248,249}.

Table 1 | HIF-targeted genetic and pharmacological interventions in CKD models.

Model of kidney disease	Target	Intervention	Effect on the kidney	Refs
<i>Effects on fibrosis</i>				
Normal kidney (mouse)	Tubular cells	HIF activation by <i>Vhl</i> knockout; transgenic <i>Hif2a</i> overexpression	Induction of fibrosis	209 , 35
Unilateral ureteral obstruction (mouse)	Tubular cells	<i>Hif1a</i> knockout	Decreased fibrosis	216
Unilateral ureteral obstruction (mouse)	Myeloid cells	<i>Hif1a</i> knockout	Decreased fibrosis	217
Adenine-induced fibrosis (mouse)	Tubular cells	Transgenic <i>Hif2a</i> overexpression	Stage-dependent effects: early activation aggravated fibrosis, whereas late activation reduced fibrosis	218
AKI-associated fibrosis (mouse)	Systemic	Pharmacological HIF activation by PHD inhibitor	Decreased fibrosis	204
Remnant kidney (mouse)	Tubular cells	HIF activation by <i>Vhl</i> knockout	Increased fibrosis	209
Remnant kidney (rat)	Systemic	Pharmacologic HIF activation by cobalt(II) chloride	Reduced tubulo-interstitial injury; increased angiogenesis	225
Remnant kidney (rat)	Systemic	Pharmacologic HIF activation by cobalt(II) chloride or DMOG	Improved function	227

Remnant kidney (rat)	Systemic	Pharmacologic HIF activation by L-mimosine	Profibrotic and antifibrotic effects dependent on timing of drug administration	228
Progression of cystic disease				
Normal kidney (mouse)	Tubular cells	HIF activation by <i>Vhl</i> knockout; transgenic <i>Hif2a</i> overexpression	Cyst development	210 , 35
Polycystin-1 deficiency (mouse)	Tubular cells	<i>Hif1a</i> knockout	HIF promoted cyst growth	221
Polycystin-1 deficiency (mouse)	Systemic	HIF activation by PHD inhibitor	HIF promoted cyst growth	221
Fumarate hydratase deficiency (mouse)	Tubular cells	<i>Hif1a</i> knockout	Increased cyst growth	222
Progression of diabetic nephropathy				
Streptozotocin-induced diabetic nephropathy (rat)	Systemic	HIF activation by cobalt(II) chloride	Improved function; reduced proteinuria	223
Diabetic nephropathy in hypertensive–obese rats	Systemic	HIF activation by cobalt(II) chloride	Reduced glomerulosclerosis; reduced proteinuria	224
Diabetic nephropathy in genetically obese (db/db) mice	Systemic	<i>Hif1a</i> downregulation by fasudil	Improved function; reduced proteinuria	230
Glomerular disease				

Normal kidney (rat)	Systemic	HIF activation by cobalt(II) chloride	Proteinuria; impaired podocyte function	229
Rapidly progressive glomerulonephritis (mouse)	Podocytes	HIF activation by <i>Vhl</i> knock-out	Disease trigger	212
Rapidly progressive glomerulonephritis (mouse)	Podocytes	Transgenic <i>Hif2a</i> overexpression	Disease trigger	214
Nephrin-promoted CD25 model of glomerulosclerosis (mouse)	Podocytes	<i>Hif1a</i> knockout	Reduced glomerulosclerosis	215
Thy-1 nephritis (rat)	Systemic	HIF activation by cobalt(II) chloride	Improved function	226
Glomerular development (mouse)	Podocytes	HIF activation by <i>Vhl</i> knockout	Glomerulomegaly and glomerulosclerosis	213

AKI, acute kidney injury; CKD, chronic kidney disease; DMOG, dimethyloxallylglycine; PHD, prolyl hydroxylase domain.

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