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Review

Hypoxic regulation of erythropoiesis and iron metabolism

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Haase VH. Hypoxic regulation of erythropoiesis and iron metabolism. *Am J Physiol Renal Physiol* 299: F1–F13, 2010. First published May 5, 2010; doi:10.1152/ajprenal.00174.2010.—The kidney is a highly sensitive oxygen sensor and plays a central role in mediating the hypoxic induction of red blood cell production. Efforts to understand the molecular basis of oxygen-regulated erythropoiesis have led to the identification of erythropoietin (EPO), which is essential for normal erythropoiesis and to the purification of hypoxia-inducible factor (HIF), the transcription factor that regulates EPO synthesis and mediates cellular adaptation to hypoxia. Recent insights into the molecular mechanisms that control and integrate cellular and systemic erythropoiesis-promoting hypoxia responses and their potential as a therapeutic target for the treatment of renal anemia are discussed in this review.

erythropoietin; hypoxia; hypoxia-inducible factors; iron; red blood cells

ONE OF THE MOST EXTENSIVELY studied systemic adaptations to hypoxia is the stimulation of red blood cell (RBC) production. Over 100 years ago, Paul Bert and Denis Jourdanet observed the association between reduced atmospheric oxygen pressure and elevated RBC numbers in the blood of animals and humans (12, 13, 64). Francois-Gilbert Viault then demonstrated that ascent to a high altitude provided an acute and direct physiological stimulus for RBC production during his 1890 expedition to the Peruvian Andes by measuring the RBC increases in his own and the blood of his companions (156). It was the interest in understanding the physiological and molecular basis of this erythropoietic response that led to the discovery of erythropoietin (EPO) and that paved the way for the identification of the molecular machinery that senses oxygen and controls a wide spectrum of tissue-specific and systemic responses to hypoxia.

The hypoxic induction of *EPO* serves as a paradigm of oxygen-dependent gene regulation, and the search for the transcription factor that mediates this induction led to the discovery of the hypoxia-inducible factor (HIF) as a key mediator of cellular adaptation to low oxygen. Recent experimental evidence suggests that HIF promotes erythropoiesis through coordinated cell type-specific hypoxia responses, which include increased EPO production in the kidney and liver, enhanced iron uptake and utilization, as well as changes in the bone marrow microenvironment that facilitate erythroid progenitor maturation and proliferation. Because of its central role in the hypoxic regulation of erythropoiesis, pharmacological targeting of the HIF oxygen-sensing pathway has the potential to become an effective, novel therapy in the treatment of anemia that is associated with inadequate EPO production. This review provides an overview of recent insights into the molecular mechanisms that underlie oxygen-dependent regulation of EPO synthesis, iron metabolism, and erythroid progenitor maturation and discusses their relevance to clinical disorders.

Oxygen-Dependent Regulation of EPO Synthesis: A Paradigm of Hypoxic Gene Regulation

The human *EPO* gene encodes a glycoprotein hormone, which consists of 165 amino acids in its circulating form. Serum EPO is heavily glycosylated and has a molecular mass of ~30 kDa, 40% of which is derived from its carbohydrate portion. Its major action is the prevention of apoptosis in EPO-dependent colony-forming unit-erythroid cells and erythroblasts that have not begun hemoglobin synthesis. Its receptor (EPO-R), which is also hypoxia inducible (26, 91, 167), lacks intrinsic enzymatic function and associates with the tyrosine kinase Janus kinase 2 (JAK2), which phosphorylates EPO-R at multiple sites upon ligand binding, thus providing docking sites for signal-transducing molecules that contain src homology 2 domains. EPO-R signals through multiple pathways. These include the signal transduction and activator of transcription (STAT) 5 pathway, the phosphatidylinositol 3-kinase/protein kinase B (PI-3K/AKT) and MAPK/ERK pathways, and PKC (62).

Hypoxia is the primary physiological stimulus for EPO production, which, depending on the hypoxic condition, increases serum EPO levels up to several hundred-fold (33). Studies in hepatoma cells aimed at isolating the transcriptional activator responsible for the hypoxic induction of *EPO* identified the heterodimeric basic helix-loop-helix transcription factor HIF-1 as the transcriptional regulator that binds to the hypoxia-sensitive enhancer located in the 3′-prime region of the *EPO* gene (159, 160). HIF-1 belongs to the PAS [PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/single minded (SIM)] family of transcription factors and consists of

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an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, also known as ARNT (69, 138, 163). Together with HIF-2α (also known as EPAS-1 or HLF), HIF-1α facilitates oxygen delivery and cellular adaptation to hypoxia by stimulating multiple biological processes, such as erythropoiesis, angiogenesis, and anaerobic glucose metabolism (137). HIFs regulate gene expression by binding to specific DNA recognition sequences, referred to as hypoxia-response elements (HREs) (Fig. 1). All three known HIF α-subunits, HIF-1α, HIF-2α, and HIF-3α, are targeted for rapid proteasomal degradation under normoxia by the von Hippel-Lindau tumor suppressor pVHL, which acts as the substrate recognition component of an E3 ubiquitin ligase complex (99, 101). Whereas HIF-1α and HIF-2α heterodimers function as transcriptional activators, splice variants of HIF-3α have been shown to be inhibitory (90, 100). Although HIF-1 and HIF-2 share many common transcriptional targets, they also regulate unique targets and have specific biological functions. Anaerobic glycolysis, for example, appears to be predominantly controlled by HIF-1 (55), whereas HIF-2 has emerged as the main regulator of EPO production in the adult (48, 107, 126, 135). In addition to HRE-mediated transcriptional regulation, which requires heterodimerization with ARNT, HIF-α modulates cellular signaling pathways through functional interaction with proteins that do not contain PAS domains. These include, among others, tumor suppressor protein p53, the c-Myc proto-oncogene, and the Notch intracellular domain (2, 49, 72, 127).

Under normal oxygen conditions, HIF-α-subunits are rapidly degraded following ubiquitylation by the pVHL-E3 ubiquitin ligase, which precludes the formation of transcriptionally active heterodimers. pVHL-mediated polyubiquitylation of HIF-α requires hydroxylation of specific proline residues (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in human HIF-2α) within its oxygen-dependent degradation domain (20, 35, 53, 56, 58, 94, 169). Hydroxylation of HIF-α is carried out by three major 2-oxoglutarate-dependent dioxyge-

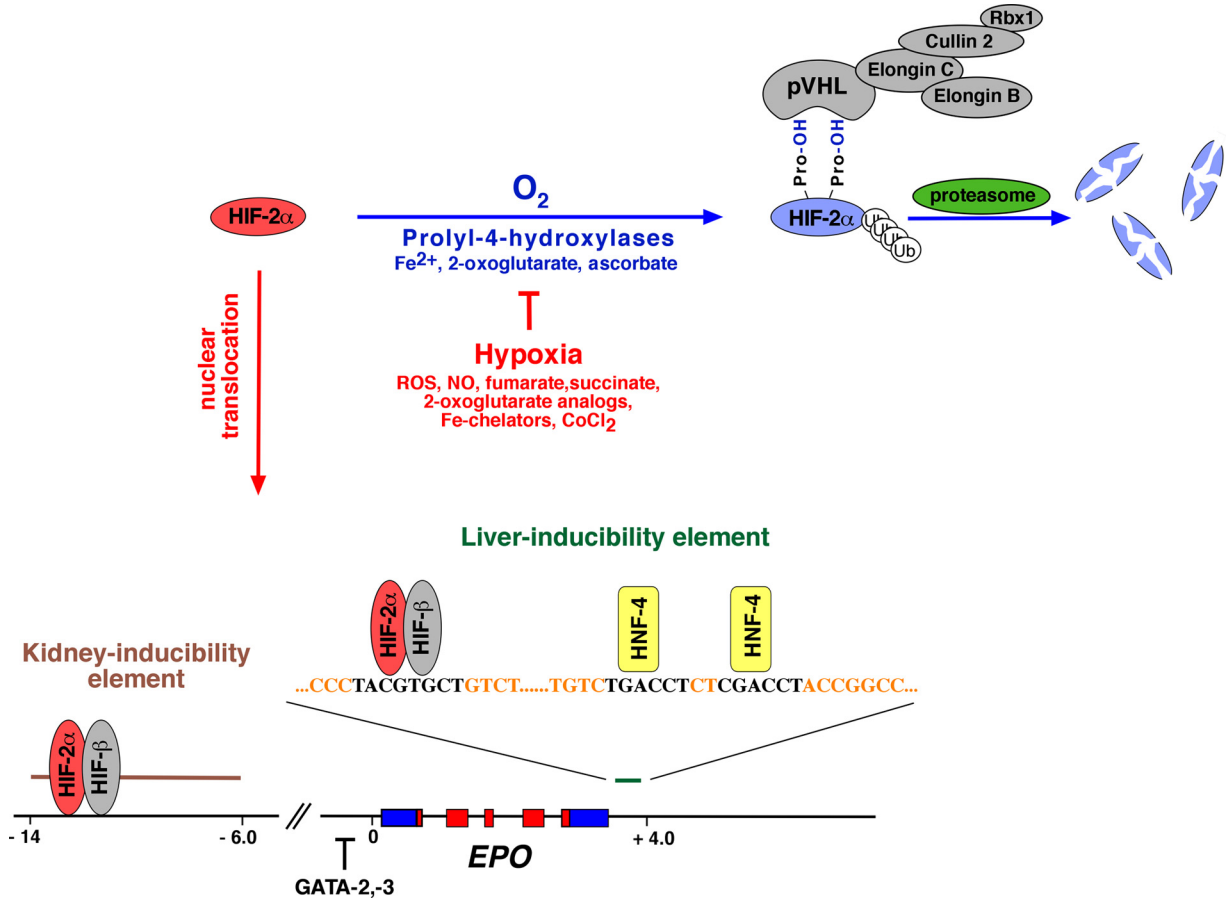

Fig. 1. Hypoxia-inducible factor (HIF)-2 regulates erythropoietin (*EPO*). Shown is an overview of *EPO* gene regulation by the von Hippel-Lindau (VHL)/HIF/prolyl-4-hydroxylase domain (PHD) oxygen-sensing pathway. Proteasomal degradation of HIF-2α by the VHL tumor suppressor (pVHL)-E3-ubiquitin ligase complex (shown are key components of this complex) requires hydroxylation by oxygen- and iron-dependent PHDs. Binding to hydroxylated HIF-α occurs at the β-domain of pVHL, which spans amino acid residues 64–154. The C-terminal α-domain links the substrate recognition component pVHL to the E3 ubiquitin ligase via elongin C. In the absence of molecular oxygen, HIF-2α is not degraded and translocates to the nucleus where it forms a heterodimer with HIF-β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-2α/β heterodimers bind to the HIF consensus binding site 5′-*RCGTG*-3′ and increase *EPO* transcription in the presence of transcriptional coactivators, such as CREB-binding protein (CBP) and p300. Hypoxic induction of *EPO* in the liver is mediated by the liver-inducibility element located in the 3′-end of the *EPO* gene and in renal interstitial fibroblast-like cells by the 5′-kidney-inducibility element, which is located 6–14 kb upstream of its transcription start site. Nitric oxide, reactive oxygen species, Krebs cycle metabolites succinate and fumarate, cobalt chloride ($CoCl_2$), and iron chelators such as desferrioxamine inhibit HIF PHDs in the presence of oxygen, resulting in increased *EPO* transcription. *EPO* mRNA is encoded by 5 exons depicted by boxes. Coding sequences are shown in red. Nontranslated regions are shown in blue, and numbers indicate distance from the transcription start site in kb (not drawn to scale). Also shown are binding sites for hepatocyte nuclear factor (HNF)-4 in the 3′-liver-inducibility region. Fe^{2+}, ferrous iron; NO, nitric oxide; ROS, reactive oxygen species; ub, ubiquitin.

nases [prolyl-4-hydroxylase domain (PHD) proteins PHD1, PHD2, and PHD3 also known as egl nine homolog (EGLN)2, EGLN1, and EGLN3, respectively] and requires molecular oxygen, ferrous iron, and ascorbate (133). To add complexity to the regulation of this pathway, HIF increases transcription of *PHD2* and *PHD3*. Furthermore, protein turnover of PHD1 and PHD3 is hypoxically regulated by Siah proteins, which themselves are hypoxia inducible (111, 112). An additional hypoxic switch operates in the carboxy-terminal transactivation domain of HIF-α with oxygen-dependent asparagine hydroxylation via factor inhibiting HIF (FIH), which blocks binding of transcriptional coactivators CREB-binding protein (CBP) and p300. Conversely, FIH inactivation facilitates CBP/p300 recruitment to the HIF transcriptional complex and results in increased HIF target gene expression under hypoxia or in *VHL*-deficient cell lines (78, 79, 89, 148).

HIF-2 Dependence of EPO Synthesis

Although HIF-1 was isolated from human Hep3B cells utilizing the HIF binding sequence from the *EPO* 3′ hypoxia enhancer, both in vivo and in vitro evidence now suggest that HIF-2 is the key mediator of the hypoxic induction of EPO in the adult kidney and liver (Fig. 1). This notion is consistent with histological studies, which demonstrated that the location of HIF-2α-expressing renal cells coincided with the location of EPO-producing renal interstitial fibroblast-like cells (129). While HIF-1α is widely expressed, the expression of HIF-2α is more restricted. HIF-2α was initially identified as an endothelial-specific HIF; however, subsequent studies found HIF-2α expression also in hepatocytes, cardiomyocytes, glial cells, type II pneumocytes in the lung, and in renal peritubular interstitial cells (129, 164). In contrast to EPO-producing renal interstitial cells, hypoxia responses in pVHL-competent renal tubular epithelial cells are mediated by HIF-1 under physiological conditions (50).

The analysis of HIF-1α and HIF-2α knockout mice provided the first major insights into the functional differences between HIF-1 and HIF-2. Mice that lack both copies of HIF-1α die in utero between embryonic *day 8* (*E8*) and *E11* from neural tube defects, increased cell death in the cephalic mesenchyme and cardiovascular malformations (57, 130). Mice with homozygous deletion of HIF-2α die in utero or around birth, unless bred in a mixed genetic background (134). Three different phenotypes have been described for homozygous HIF-2α germ line inactivation: *1*) defective catecholamine synthesis in the organ of Zuckerkandl, leading to heart failure and midgestational death (154); *2*) abnormal VEGF-mediated lung maturation, resulting in perinatal death (28); and *3*) severe vascular defects in the yolk sac and embryo proper, resulting in death between *E9.5* and *E13.5* (116). When bred in a mixed genetic background, HIF-2α knockout mice survived into adulthood but developed hepatic steatosis, skeletal myopathy, and cardiac hypertrophy, which were associated with mitochondrial abnormalities and deficiencies in reactive oxygen species scavenging and an inadequate production of SOD (134). Hematological analysis revealed pancytopenia and was associated with hypocellularity in the bone marrow (136). Since renal EPO synthesis was decreased and RBC numbers were normalized following treatment with recombinant EPO (135), anemia in HIF-2α mutants resulted from inadequate EPO production in the kidney, and not from a cell-autonomous defect in erythroid precursor maturation. While Morita et al. (107) had already suggested a role for HIF-2 in the synthesis of retinal EPO, Scortegagna and colleagues (135, 136) established that HIF-2 was essential for the maintenance of systemic EPO homoeostasis. Due to embryonic lethality, the relative contribution of HIF-1 and HIF-2 to the hypoxic induction of EPO was difficult to define in germ line knockout mice, since a direct comparison was not possible. Nevertheless, analysis of HIF-1-deficient embryos at *E9.5* demonstrated a reduction in myeloid multilineage cells and committed erythroid progenitors. *EPO* mRNA levels were found to be decreased in the embryo proper but not in the yolk sac. In contrast *EPO-R* mRNA was decreased in both tissues (167). In adult mice with heterozygous HIF-1α deficiency, Yu and colleagues (168) reported delayed erythrocytosis following exposure to chronic hypoxia (10% O_2 over a period of up to 6 wk; serum EPO or tissue *EPO* mRNA levels were not measured). Furthermore, Cai and colleagues (22) observed that renal *EPO* mRNA did not rise in HIF-1α heterozygotes exposed to 1 h of intermittent hypoxia, thus supporting a role for HIF-1 in the hypoxic induction of renal EPO. Given recent findings regarding the role of dermal oxygen-sensing in the regulation of EPO synthesis, it is plausible that in the former study, reduced expression of HIF-1α in the skin may have indirectly affected renal EPO responses through alterations in blood flow (63). Nevertheless, the degree to which individual HIF-α subunits contributed to EPO production, that is, whether *EPO* was coregulated by HIF-1 and HIF-2 or whether it was preferentially regulated by one HIF or the other, could not be determined in these animal models. Warnecke and colleagues (161) addressed this issue in Hep3B and Kelly cells using small interfering RNAs directed against HIF-1α or HIF-2α and found that the hypoxic induction of *EPO* was HIF-2 dependent, while HIF-1 played only a minor role. Similar observations were made in EPO-producing cultured cortical astrocytes (24), whereas studies in other cell lines suggested HIF-1 dependence (37).

The most compelling support for the notion that HIF-2 is the main regulator of adult EPO synthesis comes from conditional knockout studies in mice using a tamoxifen-inducible ubiquitously expressed Cre-recombinase transgene, which produced a widespread and efficient, albeit not complete, recombination of HIF-α conditional alleles. Gruber and colleagues (48) reported that postnatal global ablation of HIF-2α but not of HIF-1α, resulted in anemia, which, similar to HIF-2α germ line deletion, was correctable with recombinant EPO. While the increase in renal EPO following phenylhydrazine treatment (phenylhydrazine induces hemolytic anemia) was blunted in HIF-2α-ablated mice, postnatal deletion of HIF-1α had no effect on renal EPO production. Although the effects of chronic hypoxia were not examined in these mice, this study succeeded in directly comparing both HIFs and could not find a significant role for HIF-1 in the regulation of systemic EPO homeostasis at baseline or in response to acute anemia (48).

Our laboratory used cell type-specific knockouts to investigate the differences between HIF-1 and HIF-2 in the regulation of liver and renal EPO synthesis. Inactivation of HIF-2α in the kidney alone completely ablated the renal EPO response in mice subjected to normobaric hypoxia (10% O_2 for 10 days), phlebotomy-induced anemic hypoxia, or to treatment with a HIF prolyl-4-hydroxylase inhibitor (Kapitsinou PP, Liu Q,

Unger TL, Rha J, Davidoff O, Keith B, Epstein JA, Moores SL, Ercikson-Miller C, Haase VH, unpublished observations). Cell type-specific inactivation of pVHL in ~20–30% of hepatocytes resulted in HIF-2- but not HIF-1-dependent erythrocytosis, while pharmacological inhibition of HIF prolyl-4-hydroxylases caused a HIF-2-dependent increase in liver *EPO* mRNA levels (126). More importantly, inactivation of hepatocyte-derived HIF-2 and not HIF-1 resulted in postnatal anemia that resolved in adulthood (the liver is the main tissue source of EPO during embryonic development), and HIF-2 but not HIF-1 induced liver EPO in response to anemia (126). Consistent with our findings are studies with a nondegradable form of HIF-1α or HIF-2α in hepatocytes. Overexpression of HIF-2α produced erythrocytosis, whereas overexpression of HIF-1α did not (70). Taken together, conditional knockout studies have now established that, in the adult, renal and hepatic EPO synthesis is HIF-2 and not HIF-1 dependent, identifying HIF-2 as a key pharmacological target for the treatment of anemia that is associated with inadequate EPO production.

The molecular basis for HIF-2 dependence of EPO is not well understood. Chromatin immunoprecipitation assays in Hep3B cells demonstrated that HIF-2 and not HIF-1 associated with the endogenous *EPO* HRE (126), while HIF-1 preferentially bound to the unmodified *EPO* HRE fragment in vitro, which is consistent with its purification from hypoxic Hep3B extracts using a 18-nucleotide fragment (142). It is likely that HIF-2 binding to the *EPO* HRE requires additional nuclear factors that associate with the *EPO* gene, as HIF-2-mediated expression of an *EPO* HRE-luciferase construct required a 223-bp enhancer fragment, which contained additional transcription factor binding sites (142, 161). One of these sites includes a DR-2 element, which is a binding site for members of the nuclear hormone receptor family. Hepatocyte nuclear factor-4 (HNF-4) binds to this element and is a potential candidate factor that may specifically cooperate with HIF-2 (Fig. 1) (161). Similar to HIF-2, HNF-4 expression coincides with sites of EPO production in the liver and renal cortex and is required for the hypoxic induction of *EPO* in Hep3B cells (15, 42, 161). The notion that binding of HIF-2 to regulatory sequences in certain genes depends on the availability and cooperation with other transcription factors has been previously suggested; however, factors that are required for HIF-2-dependent hypoxic induction of *EPO* have not been identified (7).

Posttranslational Modifications of HIF that Control EPO Production

Several posttranslational modifications of HIF-2α have been identified that modulate the systemic EPO response. HIF-2α is acetylated during hypoxia and deacetylated by sirtuin 1, a NAD^+-dependent protein deacetylase, which increases HIF-2-dependent EPO synthesis in vitro and in vivo, thereby linking cellular redox and energy state to systemic hypoxia responses (32). Sirtuin 1-deficient mice produced significantly lower amounts of fetal liver *EPO* mRNA and during adulthood less renal *EPO* in response to hypoxia (6% O_2). Interestingly, caloric restriction, which induces sirtuin 1 activity, suppresses EPO production in the liver (27, 66). Further studies are needed to reconcile these contradictory findings.

An additional posttranslational modification with direct impact on EPO synthesis and hypoxia-induced erythropoiesis is SUMOylation. SUMO (small ubiquitin-like modifier) proteins are structurally related to ubiquitin and reversibly modify function and cellular localization of targeted proteins. One of the enzymes, which remove SUMO, is sentrin/SUMO-specific protease (SENP). SENP1 knockout mice developed severe anemia and died during midgestation (25). In this model, de-SUMOylation under hypoxic conditions did not occur in the absence of SENP and prevented the activation of HIF signaling in the nucleus. Instead, SUMOylated HIF-α was targeted for proteasomal degradation in a pVHL- and ubiquitin-dependent, but PHD-independent manner (PHD enzymatic activity is dependent on molecular oxygen), which resulted in a strong reduction of hepatic *EPO* mRNA levels (25). Although SUMOylation of HIF-α was specifically investigated with regard to hypoxic HIF-1 signaling, the presence of anemia strongly suggests that SENP de-SUMOylates HIF-2α as well.

Cellular Sources of EPO

Experiments in animals in which various organs were surgically removed identified the kidney as the major site of EPO production (60), where interstitial peritubular fibroblast-like cells in the inner cortex and outer medulla synthesize EPO (9, 73, 75, 76, 98, 114). *EPO* expression in other renal cell types, such as tubular epithelial cells, appears to be suppressed by GATA transcription factors, in particular GATA-2 and GATA-3. Mutations in the core GATA binding site in EPO-green fluorescent protein (GFP) transgenic animals resulted in GFP expression in distal tubular cells under normoxic and hypoxic conditions, indicating that GATA transcription factors participate in regulating cellular restriction of renal EPO expression (114). It is also interesting that the kidney does not respond to hypoxia by dynamically increasing *EPO* message levels in individual cells, but rather by recruiting additional cells capable of generating a fixed amount of EPO in a P_{O_2}-dependent fashion. In this regard, renal EPO production differs from EPO synthesis in hepatocytes, hepatoma, neuroblastoma, or melanoma cell lines (75, 114). HIF-mediated induction of EPO in the liver and kidney is also controlled by distinct regulatory elements, which are located on the opposite ends of the *EPO* gene, the kidney-inducibility element in the 5′-region and the liver-inducibility element in the 3′-region, first described by Semenza and colleagues (139–141) (Fig. 1).

The isolation and establishment of EPO-producing cells from the kidney have been largely unsuccessful, although hypoxic induction of EPO synthesis was reported in 4E cells, a mesenchymal cell clone with stromal cell characteristics isolated from the adult kidney (125). EPO-producing renal cells, however, have been successfully tagged with GFP (114), using BAC transgenes, in which a segment of the murine *EPO* gene was replaced with *GFP* cDNA, bringing GFP under the control of *EPO*-regulatory elements (114). GFP expression in transgenic mice was induced by anemia in renal peritubular interstitial cells specifically, and in hepatocytes surrounding the central vein, supporting the notion that these two cell types are the major sources of EPO production under hypoxic conditions. In the kidney, GFP-positive interstitial cells were unique in their morphological appearance, possessed dendrite-like processes, and expressed neuronal-specific markers, such

as microtubule-associated protein 2 and neurofilament protein, light polypeptide. This finding suggested that renal EPO-producing cells might be derived from progenitor cells related to the neural lineage. In keeping with this notion, Frede and colleagues (39) recently described the establishment of an EPO-producing renal tumor cell line with similar morphological and molecular characteristics. These exciting findings will certainly stimulate further interest and investigation into the histogenetic origin of renal EPO-producing cells.

While the kidney is the primary physiological site of adult EPO synthesis, the liver is the main source during embryonic development. In adults, however, the liver is the major source of extrarenal EPO production following stimulation with moderate to severe hypoxia. While hepatocytes have been identified as the primary cell type responsible for EPO synthesis in the liver, *EPO* has also been detected in hepatic stellate cells, which are also known as ITO cells (74, 97). The onset of the transition from liver to the kidney as the primary site of EPO production is species dependent and usually occurs in late gestation or around birth (30, 34, 108, 170). Despite extensive efforts by many laboratories, the molecular mechanisms that underlie this switch are poorly understood and may involve transcriptional repression and/or reduced expression of certain transcriptional activators such as transcription factor GATA-4 (31). In the adult, liver *EPO* mRNA levels, which are very difficult to detect at baseline, rise substantially following stimulation with moderate to severe hypoxia and account for most, if not all, physiologically relevant systemic EPO of extrarenal origin (40, 41). However, hypoxia-stimulated liver EPO production is usually not sufficient to compensate for the loss of renal EPO in the setting of nephrectomy or advanced chronic renal failure. Because of the recent advances in understanding the molecular basis of HIF oxygen sensing, pharmacological stimulation of HIF signaling through prolyl-4-hydroxylase inhibition, with the goal of stimulating hepatic EPO synthesis, offers great potential as a therapeutic strategy for the treatment of renal anemia or other conditions associated with inadequate EPO production.

Aside from the kidney and liver as the two major sources of synthesis, *EPO* mRNA expression has also been detected in the brain (neurons and glial cells), lung, heart, bone marrow, spleen, hair follicles, and the reproductive tract (10, 16, 29, 30, 38, 71, 88, 92, 93, 96, 166). Of those cell types, glial cells in the mouse brain have recently been shown to contribute to the serum EPO pool in response to acute anemia (162). EPO synthesized in other organs appears to act locally, modulating, for example, regional angiogenesis and cellular viability and does not seem to contribute to erythropoiesis (for a overview of the nonhematopoietic actions of EPO, see Ref. 61).

Indirect Regulation of EPO Synthesis

Although increased EPO synthesis in kidneys and liver is a consequence of their ability to directly sense changes in regional oxygen tension, some studies have suggested that under certain experimental conditions, renal EPO production can be activated by extrinsic signals. For example, Wussow and colleagues (158) postulated the existence of an O_2 sensor in the brain stem, which triggers renal EPO production through release of yet to be identified humoral factors (isolated brain stem hypoxia was generated by increased intracranial pressure). More recently, Boutin and colleagues (17) demonstrated that the skin has the potential to modulate renal and hepatic EPO synthesis indirectly through HIF-1- and nitric oxide (NO)-mediated effects on dermal blood flow (Fig. 2). However, this would still require intact oxygen-sensing mecha-

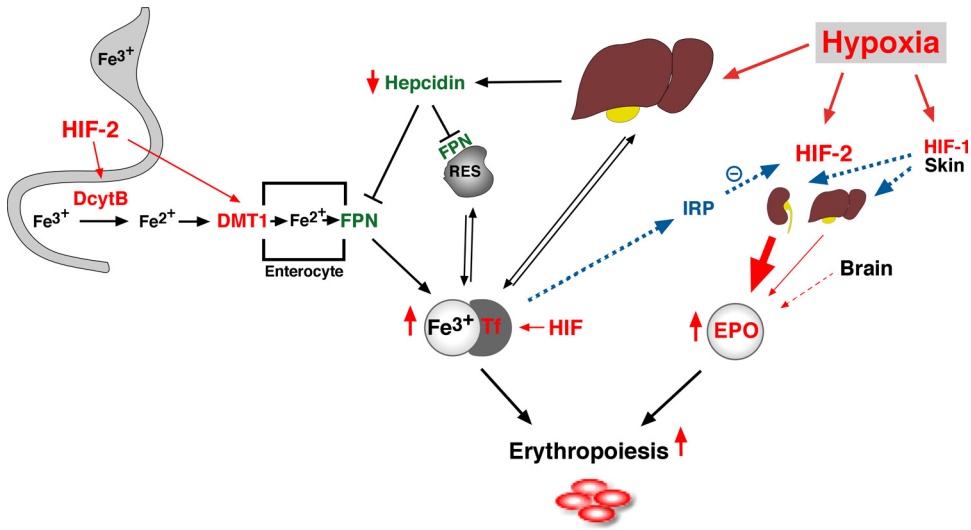

Fig. 2. Hypoxia coordinates EPO synthesis with iron metabolism. Shown is a simplified overview of hypoxic and HIF-mediated effects on iron metabolism. HIF-2 induces renal and hepatic *EPO* synthesis in response to hypoxia, which results in increased serum EPO levels (circle), stimulating erythropoiesis. Renal and liver EPO responses are modulated by dermal HIF-1 (see the text). Also, included in this schematic is the recently described contribution of glial cell-derived EPO to the serum EPO pool. An adjustment of iron metabolism is needed to satisfy increased iron demand in the bone marrow. In the duodenum, duodenal cytochrome *b* (DcytB) reduces ferric iron (Fe^{3+}) to its ferrous form (Fe^{2+}), which is then transported into the cytosol of enterocytes (square) by divalent metal transporter-1 (DMT1). *DcytB* and *DMT1* are both hypoxia inducible and HIF-2 regulated. Absorbed iron is released into the circulation by ferroportin (FPN) and is then transported in complex with transferrin to liver, reticuloendothelial cells, bone marrow, and other organs. Transferrin (Tf) is HIF regulated, and hypoxia increases its serum levels. Hypoxia, low serum iron levels, and increased "erythropoietic drive" inhibit hepcidin synthesis in the liver, resulting in diminished FPN cell surface expression in different tissues. As a result, more iron is released from enterocytes, hepatocytes, and reticuloendothelial cells (RES). When intracellular iron levels are low, iron regulatory protein (IRP) inhibits HIF-2α translation and diminishes hypoxia-induced erythropoiesis.

nisms at the sites of renal and hepatic EPO production. Keratinocyte-specific inactivation of HIF-1α, but not HIF-2α resulted in decreased renal EPO synthesis in response to hypoxia (9% O_2 for 14 h), which was associated with increased blood flow to the kidney. It was postulated that this caused increased renal oxygenation, leading to suppression of EPO synthesis (17). Conversely, constitutive activation of HIF signaling in keratinocytes produced NO-dependent dermal vasodilatation, which redirected blood flow to the skin and away from the kidneys and liver. This caused increased EPO production due to tissue hypoxia, which resulted in polycythemia. Although this study did not detect *EPO* mRNA in skin samples, a direct contribution of dermal EPO to total serum EPO cannot be completely ruled out, given more recent findings of EPO synthesis in hair follicles (16). Interestingly, experimental renal artery stenosis causes only a small linear rise in serum EPO, as opposed to large exponential increases in response to anemia (115). This is most likely due to the functional link between renal blood flow (RBF) and glomerular filtration rate (GFR). A decrease in GFR as a result of diminished RBF would reduce tubular workload and oxygen consumption and thus offset the negative effects of reduced RBF on tissue oxygenation. As a result, tissue oxygen levels are relatively independent of RBF alterations that occur within the physiological range (36). In light of these considerations, additional studies are warranted to determine whether changes in RBF alone can completely account for the observed changes in renal EPO synthesis in the skin knockout model.

Furthermore, the skin seems to have the ability to independently sense alterations in environmental O_2, to which it responds with changes in vascular tone, that is, vasoconstriction and a reduction in blood flow under conditions of acute hypoxia. This was demonstrated experimentally by delivering different oxygen concentrations to the lungs and skin of mice for a short period of time (5 h); that is, mice were breathing hypoxic gas while their body, i.e., skin, was exposed to either normoxic (21% O_2) or hypoxic gas (10% O_2). Mice breathing hypoxic gas with their skin exposed to normoxia had enhanced renal EPO responses, while the renal EPO response in mice whose lung and skin were exposed to the same degree of hypoxia was diminished. The latter response was associated with HIF-1-dependent dermal vasoconstriction and a relative increase in RBF.

Hypoxic Regulation of Iron Homeostasis and Bone Marrow Environment

The availability of sufficient amounts of iron is critically important for normal and stress-induced erythropoiesis. Serum iron levels depend on intestinal absorption from the diet, transport capacity in the blood, recycling of iron released from phagocytosed erythrocytes, and the release of iron from other tissue stores, such as the liver. Most of the iron used for normal erythropoiesis is recycled from phagocytosed erythrocytes (~20 mg /day) (for a review see Ref. 44). When erythropoiesis is stimulated by hypoxia, iron demand in the bone marrow increases. This necessitates increased intestinal iron uptake, an augmentation of serum iron binding capacity and enhanced mobilization of iron from internal stores. Therefore, it is not surprising that some key proteins involved in iron metabolism are oxygen regulated. Bona fide HIF targets involved in maintaining iron homeostasis include transferrin, which transports serum iron in its ferric form (Fe^{3+}) to target organs; its high-affinity receptor transferrin receptor-1 (TfR1) (85, 128, 149); ceruloplasmin, which oxidizes Fe^{2+} to Fe^{3+} and is also important for iron transport (110); the divalent metal transporter-1 (DMT1) (95, 143), which transports iron into the cytoplasm of cells; duodenal cytochrome *b* (DcytB) (143), which reduces ferric iron to its ferrous form (Fe^{2+}); and heme-oxygenase-1, which is important for the recycling of iron from phagocytosed erythrocytes (80).

The intestinal uptake of dietary iron is oxygen sensitive and is mediated by DMT1, which moves iron across the cell membrane in its ferrous form (Fig. 2). Before this can occur, it is necessary to reduce dietary iron from its ferric form to Fe^{2+}, which is accomplished by DcytB. HIF-2 has been shown to increase cellular expression of DMT1 and DcytB and to enhance intestinal iron uptake (Fig. 2) (95, 143). Following uptake by enterocytes, iron is then released into the circulation via ferroportin. Ferroportin is the only known membrane-bound iron transporter that exports iron out of cells. It is also expressed in hepatocytes and macrophages. The number of ferroportin molecules on the cell surface determines how much iron is exported, that is, how much iron is either taken up from diet, or released from hepatic or reticuloendothelial iron stores. Ferroportin surface expression is negatively regulated by hepcidin, a small polypeptide produced in the liver, which in the active form of 25 amino acids promotes its internalization and degradation (Fig. 2). In physiological settings, hepcidin production is suppressed in low-iron states and under conditions of increased erythropoietic activity. Its synthesis is increased in inflammatory states (interleukin-6 induces *hepcidin* transcription through JAK/STAT), lending support to the notion that hepcidin has a key role in the pathogenesis of the anemia of chronic disease (43). Chronically elevated serum hepcidin levels result in decreased release of iron from enterocytes, hepatocytes, or macrophages, leading to hypoferremia. Conversely, constitutively low expression of hepcidin is associated with the development of hemochromatosis (3).

In vitro and in vivo studies have demonstrated that hepcidin synthesis is hypoxia regulated (113). Several mechanistic explanations for the hypoxic suppression of hepcidin have been proposed, the simplest model being that HIF-2 increases renal EPO production and "erythropoietic drive," thereby indirectly suppressing hepcidin. It has been postulated that enhanced erythropoiesis produces a bone marrow-derived systemic signal that leads to hepcidin suppression in the liver, thus permitting increased ferroportin surface expression, which in turn increases iron availability for erythropoiesis. A candidate factor for such signal is growth and differentiation factor 15 (GDF15), an iron- and oxygen-regulated (HIF-independent) member of the TGF-β superfamily (77, 153). Studies with liver-specific knockout mice have also suggested that hepatic HIF-1 suppresses *hepcidin* directly through HRE-dependent mechanisms (123). However, this is debatable and experimental evidence exists that contradicts these findings (18, 157). Another model is based on the effects of hypoxia on iron-dependent regulatory pathways that control *hepcidin* transcription. Regulation of hepcidin expression in the liver involves HFE, a protein, which is mutated in patients with hereditary hemochromatosis, TfR1, and TfR2 (3, 45), and hemojuvelin (HJV), which acts as a coreceptor for bone morphogenetic

protein-6 (BMP6) and induces *hepcidin* transcription in a SMAD-dependent fashion (4, 8, 103). HIF-1 regulates furin, a proprotein convertase that cleaves HJV, generating soluble HJV (sHJV). sHJV reduces hepcidin synthesis by competing for BMP6 binding, thereby antagonizing signaling through membrane-bound HJV (102, 144).

To add more complexity to the cross talk between iron metabolism and HIF signaling, iron is necessary for HIF prolyl-4-hydroxylation, and its oxidation state and abundance affect the activity of PHDs and the efficiency by which normoxic HIF degradation occurs. An additional feedback loop has been proposed that links intracellular iron levels to HIF-2α translation and would limit HIF-2-induced EPO synthesis when intracellular iron levels are low. The 5′-untranslated region of *HIF-2α* mRNA contains an iron-regulatory element (IRE), which is a stem-loop structure that binds iron-regulatory protein (IRP) in the presence of reduced intracellular iron levels (Fig. 2) (109). IRPs (IRP1 and IRP2) function as intracellular iron sensors that control the expression of classic iron-sensitive genes, such as TfR1, ferritin, and DMT1 (132, 171). With regard to IRP1 regulation, iron is incorporated into an iron-sulfur cluster at the center of the protein and converts IRP1 to an enzyme with aconitase activity. In its aconitase form, IRP1 does not bind to the IRE. In contrast, IRP2 does not convert to an aconitase and is regulated via iron-dependent proteasomal degradation (109, 131, 155). Depending on the location of the IRE stem loop, the IRP/IRE complex either inhibits translation (5′-IRE), for example in the case of *ferritin*, or it stabilizes mRNAs, which is the case for *TfR1* where the IRE is located in the 3′-end (*TfR1* mRNA levels increase when intracellular iron is low). The IRE in *HIF-2α* is located in its 5′-untranslated region. When intracellular iron levels are low, the IRP/IRE complex inhibits translation of HIF-2α. This in turn limits EPO synthesis, adjusting the hypoxic inducibility of erythropoiesis to iron availability.

In addition to increasing iron availability, hypoxia promotes erythropoiesis through modulation of erythroid progenitor maturation and proliferation, and through its direct effects on the bone marrow microenvironment (1, 165). Hypoxia stimulates EPO receptor expression and regulates components of the hemoglobin synthesis pathway (26, 52, 84, 91, 167). It modulates the interaction of erythroid progenitors with other cell types, thereby affecting stem cell maintenance, lineage differentiation, and maturation. Recent studies have highlighted a role for endothelial HIF-2 in facilitating hypoxia-stimulated erythropoiesis (165). Mice with globally reduced HIF-2α expression developed a defect in erythroid maturation (mutant mice had increased numbers of immature erythroblasts), which appeared to depend on HIF-2-mediated expression of vascular cell adhesion molecule (VCAM)-1, an integrin receptor that binds very late antigen-4 (VLA4) on erythroblasts supporting erythroid maturation. This finding together with its emerging role in the regulation of iron metabolism underscore the notion that HIF-2 has a central function in oxygen-regulated erythropoiesis, which extends beyond the induction of EPO synthesis.

Genetic Alterations in VHL/HIF/PHD Pathway that Lead to Erythrocytosis

Clinically, even mild to moderate perturbations in the VHL/HIF/PHD oxygen-sensing pathway lead to the development of erythrocytosis. Whereas serum EPO levels are suppressed in primary erythrocytosis, where the molecular defects reside within the erythroid progenitors themselves (47, 81), secondary forms result from increased EPO production. Secondary erythrocytosis occurs in response to chronic hypoxic conditions, such as chronic obstructive pulmonary disease, right-to-left cardiac shunts, and high altitude, or can be due to EPO-producing tumors or genetic alterations in the molecular machinery that controls EPO synthesis.

Abnormalities in the VHL/HIF/PHD pathway were initially observed in patients with Chuvash polycythemia. Chuvash polycythemia is a rare autosomal recessive form of secondary erythrocytosis, endemic to Chuvashia, a republic in central European Russia, and is associated with a homozygous mutation in the VHL tumor suppressor at codon 200 [C598T ⇒ R200W; compound heterozygotes have also been described (5, 83, 120)]. More recently, this and similar *VHL* mutations have also been found in patients with idiopathic erythrocytosis who are ethnically distinct from Chuvash patients (23, 122). Codon 200 is located in the C terminus of pVHL and lies outside the central binding groove for hydroxylated HIF-α (β-domain core) and the helical α-domain (amino acid residues 157–189), which interacts with elongin C (147). Genetic alterations in these two core regions are strongly associated with the development of VHL disease, an inherited autosomal dominant tumor syndrome, where individuals who carry one defective *VHL* allele in their germ line are predisposed to the development of highly vascularized tumors, which include renal cell carcinoma, hemangioblastomas of the CNS and retina, and pheochromocytomas (86). Chuvash patients, who are homozygous for the R200W allele, are not predisposed to the development of these tumors. The pVHL-R200W mutation targets HIF-1α more efficiently than HIF-2α, while the overall ability to capture hydroxylated HIF-α for proteasomal degradation is impaired. This is most likely due to changes in protein stability or conformation that could impinge on the pVHL-HIF-α interaction (51). Although individuals with Chuvash polycythemia are not prone to tumor development, they suffer from premature morbidity and mortality due to pulmonary hypertension, cerebrovascular accidents, and vertebral hemangiomas (21, 46). Evaluation of cardiopulmonary function in a small group of Chuvash patients revealed significant abnormalities in respiratory and pulmonary vascular regulation at baseline and in response to hypoxia. Basal ventilation and pulmonary vascular tone were elevated, and increases in heart rate and ventilation, as well as pulmonary vasoconstrictive responses to mild or moderate hypoxia, were considerably enhanced, indicating that tight regulation of the VHL/HIF/PHD axis is required for normal cardiopulmonary physiology (145).

The link between *VHL* mutations and familial erythrocytosis has stimulated further search for genetic defects in other components of the VHL/HIF/PHD oxygen-sensing pathway. This has led to the identification of families with heterozygous mutations in HIF-2α or PHD2. Interestingly, mutations in HIF-1α have not been found to date, underscoring the importance of HIF-2 in the regulation of EPO synthesis in humans. Point mutations that have been found in HIF-2α convert glycine to either tryptophan or arginine at position 537, and methionine to valine at position 535 (117, 119). These codons are close to proline residue 531, which is one of the main hydroxylation sites for PHDs (the other major hydroxylation

site is proline 405). Biochemical analysis demonstrated that the G537W change impaired recognition, as well as hydroxylation of HIF-2α by PHD2, resulting in a partial gain of function. Conversely, several PHD2 mutations have been identified. These are associated with diminished prolyl-4-hydroxylase activity and result in increased RBC production associated with inappropriately normal serum EPO levels (serum EPO levels should be completely suppressed in the setting of elevated RBC counts) (118, 121). PHD2 mutations include an amino acid change at position 317, which is in the direct vicinity of two iron-chelating residues (position 313 and 315) that are critical for PHD catalytic activity.

It is important to point out in this context that only ~5% of renal cancers are associated with erythrocytosis (68). Most of these cancers are *VHL* deficient and stabilize HIF-1α and HIF-2α constitutively, resulting in increased expression of many HIF-regulated genes; however, *EPO* transcription is repressed, despite the very high levels of HIF-2 activity. The molecular basis for EPO repression in this setting is not clear but may involve certain transcriptional regulators, such as GATA-2 and GATA-3 (114).

VHL/HIF/PHD Axis as a Therapeutic Target for Treatment of Anemia

Miyake and colleagues (106) purified EPO from large quantities of urine obtained from severely anemic patients. This permitted determination of its protein sequence and subsequent cloning of its cDNA (59, 82). Recombinant EPO was approved by the US Food and Drug Administration (FDA) for clinical use in renal patients in 1989 and has since then become the standard therapy for renal and cancer-associated anemia (FDA approval in 1993). EPO therapy eliminates the need for red cell transfusions, improves cardiovascular function and cognitive abilities, and over the last 20 years has transformed the lives of millions of patients. Despite its clinical effectiveness, recent randomized controlled trials and meta-analyses of clinical trials have reassessed the risks and benefits of recombinant EPO use in predialysis and cancer patients and have raised serious safety concerns, resulting in a black box warning issued by the FDA. These studies indicate that there is increased risk of serious cardiovascular complications or adverse composite outcomes in kidney disease when higher than recommended hemoglobin levels (<13 g/dl) are achieved and that use of recombinant EPO in cancer patients can promote tumor growth and increase overall mortality (for recent reviews on this topic, see Refs 14 and 146). Although the molecular mechanisms that underlie these clinical observations are not understood, patient safety concerns have prompted clinicians to reevaluate their EPO-prescribing practices. With regard to renal anemia, which is associated with increased risk of cardiovascular events, a major current debate revolves around the issue of whether and when predialysis patients should receive recombinant EPO and which hemoglobin levels should be targeted, as the overall clinical benefits of EPO therapy in moderately anemic patients with diabetes and chronic kidney disease are uncertain (124). Nevertheless, the clinical success of EPO therapy has been a major incentive for the design of new erythropoiesis-stimulating agents, including longer-acting versions of recombinant EPO, and has stimulated the development of novel therapeutic strategies aimed at boosting synthesis of endogenous EPO (87).

Pharmacological inactivation of PHD enzymes using 2-oxoglutarate analogs increases serum EPO levels in animal models and in humans and has the potential to benefit patients with anemia resulting from inadequate EPO production (11, 54). In vitro and in vivo studies have indicated that individual PHDs are functionally different with regard to EPO synthesis and that pharmacological targeting of specific PHDs may improve specificity. Inducible, global deletion of PHD2 in adult mice resulted in severe erythrocytosis (HCT values >80%) and multiple other organ pathologies, resembling phenotypes that are associated with pVHL inactivation, in particular when PHD3 is deleted simultaneously (104, 105, 150–152). Erythrocytosis in conditional PHD2 knockout mice was associated with a dramatic increase in renal *EPO* mRNA expression. PHD1- and PHD3-deficient mice, which in contrast to PHD2 germ line knockout mice survive into adulthood, developed erythrocytosis only when both enzymes were inactivated simultaneously, as shown in one report (150), while erythrocytosis was not found in another study (reported as unpublished data in Ref. 104). In PHD1/PHD3 double knockout mice that developed erythrocytosis, hematocrits were moderately increased to 67% compared with 53% in controls, with the source of EPO being the liver and not the kidney, as hepatic and not renal *EPO* mRNA levels were elevated (150). Surprisingly, serum EPO levels were suppressed (150). Taken together, these in vivo findings illustrate that among the PHDs, PHD2 is the most critical regulator of HIF proteolysis under normoxic conditions and that complete inactivation of PHD2 results in a major disruption of oxygen sensing, which is associated with a dramatic upregulation of HIF target genes that leads to embryonic death (152). PHD1 and PHD3, on the other hand, play distinct roles in oxygen-dependent HIF proteolysis and may be better suited as drug targets with regard to boosting EPO synthesis. Functional diversity between individual PHDs is expected, because of differences in cellular localization, hypoxia inducibility, and biochemical behavior. For example, only PHD2 and PHD3 are hypoxia inducible, and PHD3 does not hydroxylate proline 402 in HIF-1α, which, although not directly proven, is most likely also the case for the corresponding residue in HIF-2α (for a review, see Refs. 19 and 65). Furthermore, PHD1 and PHD3 appear to have a preference for HIF-2α in vitro and in vivo (6, 150). PHD1/PHD3−/− mice upregulate HIF-2α but not HIF-1α in the liver, while PHD2 inactivation increases both HIFs (104, 150). A preference of PHD2 for HIF-1α, however, has been suggested by in vitro studies (6).

Pharmacological PHD inhibition with the goal of boosting endogenous EPO synthesis may be an attractive alternative to therapy with recombinant EPO because of its potential beneficial effects on iron homeostasis. However, HIF transcription factors regulate a multitude of biological processes, and intermittent HIF activation over prolonged periods of time may lead to profound changes in cellular metabolism, growth, and differentiation, warranting words of caution. Whether long-term or short-term use of HIF-stabilizing compounds for the treatment of anemia is safe will have to be carefully evaluated.

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