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Nonrenal regulation of EPO synthesis

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Erythropoietin (EPO) is a circulating glycoprotein hormone whose principal function is thought to be red blood cell production. It is a classic example of a hypoxia-inducible gene, and studies of the induction of EPO synthesis by low oxygen led to the discovery of a widespread system of hypoxia-inducible transcription factors. Tissue-specific expression of the EPO gene is tightly controlled, and in the adult organism the kidney produces around 90% of systemic EPO. Before birth, the liver is the main site of EPO production; factors contributing to the liver-to-kidney switch are still elusive, but may provide clues to the tissue-specificity of EPO gene expression. EPO has also been detected in non-erythropoietic tissues such as the brain, where it is suggested to exert local protective effects. Apart from classical ways of regulating renal EPO during hypoxia and anemia, novel pathways have been discovered that demonstrate that other organ systems in the adult might not only be important for the production of EPO but also for modulating the hypoxic EPO response. Knowledge of the molecular bases of these non-renal pathways will eventually help to develop pharmacological strategies to induce endogenous EPO production when the main source, the kidney, is significantly impaired. This review will provide an overview of the molecular aspects of *EPO* gene regulation by hypoxia-inducible transcription factors and of the tissue-specific regulation of EPO production in adult mammals. Insights into the biology of EPO production in genetically modified animals, with an emphasis on recent advances in the understanding of non-renal EPO regulation, will be discussed.

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REGULATION OF EPO BY HYPOXIA AND HYPOXIA-INDUCIBLE TRANSCRIPTION FACTORS

That a humoral factor might exist that controls erythropoiesis is a concept that dates back more than 100 years. In 1906, Carnot and Deflandre[1] observed a rise in the number of red blood cells in animals when injected with serum from anemic donor animals. Before that observation, in 1890, Viault described the acute effect of hypoxemia on erythropoiesis, in the first linkage of hypoxia and red blood cell production. Subsequently, erythropoietin (EPO) was purified in 1977, the *EPO* gene was cloned 1985, and the *EPO* receptor (*EPOR*) was cloned in 1989 (see the review by Jelkmann[2]). Various studies *in vivo* showed that EPO mRNA levels could be induced by orders of magnitude by anemia and subsequent tissue hypoxia.[3] One clue to this oxygen-dependent regulation seemed to be inherent in the structure of the *EPO* gene: key regulatory elements, such as the promoter, the first intron and parts of the 3′-flanking region are highly homologous between species.[4] This indicated that both the capability of certain tissues to express EPO, and the regulation of *EPO* gene expression are most likely conserved. As EPO is not stored, increased production correlates with an increase of mRNA through increased transcription and increased stability. Detailed characterization of the 3′-regulatory region of the *EPO* gene defined it as critical for hypoxic induction[5] and led to the identification of the transcription factor, hypoxia-inducible transcription factor (HIF).[6]

HIF is a heterodimeric DNA-binding complex composed of two basic helix-loop-helix proteins of the PAS family: the constitutive non-oxygen-responsive subunit HIF-1β (also termed ARNT) and one of either the hypoxia-inducible α-subunits, HIF-1α or HIF-2α. HIF-α subunits are rapidly degraded in normoxia but highly inducible by hypoxia (for review of HIF-α function, see the review by Wenger[7]). The interface between oxygen and the HIF-α subunit is provided by distinct enzymatic reactions: the hydroxylation of two prolyl residues in the oxygen-dependent degradation domain of the α-subunits. This oxygen-dependent hydroxylation regulates the interaction with the von Hippel–Lindau tumor suppressor protein, which targets HIF-α for proteolysis by the ubiquitin–proteasome pathway.[8] Under hypoxic conditions, prolyl hydroxylation is suppressed, HIF-α protein escapes proteasomal destruction, accumulates, and translocates to the nucleus. There, the α/β-heterodimeric transactivating complex HIF binds to promoter or enhancer sequences of target genes that

control important biological functions, such as glycolysis, angiogenesis, and erythropoiesis.

The HIF-modifying enzymes are termed prolyl hydroxylase domain (PHD) enzymes (PHD1–3) (see the review by Schofield and Ratcliffe[9]). The PHDs are nonheme Fe (II) and 2-oxoglutarate-dependent dioxygenases that split molecular oxygen; thus, in the absence of oxygen, the HIF prolines remain unmodified. The effects of hypoxia can be mimicked by iron chelation, use of 2-oxoglutarate analogs, or substitution of Fe (II) by metal ions, such as cobalt. Therefore, inhibition of the PHDs represents an attractive target for pharmacological manipulation of the HIF pathway, as discussed below.

In contrast to regulation of HIF-α stability by the PHDs, transcriptional activity is further regulated by the hydroxylation of an asparagine residue. The hydroxylation reaction is carried out by an asparaginyl hydroxylase, termed factor inhibiting HIF (FIH), and this modification prevents interaction of HIF-α with the transcriptional coactivator p300.[10]

The transcription factors HIF-1α and HIF-2α share significant sequence homology and are both regulated post-translationally by protein degradation. HIF-2α, originally termed endothelial PAS domain protein 1 (EPAS1) because of its expression in endothelial cells,[11] exhibits a more restricted expression pattern than HIF-1α.[12] Although both HIF-α-subunits are able to bind the consensus HRE, they seem to regulate a different set of target genes depending on the cellular context and the oxygen concentration. The factors and molecular mechanisms that potentially determine this isoform-specific target gene selectivity remain poorly defined (see the review by Patel and Simon[13]). Interestingly, as discussed below, although HIF-1α was originally identified to bind to the HRE in the 3′-enhancer of the EPO gene,[6] there is now considerable evidence, both *in vitro* and *in vivo*, that HIF-2α is the main HIF-α-subunit controlling EPO gene expression (Figure 1).

EPO EXPRESSION IN MAMMALIAN TISSUE: RENAL AND NONRENAL TISSUE

Although far from being completely elucidated, the mechanisms underlying the hypoxia-inducible upregulation of the *EPO* gene are understood in considerable detail. The regulatory pathways determining tissue specificity of EPO expression, however, are much more opaque at this point in history.

EPO is expressed in the kidney, liver, brain, spleen, lung, and testis (see the review by Fandrey[14]). No structural or functional differences of EPO coming from these different sources have been described. The kidney is the major site of EPO production in adult animals. In most patients with significantly impaired renal function, EPO production is substantially reduced. Studies in adult rats conducted as early as 1957 showed that after nephrectomy, plasma EPO levels decline sharply and can no longer be induced by phlebotomy.[15] Attempts to identify EPO-producing cells in the kidney yielded contradictory results initially, but two important studies provided convincing evidence that the renal EPO-producing cells are peritubular fibroblast-like type-1 interstitial cells, located in the renal cortex and outer medulla.[16,17] Immunohistochemical studies indicated that

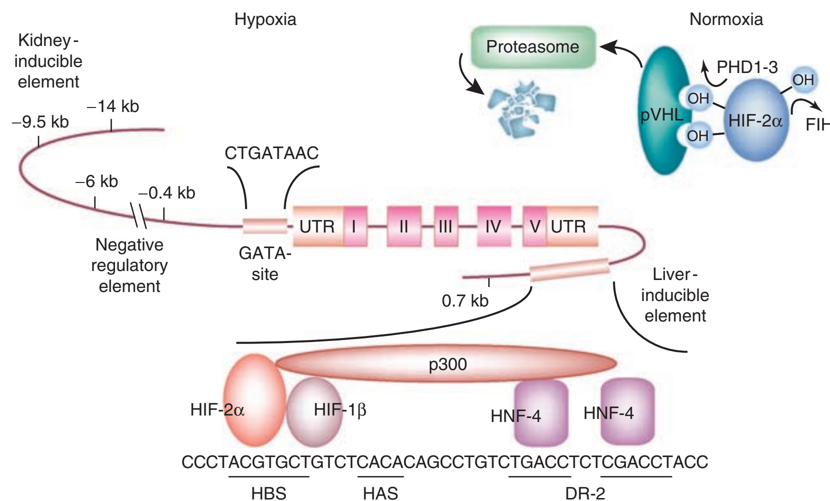

Figure 1 | Schematic representation of the *EPO* gene, its important regulatory sequences, and the factors controlling hypoxic EPO induction: exons are depicted as red numbered boxes, and UTRs are depicted as open boxes. The kidney-inducible element lies far upstream (between −9.5 and −14 kb). A negative regulatory element preventing ectopic expression lies nearer, between −6 and −0.4 kb. The functional GATA site, which has been mapped to the minimal promoter, is expanded. Parts of the 3′-liver-inducible element are shown in detail: the hypoxia response element (HRE) with the HIF-α-binding site (HBS) and the CACA-repeat (HIF ancillary sequence), which is necessary for full hypoxic induction. The nuclear receptor half-site (DR-2), where hepatic nuclear factor 4 (HNF4) can bind, is adjacent. The induction of EPO in hypoxia is shown: HIF-2α is stabilized, can dimerize with HIF-1β, and translocates to the nucleus where it binds to the HRE in the 3′-enhancer. P300 is recruited for full activity (and potential interaction with the basal transcriptional machinery in the minimal promoter). Upper right: in normoxia, HIF-2α is hydroxylated by prolyl hyroxylases (PHD) and thus recognized by von Hippel–Lindau tumor suppressor protein, which serves as a recognition component of a ubiquitin ligase that targets HIF-α for degradation by the proteasome. HIF-α is also hydroxylated by factor-inhibiting HIF (FIH), which prevents binding of p300 and reduces transcriptional activity.

HIF-2α might be the dominant subunit driving hypoxic EPO expression in these cells.[18] Unfortunately, all efforts to generate EPO-expressing renal cell lines for further detailed studies have been unsuccessful thus far. Therefore, hepatoma cell lines are continued to be used to investigate the molecular bases of EPO regulation. In those cells, HIF-2α was found to be the HIF-α isoform predominantly responsible for EPO regulation.[19]

The kidney is not the only major EPO-producing tissue in adults (Figure 2); in fact, early estimates indicated that around 10% of the circulating EPO originates from nonrenal tissue.[20] During fetal development, the main source of EPO is the liver rather than the kidney. A comprehensive study in sheep showed that the fetal liver produces the majority of prenatal EPO and that a liver-to-kidney shift toward renal EPO production occurs in late gestation.[21] This was later confirmed for other species as well, with differences in the timing of the onset[22] and in the remaining contribution of the adult liver to the overall EPO production. Efforts to quantify the contribution of hepatic EPO to the total production yielded high estimates in rats: around 40%, with potential to increase the proportion to 80% after nephrectomy.[23] More intriguing than the actual percentage is the fact that nonrenal EPO is still produced in a significant amount in adult mammals. The biological principle behind that observation, and why extrarenal sites cannot compensate for the decreased levels of EPO in kidney disease is, however, still not resolved.

In contrast to the kidney, two hepatic cell types are capable of producing EPO: hepatocytes and Ito-cells, as determined by *in situ* hybridization and the use of transgenic animals.[24,25] Another interesting and not yet resolved difference between renal and hepatic EPO regulation is the fact that hepatocytes seem to be capable of increasing the EPO expression at a cellular level, whereas in the kidney, upregulation is achieved by increased recruitment of a larger number of cells with a fixed amount of EPO mRNA.[24,26]

The molecular mechanisms underlying the liver-to-kidney switch are still obscure. The differential timing between species, however, suggests that it might not be determined by physiological changes in circulation or oxygenation during birth. Studies in transgenic animals containing a copy of the human *EPO* gene, including large portions of adjacent regulatory regions, were performed to identify sequences necessary for tissue-specific *EPO* regulation (Figure 1). These experiments suggested that regulatory elements for hypoxic induction in the liver are between −0.4 kb upstream and 0.7 kb downstream of the *EPO* gene-coding sequences. Apart

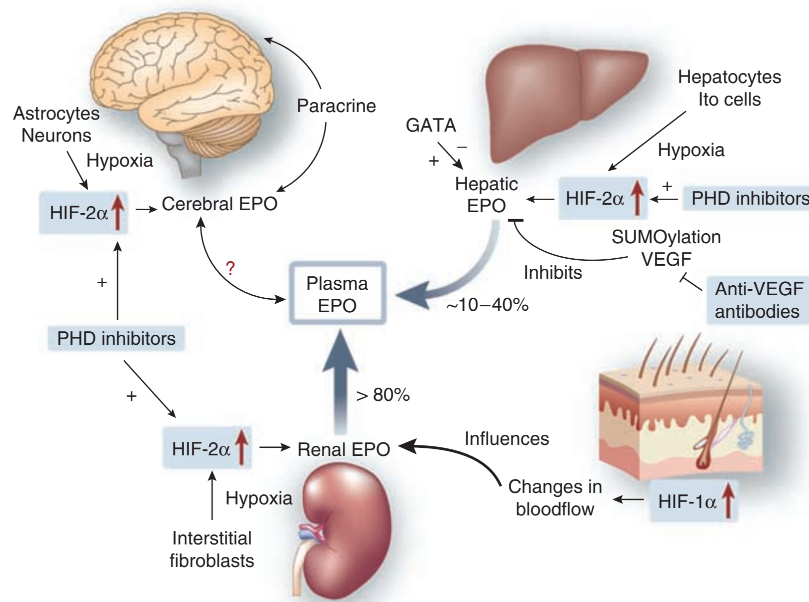

Figure 2 | The three main sites of EPO production in mammals: the central nervous system, the liver, and the kidney. In the central nervous system, EPO produced by astrocytes and neurons is suggested to exert paracrine neuroprotective effects. Controversy exists as to how much EPO can cross the intact blood–brain barrier in both ways. The majority of the plasma EPO in adult mammals is produced by interstitial fibroblasts in the kidneys. Renal EPO expression has been shown to be influenced by the hypoxic response of the skin, involving HIF-1α (rather than HIF-2α) and, during the acute response, HIF-independent pathways. The contribution of the adult liver to the total EPO production is difficult to quantify: estimates show species differences but can range up to ~ 40% of the total EPO. Hepatic EPO is produced in Ito-cells and in hepatocytes, where HIF-2α is the main regulator of hypoxic EPO induction. GATA transcription factors have been described to negatively, but also positively, influence hepatic EPO expression. Novel inhibitory pathways of hepatic EPO expression are described for VEGF and SUMOylation of HIF-α. Given the central role of HIF in the EPO regulation, targeted pharmacological induction of HIF through PHD-inhibitors can potentially induce endogenous EPO in the kidney (diseased or healthy) as well as in the two other nonrenal sites.

from the HIF-binding site, the 3′-enhancer contains a direct repeat DR-2 element, which is the binding site for nuclear hormone receptor family members. Hepatic nuclear factor-4 binding to this site is required for full functionality.[27] Moreover, a repressing element preventing promiscuous expression of *EPO* is present between −0.4 and −6 kb upstream of the gene[28] and an element directing expression to the kidney lies even further upstream, between −9.5 and −14 kb.[29] Specific *trans*-acting factors or the precise sequences of the *cis*-acting elements are, however, still elusive. Recently, certain GATA transcription factors that bind to a GATA site in the minimal promoter have been implicated in the repression of hepatic EPO *in vitro* and *in vivo*.[30] In contrast, GATA-4 has been shown to be critical for *EPO* transcription *in vitro*, and expression levels of GATA-4 are high in fetal liver.[31] Tissue-specific expression of different GATA factors in a defined temporal–spatial pattern might, therefore, be involved in the liver-to-kidney switch. Taken together, no clear data exist to date as to why EPO production is switched from the liver to the kidney, which factors are involved, and whether this switch is irreversible. Basal and inducible hepatic EPO production persists after the switch, although at a much lower amplitude than before. If extrarenal sources of endogenous EPO are to be exploited in the future, it will be of great importance to understand the molecular mechanisms behind this switch in more detail.

The third main tissue known to express *EPO* and *EPOR* is the central nervous system (Figure 2). EPO mRNA is constitutively expressed in the cortex and hippocampus of murine and human brains (see the review by Marti[32]). On a cellular level, there is evidence that neurons and astrocytes are the main source of cerebral EPO.[33,34] Cerebral EPO is separated from the systemic circulation by the blood–brain barrier. It has therefore been suggested that the physiological role of EPO is not important for erythropoiesis; however, a contribution of cerebral EPO for other systemic responses has not been ruled out either. Controversy exists as to whether recombinant EPO (or endogenous EPO) can actually cross the intact blood–brain barrier, as suggested by Brines *et al.*[35] Others have reported that in the absence of ischemic or traumatic damage, only high does of EPO seem to be able to cross the adult blood–brain barrier.[36] In summary, although knowledge is still incomplete, the proposed function of cerebral EPO is more paracrine then endocrine: it is critical for brain development, acts as an endogenous protective agent against ischemia and hypoxia, and as a neurotrophic factor promoting neuronal survival (see the reviews by Marti[32] and Dame *et al.*[37]). It is interesting to note that the EPO production in the brain seems to be constant, and no significant changes in EPO expression have been reported for cerebral EPO during different developmental stages.[37] Thus, in contrast to the liver, putative repressors might act differently in the brain, or may not exist. As for other putative tissue-specific regulatory regions: the determinants of continuous CNS EPO expression remain unknown.

EPO mRNA expression has been detected in the lung, spleen, testis, and placenta; however, the levels of expression are low in the lung and spleen.[23,38] The role of these organs for local and systemic EPO homeostasis is therefore still largely obscure. It is interesting to note, however, that in the testis, as in the brain, EPO is separated from the systemic circulation by a blood–testis barrier, potentially obstructing free diffusion of the hormone and indicating a paracrine role in this organ as well.

INSIGHTS INTO NONRENAL EPO EXPRESSION BY THE USE OF GENETICALLY MODIFIED ANIMALS

When kidneys fail to produce substantial levels of EPO, adult hepatic EPO expression does not compensate for the loss of renal EPO (for example, as observed in uremic rats[23]). Thus, repression of (or the inability to upregulate) the *EPO* gene in the adult liver is still dominant even in situations with increased systemic demand for the hormone after the fetal liver-to-kidney switch. By elucidating detailed mechanisms of hepatic EPO gene regulation, researchers seek to find potential targets for therapeutic intervention.

As pointed out, GATA transcription factors may play a role in hepatic EPO repression.[30] GATA inhibitors have already been shown to restore erythropoiesis in a mouse model of anemia of chronic disease,[39] but it has not been shown that these compounds are able to turn on hepatic EPO synthesis specifically when the kidneys are unable to produce EPO.

The von Hippel–Lindau gene (*VHL*), the negative regulator of both HIF-α isoforms, is frequently mutated in renal cancer (see the review by Kaelin[40]). Loss of *VHL* leads to HIF-α stabilization, and occasionally to paraneoplastic polycythemia, due to excessive EPO production of carcinomatous renal tissue.[41] Studies with animals with Cre-loxP-mediated deletion of *VHL* in hepatocytes have shown that chronic inactivation of *VHL* stabilizes HIF-α and is capable of overriding hepatic repression of the EPO gene.[42] Erythrocytosis was not rescued in double-knockouts of *VHL* and *HIF1A*, suggesting that HIF-2α is a primary regulator of this effect. This was subsequently shown by the same group,[43] thus the bulk of evidence now suggests that, *in vivo*, HIF-2α regulates hepatic hypoxic EPO induction. Obviously, loss of *VHL* results in various other pathologies, some related to HIF-α activation, whereas others are HIF-α independent. But these studies have shown that through chronic stimulation of the HIF-α pathway, hepatic EPO expression was inducible, overriding other potential tissue-specific repressors. Of note, loss of *VHL* in a cell type previously not capable of expressing EPO is not sufficient to turn on EPO production, as shown *in vivo* in renal proximal tubular cells.[44] An important complication of the studies mentioned is the uncertain extent of the described phenotype caused by the early developmental loss of *VHL* in hepatocytes. Therefore, studies with inducible cre-mediated deletion of *VHL* in adult mouse hepatocytes are clearly needed.

For the kidney, the only published viable global *EPAS1* (HIF-2α) knockout mouse suffers from pancytopenia, among other pathologies; this is the result of reduced renal EPO production.[45] The extent of impairment of hepatic and cerebral EPO production in those mice was not examined. In line with hypoxic renal and hepatic EPO induction, the upregulation of EPO in astrocytes *in vitro* is also dependent on HIF-2α.[46]

NOVEL FINDINGS OF NONRENAL EPO PRODUCTION

Most recent research on EPO expression has focused on the VHL/HIF-α pathway in EPO-producing tissue. However, the spectrum of different levels of EPO regulation became broader recently with several unexpected findings.

An intriguing and unexpected link between the proangiogenic factor VEGF and hepatic EPO expression was reported by Tam *et al.*[47] Stringent hepatic and systemic inhibition of VEGF by various means, as well as conditional deletion of the *VEGF* gene in the liver, induced hepatic EPO expression that increased the hematocrit and led to polycythemia in rodents and, to a lesser extent, in primates (Figure 2). A VEGF-dependent repression of hepatocytic EPO was suggested as the mechanism, which involved sinusoidal endothelial cells and was potentially mediated by VEGF receptor-2 stimulation. The factors that induce EPO in the absence of VEGF are still undefined. HIF-1α did not participate as expected; however, the extent of HIF-2α's involvement in this novel pathway remains to be clarified. Intriguingly, deletion of *VHL* in hepatocytes increased hepatic VEGF expression,[42] but EPO was still upregulated by HIF-2α stabilization, indicating that the VHL/HIF-α axis might be dominant over this novel VEGF-mediated pathway.

A recent report showed yet another level of regulation of the hepatic EPO expression pathway by linking SUMOylation and HIF-α. SUMO is a small, ubiquitin-like modifier system that alters protein–protein interaction. HIF-α can also be SUMOylated; however, conflicting data exist as to whether this leads to increased or decreased stability of HIF-α.[48,49] SENP1 is a SUMO-specific isopeptidase that removes SUMO from HIF-1α, and interestingly, *SENP1* knockout animals die *in utero* due to the lack of hepatic erythropoiesis.[49] In this report, de-SUMOylation seemed to be necessary for full HIF-α acitivity, with an important role in prenatal hepatic EPO production. It remains to be determined whether the SUMO system participates in the tissue-specific regulation of EPO, and whether SUMOylation of HIF-2α is comparable with that of HIF-1α.

Renal EPO expression can also be influenced by nonrenal triggers. In work first published in 2005, a humoral factor released from the brain stem after the increase of cerebral pressure was postulated to trigger renal EPO expression.[50] The identity of this factor is still undetermined. More recently, the skin was described to play a previously unappreciated role in oxygen sensing and EPO response involving HIFs:[51] mice with a skin-specific deletion of *HIF1A*, but not *EPAS1* (HIF-2α), had diminished renal EPO mRNA induction and reduced systemic plasma EPO levels after hypoxic exposure. Skin-specific chronic activation of HIF-α by loss of *VHL* resulted in increased EPO plasma levels and polycythemia. No EPO production was detected in skin, but other HIF-α target genes, such as VEGF and inducible NO-synthase, were upregulated. The resulting excessive vasculature and high levels of the vasodilator NO triggered alterations in blood flow, to an extent such that hepatic EPO production was induced. Furthermore, by exposure of the skin of mice to normoxia while breathing hypoxic air and with pharmacological application of NO donors to the skin, it was determined that the skin of wild-type mice has the ability to significantly influence blood flow during acute and chronic hypoxia, and subsequently alter systemic EPO response (Figure 2). This novel signaling pathway established a link between cutaneous oxygen sensing and the systemic erythropoietic response.

THERAPEUTICAL IMPLICATIONS FOR NONRENAL EPO EXPRESSION

Emerging novel therapeutical approaches currently under investigation focus mainly on means to induce endogenous EPO expression, in contrast to administration of exogenous recombinant EPO products. Knowledge of the key enzymes of the oxygen-dependent degradation step of the HIF-α subunits has directed the focus to compounds that can inhibit these HIF-α PHDs. Several of such inhibiting compounds are now available, and animal studies have established the proof of concept that endogenous hepatic EPO can be upregulated by pharmacological stabilization of HIF-α subunits in nephrectomized mice.[52] The first human trials using HIF-stabilizing compounds have been conducted in anephric patients, and have shown that hepatic EPO can be upregulated in adult human subjects.[53] In another subgroup of this study, the diseased kidney could also be stimulated to produce more EPO. The same therapeutic principle has also been applied to induce cerebral EPO *in vivo* in a model of focal ischemia.[54] It has to be pointed out, however, that tailored substances are clearly needed, and caution has to be exercised in using these agents: HIF-α stabilization affects many other pathways apart from EPO regulation. Several might be desired, as HIF-α target genes are involved in iron metabolism,[55] and thus activation of HIF-α also regulates these factors synergistically for sustained erythropoiesis. Many other HIF-α targets have been shown to be protective,[56] but others clearly need to be avoided, as HIF-α signaling is also activated in neoangiogenesis, cancer, and metastasis (see the review by Semenza[57]).

In contrast to the primary therapeutic objective of inducing hepatic EPO expression, the use of anti-VEGF antibodies might also potentially enhance hepatic erythropoiesis.[47] However, no cases of polycythemia under VEGF-blocking antibody treatment in humans have been reported so far, possibly because the necessarily high degree of VEGF blockade is not achieved in clinical settings.

The observation that the skin plays a role in the systemic hypoxic response[51] indicates that this pathway might be used therapeutically. Mice treated with NO-donors through the skin showed an increased EPO response; however, as this is achieved by blood-flow alterations, dose escalation studies have to carefully weigh the benefits of this treatment in terms of stimulation of endogenous erythropoiesis, against the side effects of NO-donor treatment.

CONCLUSIONS AND OUTLOOK

The regulation of erythropoiesis mainly relies on modulation of *EPO* gene expression in response to tissue hypoxia. The ability to produce substantial amounts of EPO is highly restricted to certain tissues: the kidney, liver, and brain (Figure 2). The regulatory network controlling this spectrum of EPO expression is still incompletely understood: What precisely are the relative contributions of extrarenal EPO production, hepatic and potentially cerebral, to the systemic erythropoietic response? Is hepatic EPO production after the switch just a developmental artifact, or does it serve an important physiological purpose? How definite is repression of EPO expression, and can it be safely reversed and modulated pharmacologically?

Even if we do not have conclusive answers to these and other questions raised above, the current understanding of the diverse pathways influencing EPO gene expression offer exciting prospects for the future. The liver as a source of endogenous EPO production for systemic responses is quite attractive, as studies have shown that this tissue source can be stimulated pharmacologically by activation of HIF-α. In the central nervous system, pharmacological upregulation of endogenous EPO might augment the protective response during stroke. These are only a few of the therapeutic approaches imaginable if it becomes feasible to further specifically direct HIF-α stabilization, and subsequently target gene induction to nonrenal tissues. It will be intriguing to see if tailored responses can be achieved that are beneficial in clinical settings.

DISCLOSURE

The authors declared no competing interests.

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