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Anaemia in kidney disease: harnessing hypoxia responses for therapy

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Abstract

Improved understanding of the oxygen-dependent regulation of erythropoiesis has provided new insights into the pathogenesis of anaemia associated with renal failure and has led to the development of novel therapeutic agents for its treatment. Hypoxia-inducible factor (HIF)-2 is a key regulator of erythropoiesis and iron metabolism. HIF-2 is activated by hypoxic conditions and controls the production of erythropoietin by renal peritubular interstitial fibroblast-like cells and hepatocytes. In anaemia associated with renal disease, erythropoiesis is suppressed due to inadequate erythropoietin production in the kidney, inflammation and iron deficiency; however, pharmacologic agents that activate the HIF axis could provide a physiologic approach to the treatment of renal anaemia by mimicking hypoxia responses that coordinate erythropoiesis with iron metabolism. This Review discusses the functional inter-relationships between erythropoietin, iron and inflammatory mediators under physiologic conditions and in relation to the pathogenesis of renal anaemia, as well as recent insights into the molecular and cellular basis of erythropoietin production in the kidney. It furthermore provides a detailed overview of current clinical experience with pharmacologic activators of HIF signalling as a novel comprehensive and physiologic approach to the treatment of anaemia.

Introduction

Anaemia is a clinical hallmark of advanced kidney disease that is characterized by decreased levels of haemoglobin and haematocrit, and decreased numbers of circulating erythrocytes. Decreased erythrocyte numbers occur when fewer new erythrocytes enter the circulation than are lost or destroyed. In anaemia associated with renal failure, this decrease is rarely caused directly by increased rates of erythrocyte loss or destruction. Rather, it is caused by insufficient erythropoiesis to replace the 2×10^{11} senescent erythrocytes that are removed

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Competing interests

M.J.K. is a consultant for Keryx Biopharmaceuticals, Inc. V.H.H. has received honoraria from Daiichi Sankyo and serves on the Scientific Advisory Board of Akebia Therapeutics, a company that develops prolyl-4-hydroxylase inhibitors for the treatment of renal anaemia.

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from the circulation on a daily basis under physiologic conditions. Haemoglobin, the major iron-containing erythrocyte protein, transports oxygen from the lungs to other tissues to enable cellular respiration. In anaemia, decreased oxygen transport causes tissue hypoxia, which through activation of the hypoxia-inducible factor (HIF) system stimulates the production of erythropoietin, the principal hormonal regulator of erythropoiesis. This classic hypoxia response is greatly impaired in patients with kidney failure, as the kidney is the major site of erythropoietin production under physiologic and hypoxic conditions. Although therapy with recombinant human erythropoietin (rhEPO) alleviates renal erythropoietin deficiency, this approach has revealed iron deficiency and chronic inflammation to be additional important factors in the pathogenesis of anaemia associated with renal failure.

This Review includes novel mechanistic insights into the hypoxic regulation of erythropoiesis and renal erythropoietin production, and describes the newly discovered inter-relationships between erythropoietin synthesis, erythrocyte production, iron metabolism and chronic inflammation. The current clinical experience with pharmacologic HIF activators as an emerging physiologic approach to the treatment of renal anaemia is also discussed in detail.

Regulation of erythropoiesis

Erythroid differentiation—Erythropoiesis occurs mainly in the bone marrow, and involves the differentiation of erythroid progenitor cells from haematopoietic stem cells (HSCs). In a series of steps regulated by the transcription factors PU.1 and GATA1, HSCs and their progeny lose the ability to differentiate into cells of the lymphoid and granulocytic–monocytic lineages, and instead become bipotent megakaryocytic– erythroid progenitors (MEPs) (Figure 1a). Increased activity of erythroid Krüppel-like transcription factor (KLF1) promotes differentiation of MEPs[1,2] toward the most immature erythroid progenitors, burst-forming units-erythroid (BFU-Es),[3] which in culture, produce large colonies of human erythroblasts. The next stage of erythroid progenitor cells, colony-forming units-erythroid (CFU-Es),[3] and their progeny, the erythroblasts, adhere to a central macrophage, forming an erythroblastic island—the marrow niche of terminal erythropoiesis (Figure 1b).[4] Erythroblastic islands are the sites of cells at different stages of erythroblast differentiation, including proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatic erythroblasts (Figure 1). Orthochromatic erythroblasts enucleate to form reticulocytes, which are irregularly shaped cells that contain haemoglobin and residual organelles (the reticulum), distinguishing them from mature erythrocytes. The extruded nuclei are rapidly phagocytosed by central macro phages, which degrade the nuclei and the small amount of haemoglobin that is associated with each extruded nucleus, and recycle nucleosides and iron.[5] Reticulocytes enter the circulation, lose their residual internal organelles via autophagy, and transform into uniform, biconcave discoid erythrocytes through microvesicle exocytosis.[6] The maturation of reticulocytes to erythrocytes usually takes 1–2 days from the reticulocyte entering the circulation, but erythrocytes continually shed low numbers of microvesicles for another 110–120 days, after which they become senes-cent and are phagocytosed by splenic, hepatic or bone marrow macrophages.[7]

Haemoglobin synthesis—Haemoglobin synthesis begins in late basophilic erythroblasts and continues through to reticulocyte maturation (Figure 1). During haemoglobin production, large quantities of iron are imported into erythroblasts, but this iron is directed towards haeme synthesis, with little intracellular accumulation of iron or protoporphyrin. Large amounts of haeme produced in erythroblasts are promptly incorporated into haemoglobin with no accumulation of excess globin chains or haeme.[8] Intracellular iron and haeme control haemoglobin synthesis in erythroblasts through multiple mechanisms including the haeme-regulated acquisition of iron from endocytosed transferrin receptors;[9] the regulation of haeme synthesis by iron via activation of a 5' iron-responsive element in erythroid-specific 5-aminolevulinic acid synthase (ALAS2) to control the first step of haeme synthesis;[9] the regulation of erythroblast protein synthesis by haeme through inactivation of haeme-regulated eIF2α kinase (also known as haeme-regulated inhibitor; HRI), which phosphorylates the translation initiation factor eIF2α and prevents initiation of translation;[10] and induction of β-globin transcription by haeme, through the degradation of BACH1, a transcription repressor at the β-globin locus control region.[11,12]

Erythropoietin dependence and apoptosis—Within the erythroblastic island, CFU-Es and proerythroblasts lose responsiveness to stem cell factor and insulin-like growth factor-1 supplied by the marrow environment[13] and become dependent on erythropoietin, the principal regulator of erythropoiesis, for survival (Figure 1). In response to hypoxic stress, however, expansion of CFU-E and proerythroblast populations in the absence of differentiation is mediated by glucocorticoids,[14,15] which induce a protein that binds mRNAs involved in terminal erythroid differentiation,[16] and bone morphogenetic protein 4 (BMP4),[17] which is produced by central macrophages.[18] Binding of erythropoietin to specific transmembrane glycoprotein receptors (EPORs), which are expressed as homodimers[19] on CFU-E, proerythroblasts and early basophilic erythroblasts[20] leads to a series of events, including conform ational changes in EPORs, initiation of intracellular signalling by the EPORs and endocytosis of the erythropoietin–EPOR complexes, which are subsequently degraded.[21,22] Endocytosis and intracellular degradation of erythropoietin–EPOR complexes is a mechanism to metabolize circulating erythropoietin.[23] EPORs lack intrinsic enzyme activity, but they can initiate signal transduction pathways through Janus Tyrosine Kinase-2 (JAK2), which physically associates with the cytoplasmic portion of EPORs and chaperones them to the surface of erythroid cells.[24,25] Following activation and conformational changes in EPORs that result from erythropoietin binding, JAK2 autophos phorylates, phosphorylates EPORs, and initiates signalling through several pathways including signal transduction and activator of transcription-5 (STAT5), RAS–RAF–MAP kinase, and phosphoinositol-3 kinase/AKT kinase (protein kinase B).[26]

Erythropoietin-mediated signalling prevents apoptosis of CFU-Es, proerythroblasts and early basophilic erythroblasts.[27–30] During this erythropoietin-dependent period, individual erythroid cells have markedly different erythropoietin requirements for survival.[31] The varying susceptibility of erythropoietin-dependent erythroid progenitors to apoptosis is associated with varying expression levels of FAS (also known as CD95), a membrane protein of the tumour necrosis factor (TNF) receptor family, which triggers apoptosis after binding FAS ligand.[32] Erythropoietin decreases expression of FAS on erythroid progenitors.

Fas ligand is produced by various cell types, but in human erythroblastic islands, mature erythroblasts are major producers of Fas ligand.[33] By contrast in mice, the erythropoietin-dependent, early-stage erythroblasts are themselves the major source of Fas ligand.[34] Within the erythroblast populations, therefore, a negative feedback loop exists that is regulated by Fas ligand produced by mature erythroblasts in humans and by early erythroblasts in mice, which modulates the apoptotic rate of CFU-Es, proerythroblasts and early basophilic erythroblasts, and thereby affects the rate of erythrocyte production.[32,34] Murine early erythroblasts, which both produce and respond to Fas ligand, provide a stabilizing effect on steady state erythrocyte production and enable the rapid splenic responses that characterize stress-induced erythropoiesis in mice.[35] Based on the differences in erythroblast populations producing Fas ligand, simulations of erythrocyte recovery to baseline values following acute blood loss are consistent with a more protracted recovery observed in humans compared to mice.[36] Of note, erythropoietin signalling also promotes the survival of erythroblasts in the post-erythropoietin-dependent period by inducing the anti-apoptotic protein Bcl-X_L.[37,38]

The HIF oxygen sensing pathway

Hypoxia is a classic stimulus of erythropoiesis, and the quest to unravel the molecular underpinnings of this response led to identification of erythropoietin and HIF transcription factors. HIFs are key mediators of a broad spectrum of cellular hypoxia responses and are essential for erythropoiesis under normal and hypoxic stress conditions. Lack of HIF in genetic mouse models results in anaemia, whereas impaired regulation of its activity results in erythrocytosis.[39–43] Activating mutations in the HIF oxygen-sensing pathway have been identified in patients with previously unexplained polycythaemia.[44] Moreover certain genetic variants are found in Tibetans, who are protected from chronic mountain sickness, a deadly condition that is associated with high altitude and is characterized by excessive polycythemia and right-sided heart failure.[45,46]

HIF transcription factors—HIF transcription factors are heterodimeric basic helix-loop-helix proteins that belong to the PAS family of transcriptional regulators. They consist of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, also known as the aryl hydrocarbon receptor nuclear trans-locator (ARNT)[47–49] (Figure 2). Three HIF α-subunits have been identified—HIF-1α, HIF-2α and HIF-3α—however, investigations have largely focused on HIF-1α and HIF-2α, as these subunits have the most critical roles in the regulation of cellular hypoxia responses. Together, HIF-1 and HIF-2 facilitate oxygen delivery and cellular adaptation to hypoxia by controlling a broad spectrum of biological processes, which in addition to the regulation of erythropoiesis, include anaerobic metabolism, angio-genesis, mitochondrial metabolism, cellular growth and differentiation pathways.[50] HIF-mediated gene transcription is activated following DNA-binding and recruitment of transcriptional co-activators.[49,51] Although HIF-1 and HIF-2 coregulate many genes, erythropoietin synthesis and iron metabolism are primarily regulated by HIF-2 in adults.[40,41,43,52–55] The importance of HIF-2 in the regulation of erythropoiesis is underlined by mutations in patients with familial erythrocytosis.[44]

PHD-mediated HIF-αdegradation—Cells continuously synthesize HIF-α subunits; therefore, control of the degradation rate is key to regulating HIF activity and erythropoiesis.

Oxygen-dependent HIF degradation is initiated by three 2-oxoglutarate (2OG)-dependent oxygenases—prolyl-4-hydroxylase domain (PHD) proteins PHD1, PHD2 and PHD3 (also known as EGLN2, EGLN1 and EGLN3, respectively). These enzymes use molecular oxygen to hydroxylate HIF-α at specific proline residues (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in human HIF-2α) and belong to a larger family of proteins that couple the oxidative decarboxylation of 2OG to various chemical processes.[56–63] PHD2 is the main hydroxylase that regulates HIF activity under normoxia and inherited mutations in PHD2 that inhibit catalytic activity are associated with the development of erythrocytosis.[64,65] Proline-hydroxylated HIF-α is rapidly degraded following ubiquity lation by the von Hippel–Lindau (VHL)-E3 ubiquitin ligase complex. Under conditions of hypoxia, hydroxylation is inhibited and HIF signalling is activated[64] (Figure 2). A fourth putative HIF prolyl-hydroxylase, P4H-TM, localizes to the endoplasmic reticulum and might be involved in the regulation of renal erythropoietin.[66,67] In the kidney, which is the main physiologic source of systemic erythropoietin in adults, expression levels of PHD1, PHD2 and PHD3 vary between different cell types.[68] Nevertheless, transcripts of all three PHDs have been detected in erythropoietin-producing cells isolated by fluorescence-activated cell sorting.[69] An additional mechanism of hypoxic regulation operates within the carboxy-terminal trans-activation domain of HIF-α, where factor inhibiting HIF (FIH) catalyses the hydroxylation of an asparagine residue, modulating co-activator recruitment and transcriptional activity of the HIF-heterodimers.[64]

Hypoxic signalling: downstream effects

The HIF pathway regulates and coordinates erythropoiesis at multiple levels by stimulating renal and hepatic erythropoietin production, promoting the uptake and utilization of iron, and altering the bone marrow microenvironment to facilitate erythroid progenitor maturation and proliferation (Figure 3). Improved understanding of these actions has led to the concept that pharmacologic activation of the HIF axis could provide a physiologic approach to the treatment of renal anaemia by mimicking hypoxia responses that coordinate erythropoiesis with iron metabolism.

Hypoxia regulated erythropoietin transcription—In kidney and liver, HIF-2 mediates the hypoxic induction of erythropoietin and binds to specific regulatory elements within the *EPO* gene that control its oxygen-dependent transcription.[39–41,43,70] An 8 kb kidney-inducibility element is located in the 5'-region of the *EPO* gene and is required for renal *EPO* transcription. A liver-inducibility element is situated in the 3'-region of the *EPO* gene and contains a classic hypoxia enhancer, which is required for the hypoxic induction of *EPO* in hepatocytes as demonstrated by genetic studies in mice[71–74] (Figure 2). Although a putative and highly conserved 5'-hypoxia-response element (HRE) has been identified within the kidney-inducibility region, its role *in vivo* is unclear.[75]

Hypoxic signalling and iron metabolism—When erythropoiesis is stimulated by hypoxia via a HIF-2-induced increase in plasma erythropoietin level, the HIF–PHD oxygen-sensing pathway optimizes iron uptake and utilization to meet increased iron demand for erythropoiesis in the bone marrow. HIF-2 has a critical role in the regulation of iron uptake, as it increases transcription of the genes that encode divalent metal transporter 1 (*Dmt1*) and

duodenal cytochrome *b* reductase 1 *(Dcytb)* in animal models of iron-deficiency and haemochromatosis[54,76,77] (Figure 3). DMT1 transports iron into the cytoplasm of cells, and DCYTB reduces ferric iron to its ferrous form (Fe^{2+}) before it is taken up from the gut lumen into intestinal cells via DMT1. Other HIF-regulated factors involved in iron regulation include transferrin, which transports iron in its ferric form (Fe^{3+}) in plasma, the transferrin receptor (encoded by *TFR1*),[78–80] ceruloplasmin, which oxidizes Fe^{2+} to Fe^{3+} and is important for iron transport,[81] haeme-oxygenase-1, which is critical for the recycling of iron from phagocytosed erythrocytes,[82] and ferroportin (encoded by *FPN*),[83] the only known cellular iron exporter, which is targeted for degradation by hepcidin. The small 25 amino acid peptide hepcidin is mainly produced in hepatocytes and suppresses intestinal iron uptake and release of iron from internal stores.[84] As discussed in further detail below, hepcidin has an important role in the pathogenesis of anaemia of inflammation and in the development of iron-deficiency in patients with chronic kidney disease (CKD). Activation of the HIF pathway by hypoxia, genetic mutations or pharmacologic means suppresses hepcidin production in the liver and thereby enhances iron uptake and mobilization[85,86] (Figure 3).

As hepatic levels of *Hamp1* mRNA—the gene encoding murine hepcidin—were not completely suppressed in iron-deficient hepatocyte-specific HIF-1α knockout mice (~90% reduction in *Hamp1* levels compared to control) and chromatin immunoprecipitation studies indicated HIF-1 binding to the *Hamp1* promoter, Peyssonnaux and colleagues suggested that hepcidin is a direct transcriptional target of HIF-1.[87] However, subsequent studies in mouse models of global and liver-specific HIF activation found that HIF-induced suppression of hep cidin was completely dependent on erythropoietin-mediated stimulation of erythropoiesis.[88,89] These findings provided strong support for the notion that the suppression of hepcidin under conditions of HIF activation in the liver occurs indirectly via HIF-2-dependent induction of erythropoietin production and not through direct HIF-dependent transcriptional regulation. This notion is furthermore supported by genome-wide studies in cell lines, which found no enrichment of HIF-binding loci among genes that were downregulated by HIF, suggesting that HIF does not act as a transcriptional repressor and instead suppresses gene expression indirectly.[51]

Hypoxic signalling in the bone marrow—In addition to regulating iron metabolism, hypoxia and the HIF–PHD pathway have direct effects on the bone marrow. Hypoxia stimulates EPOR expression, regulates components of the haemoglobin synthesis pathway,[90–94] and modulates stem cell maintenance, lineage differentiation and maturation.[95,96] Yamashita and colleagues identified a specific role for endothelial HIF-2 in this regard, as mice with globally reduced HIF-2 expression developed defects in erythroid maturation.[96] This maturation defect seemed to involve vascular adhesion molecule (VCAM)-1, a surface protein that supports erythroid maturation.

Cellular sources of erythropoietin

Erythropoietin synthesis in the kidney—A 1957 study involving surgical removal of various organs identified the kidney as the main site of physiologic erythropoietin production in adult mammals.[97] Although several studies in the 1990s suggested that

erythropoietin was produced in renal epithelial cells,[98–101] *in situ* hybridization, immunohistochemistry and findings from various genetic models provide clear evidence that peritubular interstitial fibroblast-like cells are the physiologic source of erythropoietin synthesis in the kidney.[73,102–108] The term interstitial fibroblast-like cell has been used to indicate that this cell population encompasses a heterogeneous group of non-endothelial renal interstitial cells with molecular and morphologic features that resemble fibroblasts. Studies from our laboratory (V. H. Haase, unpublished work) and from other groups[109,110] indicate that the majority of peritubular interstitial fibroblast-like cells have the capacity to produce erythropoietin.

Renal erythropoietin-producing cells: The induction of erythropoietin synthesis in the kidney requires stabilization of HIF-2α, which either occurs under hypoxic conditions or when its degradation is inhibited by pharmacologic or genetic means.[41,111,112] Under normoxic conditions, a small number of renal erythropoietin-producing cells (EPCs) localize to the cortico-medullary region. Under hypoxic conditions the number of EPCs increases proportionally to the degree of hypoxia, resulting in a more wide-spread distribution throughout the entire cortex and outer medulla.[103,107,109,110] The size of the EPC pool correlates directly with the total level of renal *Epo* transcription, which in turn correlates directly with the concentration of erythropoietin in blood.[107] The number of EPCs, therefore, determines renal erythropoietin output and plasma erythropoietin levels (Figure 4a). Pathologic conditions that impede the ability of renal interstitial fibroblast-like cells to synthesize erythropoietin will lead to inadequate erythropoietin production and result in the development of anaemia.

Renal EPCs are unique in their morphologic appearance, as they display dendrite-like processes and express markers that are typically found in neuronal cells, such as microtubule-associated protein 2 (MAP2) and neuro-filament light polypeptide (NFL).[107] The notion that renal EPCs share similarities to neuronal-type cells is supported by lineage tracing studies in mice with specific genetic manipulations in neural-crest-derived cells.[113,114] Indeed, erythropoietin expression in a subpopulation of neural crest cells is critical for primitive erythropoiesis in the yolk sac during early embryonic development.[114] Other cellular surface markers that are typically associated with renal EPCs are ecto-5'-nucleotidase (CD73) and platelet-derived growth factor receptor β (PDGFRB), the latter being a marker of pericytes, indicating that renal pericytes have the potential to substantially contribute to the EPC pool.[105,113]

Renal interstitial fibroblasts and pericytes are derived from FOXD1-expressing stromal cells (Figure 4b), and morphologic and molecular criteria—for example, physical contact with endothelial cells or the basement membrane and the expression of certain molecular markers, such as desmin, α-smooth muscle actin (α-SMA) or S100 calcium-binding protein A4 (S100A4)—have been used to distinguish these cell types from one another.[115] Owing to limitations in the specificity of morphologic and molecular features, the accuracy of this classification has been questioned, however.[116] FOXD1 (also referred to as BF-2) is a forkhead family transcription factor, which, in the developing kidney, is expressed in the nephrogenic interstitium (stroma) that overlies the cap mesenchyme.[117] In addition to pericytes and interstitial fibroblasts, FOXD1-expressing cells give also rise to vascular

smooth muscle cells (VSMCs), renin-producing cells, mesangial cells and probably to a subpopulation of renal epithelial cells; *Foxd1* mRNA has also been detected in podocytes[118,119] (Figure 4). Genetic studies in mice indicate that renin-producing cells can be stimulated to synthesize erythropoietin under conditions of *Vhl* inactivation.[120,121] In this context, erythropoietin synthesis resulted from constitutive HIF-2 activation and was associated with a suppression of renin production and changes in the physiologic behaviour of the cells.[120,121] These findings raise the question of whether erythropoietin production by interstitial fibroblasts and/or pericytes under conditions of prolonged HIF-2 activation is associated with alterations in their normal cellular functions. To address this question, studies that characterize HIF responses in these cell types and the renal microvasculature are needed. Of note, *Epo* expression has not been observed in VSMCs, mesangial cells, podocytes or renal epithelial cells using *in situ* hybridization approaches (V. H. Haase, unpublished work).

Transdifferentiation into myofibroblasts: The *Foxd1* promoter has been used for lineage tracing studies to define the cellular origin of myofibroblasts, which are a main source of collagen production in chronically injured kidneys. These studies suggested that pericytes and not epithelial cells give rise to myofibro-blasts and raised the possibility that pericytes lose their erythropoietin-producing potential during transdifferentiation into myofibroblasts.[115,116,122] Indeed, genetic fate mapping studies using a *Cre*-recombinase transgene under the control of the *Epo* promoter support the concept that transdifferentiation of EPCs into myofibroblasts is the likely mechanism by which EPCs lose their ability to synthesize erythropoietin in the injured kidney[109] (Figure 5). The inability to produce erythropoietin was associated with an increase in NF-κB signalling in these cells, and treatment with corticosteroids reversed the loss of erythropoietin-producing ability in animal models of kidney injury.[109] Whether all renal pericytes have the potential to become EPCs remains to be examined.

Erythropoietin synthesis in the liver—Although the primary physiologic source of erythropoietin synthesis in adults is the kidney, erythropoietin production during embryonic development occurs in the liver. The underlying molecular mechanisms that control the switch from the liver to the kidney as the main physiologic source of erythropoietin are poorly understood. Reduced expression of transcriptional activator GATA4 has been suggested to have a key role during this process.[123] Although the liver does not contribute to the plasma erythropoietin pool under physiologic conditions in adults, perivenous hepatocytes can be stimulated to synthesize erythropoietin by moderate or severe hypoxia,[107] which accounts for most of the physiologically relevant systemic erythropoietin of extra-renal origin.[124,125] Erythropoietin has also been detected in hepatic stellate cells (also referred to as ITO cells).[126,127] Although induction of erythropoietin by the liver under hypoxic conditions does not correct anaemia in patients with CKD, hepatic HIF can be sufficiently stimulated by pharmacologic means to normalize haematologic parameters in rodent models of anaemia associated with renal failure or inflammation.[41,128] Robust pharmacologic induction of erythropoietin synthesis in hepatocytes requires inactivation of all three HIF-PHDs or inactivation of the VHL–E3 ubiquitin ligase complex.[41,43,129] In this regard, hepatocytes behave differently from renal EPCs, which are sensitive to PHD2

inactivation alone, indicating that HIF degradation in EPCs is regulated in a tissue-dependent manner.

Erythropoietin synthesis in other tissues—In addition to the kidney and liver as the two major sources of erythropoietin synthesis, erythropoietin mRNA has been detected in neurons and glial cells, lung tissue, cardiomyocytes, bone marrow cells, spleen, hair follicles, tissues of the male and female genitourinary tract and osteoblasts.[130–142] Although these cell types are not thought to contribute substantially to plasma erythropoietin levels under physiologic conditions, they might have a role in stress-induced erythropoiesis.[142,143] Furthermore, local non-renal erythropoietin might exert non-haematopoietic actions on angiogenic and cellular stress responses.[144] Nevertheless, many of the non-renal cell types have the potential to contribute to erythropoiesis under conditions of systemic HIF activation, which will be an important consideration when administering HIF stabilizing compounds systemically to patients with anaemia.[41,141–143,145]

Other cellular regulators—Non-EPC-derived signals and messengers, which include uraemic toxins, have the potential to modulate the HIF–erythropoietin axis (see discussion below).[146,147] For example, production of nitric oxide in macrophages has been identified as a positive regulator of renal erythropoietin production.[148] HIF-1-mediated and nitric-oxide-mediated effects on dermal blood flow might also modulate erythropoietin production indirectly through alterations in renal and hepatic blood flow.[149] However, this proposal has been challenged, as partial pressure of oxygen (pO_2) in renal tissue is largely independent of blood flow and is maintained at relatively constant levels under most physiologic conditions. Oxygen consumption in the kidney is physiologically linked to renal blood flow, which determines glomerular filtration rate and thus renal work load so that a potential increase in pO_2 from increased blood flow would be offset by an increase in oxygen consumption.[150,151] Nevertheless, the concept that hypoxia responses in non-renal tissues provide signals that regulate renal erythropoietin production is supported by additional studies. For example, isolated brain hypoxia triggers erythropoietin production in the kidney, probably due to the production of brain stem-derived humoral factors that modulate renal erythropoietin output.[152]

Within the kidney, cross talk between cells with EPC potential and other cells types is likely to affect the regulation of the EPC pool. Although these intercellular relationships are ill defined, studies from our group have shown that HIF-induced reprogramming of epithelial metabolism changes the size of the EPC pool partly through alterations in renal tissue pO_2 (V.H. Haase, unpublished work). These findings would be consistent with the observation that certain diuretics can modulate renal erythropoiesis, possibly through their effects on tubular work load and oxygen consumption.[153]

Pathogenesis of renal anaemia

As mentioned earlier, renal anaemia is caused by under-production of erythrocytes due to multiple factors, among which erythropoietin deficiency is most prominent and specific (Figure 6). Most patients with a glomerular filtration rate <30 ml/min/1.73 m^2 develop anaemia.[154] Patients with renal failure have much lower levels of erythropoietin than do

patients with similar degrees of anaemia but with intact kidney function.[155] However, among patients with renal failure, nephric patients have higher plasma erythropoietin and haematocrit levels than do anephric patients, indicating that the diseased kidneys achieve residual erythropoietin production.[155]

Erythropoietin deficiency associated with renal disease increases apoptosis of erythroid progenitors in the erythropoietin-dependence period (Figure 1), which leads to decreased reticulocyte production. rhEPO and other erythropoiesis-stimulating agents (ESAs), such as darbepoetin alfa and epoetin beta pegol permit exogenous erythropoietin therapy with major improvements in renal anaemia. Some patients, however, have suboptimal responses or require very high ESA doses to respond. These erythropoietin-resistant patients most commonly have considerable inflammation and/or iron deficiency, which suppresses erythropoietin production and has negative effects on erythropoietic progenitor cells.

The role of inflammation—Systemic inflammation in renal failure is often caused by autoimmune diseases and infections related to diabetes mellitus and/or use of intravascular devices. Bone marrow inflammatory cytokines are increased in patients with renal osteodystrophy[156,157]—a condition that is associated with erythropoietin resistance. A link between inflammation and decreased erythropoiesis was demonstrated by relatively decreased levels of serum erythropoietin in anaemic patients without renal failure who had inflammation caused by autoimmune disease[158] or malignancies[159] as opposed to levels in similarly anaemic patients without inflammation. In a rat model of inflammation, IL-1β acted indirectly through TNF, to suppress renal erythropoietin production.[160] Inflammation accompanying the acute exacerbation of chronic renal insufficiency in murine models transformed renal erythropoietin-producing fibroblast-like cells into proliferating myofibroblasts, with TNF signalling through NF-κB suppressing *Epo* transcription[109] (Figure 5).

In addition to their suppressive effects on erythropoietin production, several inflammatory cytokines, including IL-6, TNF and interferon-γ (IFN-γ),[161] inhibit differentiation of erythropoietic progenitors. IFN-γ induces PU.1, suppressing erythroid differentiation and promoting granulocytic–monocytic differentiation.[162] During the erythropoietin-dependence stages (Figure 1), IFN-γ enhances expression of apoptosis-inducing TNF receptor family members including receptors for TNF, FAS, TRAIL, TWEAK and RCAS1.[163–165] In later stages of erythropoiesis when iron is required for haemoglobin production (Figure 1), inflammation and/ or infection restricts iron supply to the bone marrow by inducing transcription of *HAMP* (which encodes hepcidin) via IL-6 signalling through the STAT3 pathway[166] and activin B signalling through the BMP/Smad1/5/8 pathway.[167] Hepcidin binds to the iron exporter ferroportin, leading to its internalization and degradation and thereby downregulates ferroportin expression on all cells.[168] The key cells that supply iron to erythroid cells for haemoglobin synthesis and are affected by hepcidin are macrophages, which recycle iron from phagocytosed senescent erythrocytes; hepatocytes, which are the major storage cells for iron; and duodenal enterocytes, which absorb dietary iron (Figure 3).

The effects of iron deficiency—Although inflammation restricts iron availability for haemoglobin synthesis, absolute iron deficiency occurs in CKD-associated anaemia because

haemodialysis causes recurrent blood loss. Erythrocytes, which contain two-thirds of the body's iron, are lost by trapping in dialysis apparatuses and associated tubing, post-dialysis bleeding at vascular access sites, and recurrent sampling for laboratory tests. These blood losses are four to eight-fold higher than the normal daily loss of 1–2 mg of iron that occurs via the gastrointestinal tract and skin under physiological conditions,[169–172] and lead to iron deficiency because oral iron absorption, even if supplemented, is insufficient to replace the losses.[173,174] Under normal conditions, haemoglobin-synthesizing erythroid cells are the largest consumers of iron (consuming about 25 mg per day), and macrophages, which phagocytose senescent erythrocytes and recycle iron from degraded haemoglobin, are the largest suppliers. After bleeding or haemolysis, mobilization of stored and recycled iron and absorption of duodenal iron are increased by erythroferrone, a hormone produced by erythropoietin-stimulated erythroblasts that decreases production of hepcidin.[175] In renal disease, erythropoietin deficiency and chronic inflammation result in reduced numbers of erythroblasts that are the source of erythroferrone (Figure 6).

As iron deficiency progresses, erythropoietic utilization of iron becomes limited while crucial iron-requiring processes in non-erythroid cells are preserved. Iron regulatory proteins (IRP1 and IRP2) bind to iron responsive elements (IREs) in 5'-untranslated regions (UTRs) and 3' UTRs of mRNAs that control expression of proteins involved in cellular iron import, export and storage.[176,177] In iron-replete cells, IRP1 has aconitase activity with an iron–sulphur cluster in its active site, and IRP2 is rapidly degraded. In iron-deficient cells, IRP1 and IRP2 bind IREs, as IRP1 lacks its iron–sulphur cluster and IRP2 is stabilized. IRPs bound to 5'-UTR IREs of mRNAs inhibit translation and expression, while IREs bound to 3'-UTRs of mRNAs stabilizes them, increasing translation and expression. In most cells, ferroportin mRNA is regulated by a 5'-IRE, and the resultant decrease in iron export conserves intracellular iron levels. Alternative splicing in duodenal enterocytes and erythroid precursor cells, however, produces ferroportin mRNAs without 5'-IREs,[178,179] and persistent ferroportin expression during iron deficiency enables iron absorption from the duodenum and diminishes iron accumulation within erythroid precursors.[180]

Under conditions of iron deficiency, IRPs exhibit increased binding to 5'-IREs of two other mRNAs involved in erythropoiesis: HIF-2α and ALAS2. Binding of IRP1 to a 5'-IRE in HIF-2α mRNA inhibits HIF-2α translation in renal cortical EPCs,[181–183] with relatively decreased levels of HIF-2α protein restricting erythropoietin production despite the presence of hypoxia in the renal cortex caused by decreased numbers of circulating erythrocytes. Although erythropoietin production is significantly greater in iron-deficiency anaemia than in anaemia associated with renal failure or chronic inflammation (such as that associated with malignancies, infections or autoimmune disorders), IRP-mediated restriction of HIF-2α translation is predicted to reduce renal erythropoietin synthesis in iron-deficiency anaemia relative to non-iron deficient, acquired anaemia of similar severity, such as haemolytic or megaloblastic anaemia. Iron deficiency also leads to binding of IRP to a 5'-IRE in mRNA encoding ALAS2, the rate-controlling enzyme in porphyrin synthesis,[9] which decreases accumulation of protoporphyrin and haeme in erythroblasts. Decreased haeme, in turn, increases HRI activity, which inhibits erythroblast protein synthesis.[10] Iron deficiency therefore results in less than maximal erythropoietin production, which reduces the number of erythroid progenitors surviving the erythropoietin-dependence period and HRI inhibits

erythroblast protein synthesis in the haemoglobin synth esis period, resulting in the production of fewer and smaller erythrocytes containing smaller amounts of haemoglobin.

Stabilizing HIF to treat renal anaemia

ESAs and intravenous iron supplementation represent the current mainstay of renal anaemia therapy. Although very effective in most patients, ESA therapy and variability in haemoglobin level due to ESA use has been associated with increased cardiovascular risk.[184–189] Pharmacologic activation of HIF responses offers great therapeutic potential, and is predicted to provide a more physiologic approach to treating renal anaemia than current approaches, thus potentially reducing the cardiovascular risks associated with recombinant erythropoietin therapy. Major benefits of anaemia therapy with HIF stabilizers include maintenance of plasma erythropoietin levels within a physiologic range, which would avoid the supraphysiologic increases in plasma erythropoietin levels that are often associated with intravenous ESA therapy; enhanced iron absorption and mobilization, which could lead to a reduction in the use of intravenous iron supplementation; and oral dosing with the potential for more effective titrability, which would reduce the fluctuations in haematological parameters and minimize the risk of over-reaching haematologic targets.

Development of HIF stabilizers—Although it had been known since the 1980s that *EPO* transcription and HIF responses could be activated with iron chelators and transition metals such as cobalt and nickel,[190–194] the discovery of PHDs as oxygen sensors provided a structural basis for the development of HIF activating compounds, called PHD inhibitors (PHIs). Before the discovery of the HIF pathway, cobalt, despite its potential for serious adverse effects, had been used clinically for the treatment of anaemia in patients on haemodialysis.[195–198] Cobalt acts as a nonspecific hypoxia mimic by inhibiting HIF-PHDs and other 2OG-dependent oxygenases, but its effects on gene expression overlap only partially with those induced by hypoxia (28% overlap in gene expression profiles obtained from hypoxia-treated and cobalt(II)-chloride-treated hepatoma cells).[199,200] Although no longer in clinical use, the pathologic effects of cobalt are still relevant to clinical practice, as cobalt poisoning of food and drinking water represents a serious health problem in Andean high altitude populations, and should be considered as a potential cause in cases of unexplained polycythaemia.[201]

The majority of PHIs developed through structure-based drug discovery programmes reversibly inhibit PHD catalytic activity by binding to the ferrous-iron-containing active site, thereby blocking entry of the cosubstrate 2OG.[202] Since PHDs belong to a large family of 2OG-dependent oxygenases, PHD-inhibiting compounds can theoretically affect the function of other 2OG-dependent oxygenases, such as tet proteins, jumonji domain-containing oxygenases and others, which target non-HIF substrates.[63,203] These potential off-target effects will require careful consideration in the preclinical and clinical evaluation of HIF-activating PHIs. To date, six PHIs, which aim to stimulate endogenous erythropoietin synthesis and other cellular HIF responses, are registered in clinical trials (Table 1). Data from these trials, presented in abstract form at clinical society meetings or on company websites,[204–206] indicate that pharmacological inhibition of HIF-PHDs is well tolerated by patients, is effective in raising and maintaining haemoglobin in patients with

CKD and end-stage renal disease (ESRD) who were ESA-naive or converted from an ESA to a PHI, and has the predicted beneficial effects on iron metabolism.

In a proof-of-principle study, a single dose of the PHI FG-2216 significantly increased serum erythropoietin levels in patients on haemodialysis. Suprisingly, the erythropoietin response was more pronounced in nephric patients on haemodialysis than in healthy individuals, whereas anephric patients on haemodialysis responded with lower serum erythropoietin levels compared to healthy controls or nephric patients with ESRD.[207] This enhanced serum erythropoietin response in nephric patients on haemodialysis might have resulted from changes in compound metabolism and pharmacokinetics due to impaired renal clearance, but also raises the possibility that ESRD kidneys might be more susceptible to the erythropoietin-stimulating effects of PHD inhibition. Although the underlying mechanisms behind such increased susceptibility are likely to be complex, they may involve the presence of renal hypoxia, which as described earlier, is a well-documented pathophysio-logic feature of CKD that is predicted to affect PHD catalytic activity, thus possibly enhancing the effects of pharmacologic PHD inhibition.[208,209]

In order to understand and predict the physiologic and clinical effects of PHIs in patients with CKD and anaemia, it is important to consider the degree to which individual PHDs are selectively targeted, and whether a certain compound might result in tissue-specific stimulation of erythropoietin production (that is, in the kidney versus the liver). Tissue-specific effects might result from differences in the tissue distribution of the compounds or reflect differences in PHD selectivity. Genetic studies in mice have shown that inactivation of PHD2 alone is sufficient to boost renal erythropoietin production,[111,112] whereas the combined inactivation of PHD1, PHD2 and PHD3 is required to induce robust and sustained erythropoietin synthesis in hepatocytes.[129] In terms of efficacy, the kidney contains a large subset of peritubular renal interstitial fibroblast-like cells that are sensitive to PHD2 inactivation alone, whereas inhibition of additional PHDs is required for the stabilization of HIF-2α and induction of erythropoietin in other subsets of peritubular interstitial fibroblast-like cells (V.H. Haase, unpublished work). These findings indicate that cell types with erythropoietin-producing capability display differential sensitivity to PHD inhibition, which will be an important c onsideration in the clinical evaluation of PHI therapy.

Potential disadvantages of PHI therapy—Although PHI therapy represents a promising novel approach for the treatment of renal anaemia and has several potential advantages over conventional ESA therapy, theoretical downsides need to be addressed in clinical trials. HIF-specific PHIs are administered systemically and target the oxygen-sensing machinery upstream of HIF, which will lead to various degrees of HIF-1 and HIF-2 activation depending on which PHDs, tissues and cell types are targeted. Since HIF transcription factors are involved in the regulation of a broad spectrum of biological processes, careful clinical safety and cardiovascular outcome studies will need to be conducted in patients with CKD to evaluate PHI-mediated effects on renal disease progression, metabolism, cardiovascular function, blood pressure, and other physiologic parameters.[210] HIF is involved in the regulation of vascular tone and blood pressure and has been shown to contribute to the development of pulmonary hypertension.[210–213] Furthermore, activation of HIF signalling in malignant cells has been associated with the

initiation and progression of various tumours, as well as resistance to therapy involving multiple levels of regulation, which precludes the use of HIF-stimulating compounds in patients with cancer.[214–216] Of considerable concern are the possible effects of global HIF activation on angiogenesis, particularly as it relates to the progression of proliferative diabetic retinopathy and tumour angiogenesis. Vascular endothelial growth factor (VEGF) is a HIF-regulated growth factor and systemic activation of the HIF axis has the potential to lead to increased VEGF production in multiple tissues.[217] Clinical studies will need to carefully screen for potential pro-angiogenic effects and other PHI-induced hypoxia responses that might be harmful to patients.

The clinical complexity of patients with advanced CKD, their often complex medication regimen and the possibility of broad-spectrum on-target biological effects might make it difficult to define causal relationships when severe adverse clinical events occur. Accordingly, the FDA temporarily suspended a phase II clinical trial with the HIF stabilizer FG-2216 due to a single case of fatal hepatic necrosis. Although the FDA granted permission to resume clinical trials with FG-2216 in 2008,[218] no further studies using this agent have been performed in humans.

HIF activators in clinical trials

Many HIF stabilizers originated from drug development programmes that initially developed the compounds to inhibit collagen hydroxylases for anti-fibrotic therapy. N-oxalylglycine (NOG) and dimethyl-oxalylglycine (DMOG) are examples of compounds that were developed under these programmes and have been widely used in preclinical investigations to study the effects of pharmacologic HIF activation on mammalian physiology and disease. Consequently, hydroxyisoquinoline-based compounds were developed that contain a carbonylglycine side chain.[219] Examples of this class of PHIs are the FibroGen compounds FG-2216 and FG-4592. The latter compound differs from FG-2216 by the addition of a phenoxy substituent at carbon position seven of the quinoline core (Supplementary Figure 1).[219,220]

FG-4592—FG-4592 is FibroGen's current lead anaemia compound with clinical data from over 1,000 patients presented at international scientific and clinical meetings.[221–224] FG-4592 inhibits all three HIF-PHDs, has a plasma half-life of ~12 h and has mostly been given orally in bi-weekly or tri-weekly doses ranging from 1 mg/kg to 2 mg/kg. Similar to FG-2216, FG-4592 stimulates the transcription of endogenous *EPO* and other genes involved in erythropoiesis, such as *EPOR* and genes that regulate iron absorption, transport and recycling.[221,225] Clinical studies in patients with CKD not on dialysis, as well as in those on haemodialysis or peritoneal dialysis, including erythropoietin-naive patients, demonstrate that FG-4592 is well tolerated, maintains haemoglobin levels within the target range and has beneficial effects on iron metabolism.[222] FG-4592 increases total iron binding capacity independent of route of administration, decreases serum ferritin levels and consistently lowers hepcidin levels.[222] Furthermore, the effectiveness of FG-4592 on erythropoiesis does not seem to be affected by inflammation, as total dose requirements to maintain haemoglobin levels in the target range were not associated with C-reactive protein levels.[222] Median peak serum erythropoietin levels 8–12 h after administration of 1mg/kg

FG-4592 were 115 mU/ml,[223] substantially lower than those achieved by intravenous injection of rhEPO.[226] A phase II comparison study of patients on haemodialysis treated with FG-4592 or epoetin alfa also indicated suppressive effects of FG-4592 on lipid metabolism, with a significant ~20% reduction in total serum cholesterol observed in FG-4592-treated patients compared to those treated with recombinant epoetin alfa.[224] Whether this finding represents a HIF-mediated and/or drug class effect remains to be investigated. Studies with FG-4592 have now entered phase III clinical trials (Table 1).

GSK1278863—GlaxoSmithKline's compound GSK1278863 is being tested in phase II clinical trials for renal anaemia (Table 1) and is also being investigated for potential benefits in ischaemia prevention and wound healing. Although the structure of GSK1278863 has not been published, it is probably similar to a commercially available test compound, GSK1002083A, which is a robust HIF-1 and HIF-2 activator and has been used for various preclinical proof-of-principle studies in mice.[41,227] GSK1278863 inhibits PHD2 and PHD3 with an IC_{50} of 22 nM and 5.5 nM, respectively, and leads to the stabilization of both HIF-1α and HIF-2α in Hep3B cells.[228] In mice, oral administration of 60 mg/kg GSK1278863 led to rapid induction of liver *Epo* mRNA followed by the induction of kidney *Epo* message after 6 h, which corresponded to an eightfold increase in serum erythropoietin levels from baseline 6–8 h after drug administration.[228] Daily oral gavage for 21 days was associated with increases in all erythrocyte parameters.[228] In a safety and tolerability study in healthy individuals, oral administration of single doses of 2–300 mg led to dose-dependent increases in serum erythropoietin levels (up to 1,000-fold in the 300 mg group). Doses of 150 mg and 300 mg were also associated with significant increases in serum VEGF levels compared to placebo control.[229] In a separate study, administration of 10–100 mg GSK1278863 to patients with non-dialysis stage 3–5 CKD and haemodialysis-dependent CKD led to dose-dependent changes in haema tologic parameters and decreased serum hep cidin levels,[230] with no significant change in serum VEGF levels at the investigated dose-ranges. GSK1278863 is also being evaluated in the context of wound healing and ischaemic injury associated with thoracic aortic aneur ysm repair. A multicentre, randomized, placebo-controlled phase II clinical study in patients with peripheral artery disease and symptomatic claudication has been completed but did not show benefits with regard to exercise performance at the doses studied.[231]

AKB-6548—Akebia Therapeutics has completed phase IIa and phase IIb trials of its lead anaemia therapy compound, AKB-6548, in patients with nondialysis-dependent CKD, and is currently enrolling patients on haemo-dialysis (Table 1). The structure of AKB-6548 is not published; however, it is probably based on a series of 8-hydroxyquinoline 7-carboxamide compounds.[219] AKB-6548 stabilizes HIF2α to a greater extent than HIF1α and produces dose-dependent increases in serum erythropoietin levels. Serum erythropoietin peaked ~18 h after administration of AKB-6548 and reached 32.4 mU/ml in healthy adults when daily doses of 900 mg were given in a 10-day phase Ib study.[232] Comparable increases were seen in patients with stage 3 and 4 CKD, who were given a single 500 mg dose. AKB-6548 seems to be well tolerated in patients with CKD, raises and maintains haemoglobin levels in the target range, increases total iron binding capacity and lowers both serum ferritin and hepcidin levels.[233–235] A slight transient decrease in mean arterial blood pressure and a mild

transient increase in serum uric acid level were observed in a 28-day dose escalation study.[233] An increase in serum uric acid levels is a typical finding in individuals who ascend to high altitude, supporting the notion that PHIs mimic various aspects of high altitude physiology.[236,237] In a randomized, double blind, placebo-controlled phase II study, daily doses of 240 mg, 370 mg, 500 mg and 630 mg AKB-6548 were given to patients with stage 3 and 4 CKD over a period of 6 weeks. Statistically significant increases in haemoglobin values, ranging from ~7.5 g/l to 15 g/l, were observed in all dosing groups, whereas serum erythropoietin levels were not statistically significantly different between patients who received AKB-6548 and those who received placebo,[234] indicating that PHI therapy is effective in treating renal anaemia within physiologic ranges of plasma erythropoietin.

BAY85-3934—Bayer compound BAY85-3934 (Molidustat) is structurally different from the aforementioned compounds in that it is based on a dihydropyrazolone ring structure that does not contain a carbonylglycine side chain. This compound inhibits all 3 HIF-PHDs with a moderate preference for PHD2 (IC_{50} of 480 nM, 280 nM and 450 nM for PHD1, PHD2 and PHD3, respectively)[238] and is currently in phase II clinical trials (Table 1). BAY85-3934 effectively stimulates erythropoiesis in animal models of renal failure and inflammatory anaemia and in addition, demonstrated antihypertensive and cardioprotective effects in partially nephrectomized rats.[238] In humans, administration of 5–50 mg BAY85-3934 led to a dose-dependent increase in serum erythropoietin levels.[239] A peak serum erythropoietin level of 39.8 mU/ml was observed 12 h after a single dose of 50 mg in healthy humans (compared with 14.8 mU/ml for placebo).[239]

Other HIF stabilizing compounds—Little information is available about two other compounds that are registered for phase I anaemia trials. Akros Pharma is investigating a glycinamide-based compound, JTZ-951, and Daiichi Sankyo has launched phase I clinical trials investigating DS-1093 (Table 1). Preclinical or clinical data are currently not publically available for either compound.

Conclusions

Improved insights into the oxygen-dependent regulation of erythropoiesis and the relationships between erythropoietin, iron and chronic inflammation provide fascinating opportunities for the development of novel therapeutics that could deliver physiologic and more comprehensive approaches to erythropoietin and iron deficiencies in renal anaemia. These discoveries have led to the development of various HIF stabilizing compounds that are currently being investigated in clinical trials. Although initial findings suggest that the strategy of HIF stabilization to stimulate erythropoiesis in patients with kidney disease is clinically effective, several safety concerns exist. These include the possibility of pro-angiogenic and adverse cardiovascular and metabolic effects, which need to be carefully assessed in long-term clinical trials. While some of these studies are planned or currently ongoing, knowledge of the effects of systemic HIF activation on human physiology and pathophysiology is still limited and remains an active area of research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

■ The hypoxia-inducible factor (HIF) oxygen-sensing pathway has a central role in regulating erythropoiesis; it mediates the hypoxic induction of erythropoietin and coordinates erythropoietin and erythrocyte production with iron metabolism

■ Peritubular renal interstitial fibroblast-like cells and pericytes synthesize erythropoietin in an oxygen-regulated and HIF-2-dependent manner; they lose their ability to produce erythropoietin as they transdifferentiate into myofibroblasts following kidney injury

■ In anaemia associated with renal disease, erythropoiesis is suppressed due to the combined and interrelated effects of erythropoietin deficiency, inflammatory cytokines and iron deficiency

■ The pharmacologic activation of hypoxia responses with HIF stabilizers provides a physiologic and comprehensive approach to the treatment of renal anaemia and warrants large, long-term clinical safety and efficacy trials

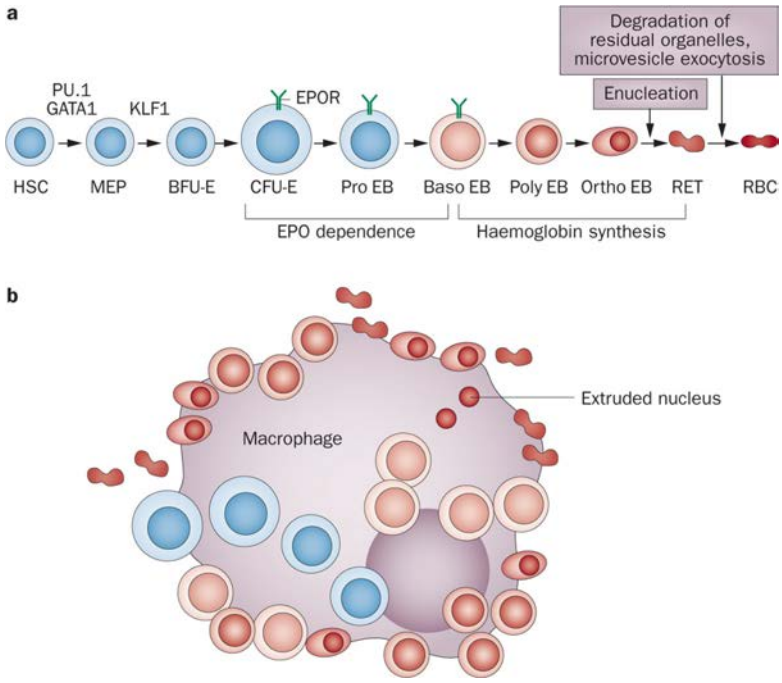

Figure 1.

Overview of erythropoiesis. **a** | Progressive stages of erythroid differentiation showing the relative sizes and presumed or known morphologic appearances of haematopoietic cells at various stages. The transcription factors PU.1 and GATA1 are important in determining whether HSCs will progress towards an erythroid or a non-erythroid fate, whereas KLF1 is important in determining whether MEPs will progress towards an erythroid or a megakaryocytic fate. PU.1 expression continues until the EPO-dependent stages, whereas GATA1 and KLF1 have important roles in differentiation throughout haemoglobin synthesis. Stages of haemoglobin synthesis show relative accumulations of haemoglobin as increasing intensity of red in the cytoplasm. The periods of EPO dependence ending at the early Baso EB stage and haemoglobin synthesis beginning in the late Baso EB stage do not overlap. **b** | An erythroblastic island. Erythroid cells from the CFU-E through to the RET stages are attached to a central macrophage. RETs detach from the macrophage before leaving the marrow and entering the blood. Two extruded nuclei created when RETs are formed from Ortho EBs are shown inside the central macrophage where they have been phagocytosed and will be degraded. Abbreviations: Baso, basophilic; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; EB, erythroblast; EPO, erythropoietin; EPOR, EPO receptor; HSC, pluripotent haematopoietic stem cell; MEP, bipotent megakaryocytic–erythroid progenitor; Ortho, orthochromatic; Poly, polychromatophilic; RBC, erythrocyte; RET, reticulocyte.

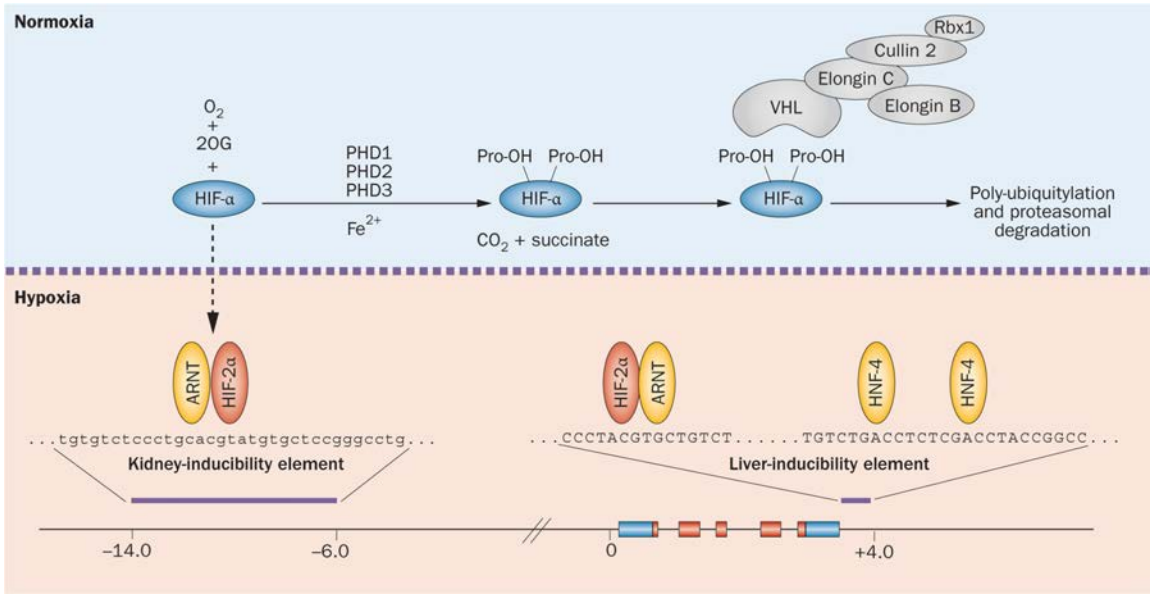

Figure 2.

Regulation of HIF degradation by PHDs and hypoxic induction of erythropoietin. HIF controls a wide spectrum of tissue-specific and systemic hypoxia responses, with HIF-2 being the principal regulator of *EPO* transcription *in vivo*. The HIF oxygen-sensing machinery targets HIF-1α, HIF-2α and HIF-3α for proteasomal degradation by the VHL–E3-ubiquitin ligase complex. In the presence of oxygen HIF-α is hydroxylated at specific proline residues (Pro-OH) by PHD enzymes. Hydroxylation of HIF-α permits binding to the β-domain of VHL, which functions as the substrate recognition component of the VHL–E3-ubiquitin ligase complex. Under hypoxic conditions, HIF-α degradation is inhibited and HIF-α translocates to the nucleus, where it forms a heterodimer with ARNT and binds hypoxia regulatory elements in *EPO*, which contain the HIF consensus binding site 5'-*RCGTG*-3'. The hypoxic induction of *EPO* in the liver is mediated by the liver-inducibility element in the 3'-end of the *EPO* gene, whereas the hypoxic induction of renal *EPO* requires the kidney inducibility element located upstream of the *EPO* transcription start site. The putative DNA sequence of the kidney inducibility element is shown in lower case letters. HNF-4 is an important coregulator of *EPO*. Boxes depict *EPO* exons. *EPO* coding sequences and non-translated sequences are depicted in red and blue, respectively. The distance from the *EPO* transcription start site is indicated in kilobases. Abbreviations: 2OG, 2-oxoglutarate; ARNT, aryl hydrocarbon receptor nuclear translocator; *EPO*, erythropoietin gene; Fe^{2+}, ferrous iron; HIF, hypoxia-inducible factor; HNF-4, hepatocyte nuclear factor 4; PHD, prolyl-4-hydroxylase domain.

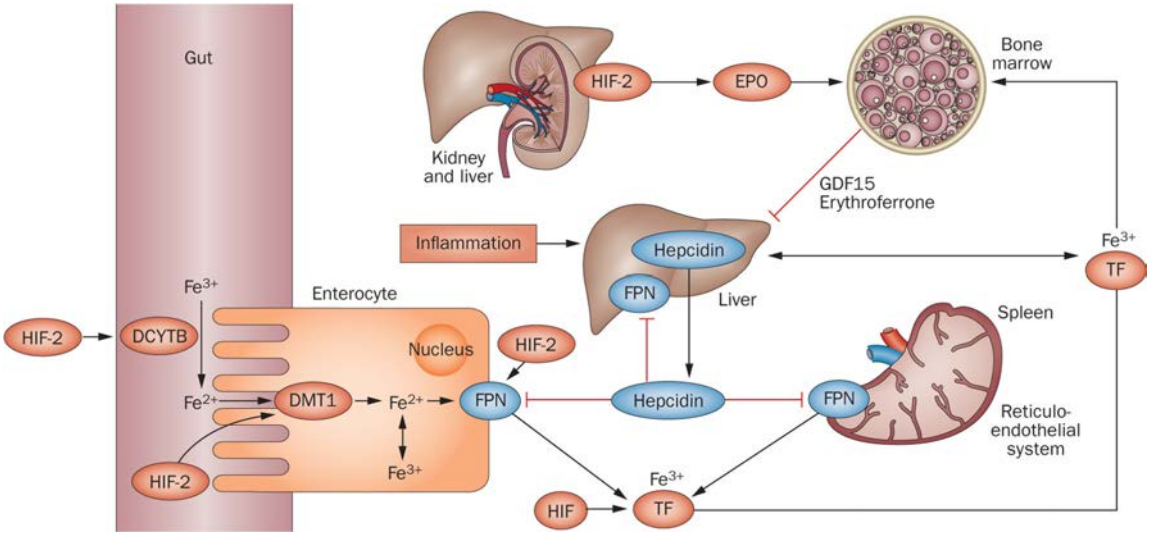

Figure 3.

HIF coordinates erythropoietin production with iron metabolism. HIF-2 stimulates renal and hepatic erythropoietin synthesis, which raises serum erythropoietin levels, stimulating erythropoiesis in the bone marrow. In the duodenum, DCYTB reduces Fe^{3+} to Fe^{2+}, which then enters enterocytes via DMT1. *DCYTB* and *DMT1* are both regulated by HIF-2. Iron is then released into the circulation via FPN, which is also HIF-2-inducible. In the circulation iron is transported in a complex with TF to the liver, bone marrow and other organs; cells of the reticuloendothelial system acquire iron through the phagocytosis of senescent red cells. TF is HIF-regulated, and hypoxia and/or pharmacologic PHD inhibition raises TF serum levels. Increased erythropoietic activity in the bone marrow produces GDF15 and erythroferrone, which suppress hepcidin in hepatocytes. Hepcidin suppression increases FPN expression on enterocytes, hepatocytes and macrophages, resulting in increased iron absorption and mobilization from internal stores. Inflammation stimulates hepcidin production in the liver and leads to reduced FPN expression and hypoferraemia. In addition to regulating hepcidin indirectly by stimulating erythropoiesis, *in vitro* studies suggest that HIF might also modulate hepcidin expression through the regulation of furin and transmembrane protease serine 6.[240–244] Abbreviations: DCYTB, duodenal cytochrome *b* reductase 1; DMT1, divalent metal transporter-1; EPO, plasma erythropoietin; Fe^{2+}, ferrous iron; Fe^{3+}, ferric iron; FPN, ferroportin; GDF15, growth differentiation factor 15; HIF, hypoxia-inducible factor; TF, transferrin.

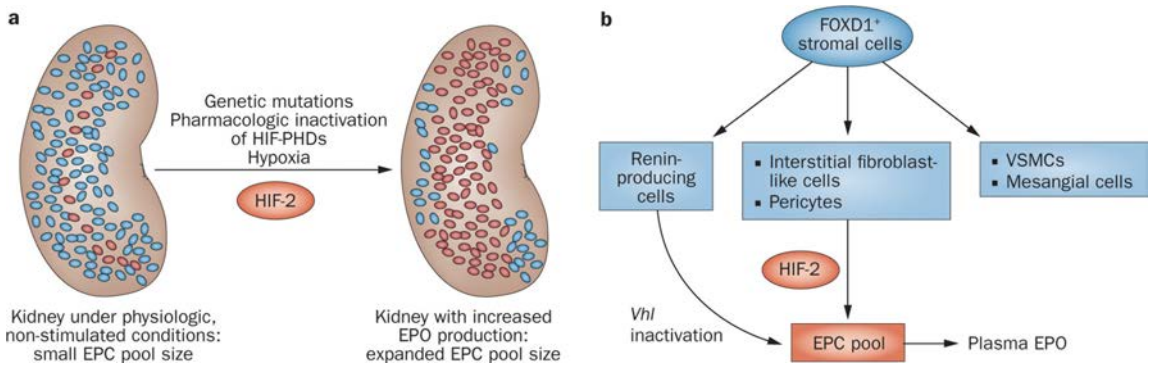

Figure 4.

The number of EPCs regulates renal erythropoietin output. **a** | Under physiologic, non-stimulated conditions a small number of renal EPCs are responsible for renal erythropoietin output. The size of the EPC pool is regulated in an oxygen-dependent manner and increases under hypoxic conditions. The expansion of the EPC pool requires HIF-2 signalling, which is activated by hypoxia, pharmacologic PHD inhibition, or as a consequence of mutations in the oxygen-sensing pathway. **b** | Renal EPCs are derived from FOXD1-expressing stromal cells, and include interstitial fibroblast-like cells, pericytes and renin-producing cells. Renin-producing cells can be induced to synthesize erythropoietin under conditions of *Vhl* gene inactivation; their role in hypoxia-induced renal erythropoietin production is unclear. Abbreviations: EPC, erythropoietin-producing cell; EPO, erythropoietin; HIF-2, hypoxia-inducible factor-2; VSMC, vascular smooth muscle cell.

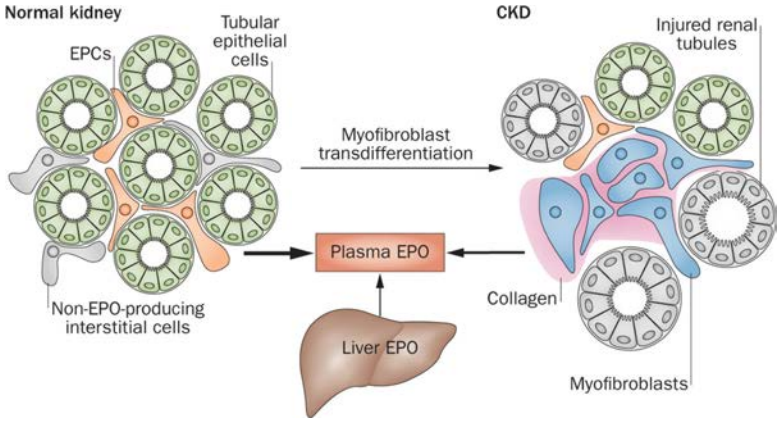

Figure 5.

Cellular basis of erythropoietin deficiency in renal failure. In the normal kidney, EPCs are recruited from peritubular interstitial fibroblast-like cells and pericytes. Tubular epithelial cells do not produce EPO. Under conditions of injury, EPCs or interstitial cells with EPC potential transdifferentiate into myofibroblasts, which synthesize collagen and lose their ability to produce EPO. In CKD, EPC recruitment is impaired, resulting in reduced renal EPO output and the development of anaemia. Under conditions of severe hypoxia or in patients with advanced CKD, the liver contributes to plasma EPO levels. Abbreviations: CKD, chronic kidney disease; EPC, erythropoietin-producing cell; EPO, erythropoietin.

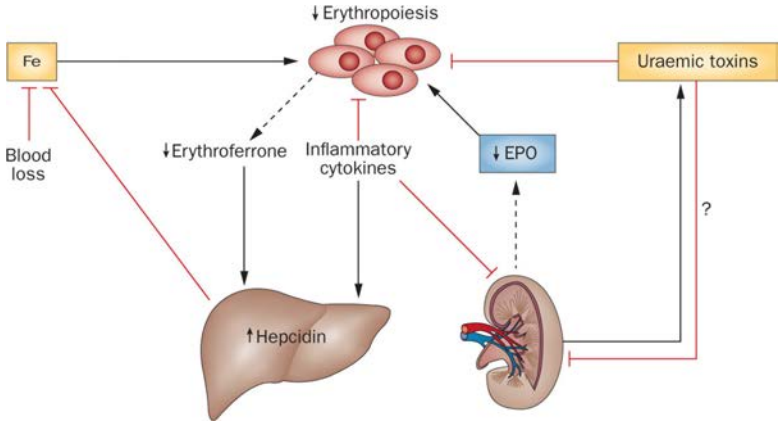

Figure 6.

Mechanisms of renal anaemia. In renal anaemia, the ability of the kidney to produce EPO is impaired. Inflammatory cytokines suppress erythropoiesis in the bone marrow, EPO production in the kidney, and stimulate hepcidin production in the liver, which negatively affects iron absorption and mobilization. Hepcidin is also maintained at higher levels by decreased erythroferrone production, which is secondary to a reduction in erythroblast numbers due to EPO deficiency. In patients with advanced CKD, the liver contributes significantly to plasma EPO levels. The contribution of uraemic toxins to the pathogenesis of renal anaemia is only poorly understood. Uraemic toxins have been shown to suppress erythroid colony formation *in vitro* as well as *EPO* transcription in hepatoma cells, the latter indicating possible suppressive effects on hepatic and renal EPO production *in vivo*.[147,245]
Abbreviations: CKD, chronic kidney disease; EPO, erythropoietin; Fe, iron.

Table 1

HIF activators in clinical trials

Compound (sponsor)	Trials registered with ClinicalTrials.gov*	Study characteristics
AKB-6548 (Akebia Therapeutics)	Healthy individuals: NCT02327546	Pharmacokinetic study to assess the effect of ferrous sulphate on bioavailability of the compound
	Nondialysis-dependent CKD: NCT01906489 (completed November 2014)	Phase II study to evaluate the haemoglobin responses, safety and tolerability of AKB-6548; 210 patients; dosing for 20 weeks
	Dialysis-dependent CKD: NCT02260193	Phase II study to evaluate the haemoglobin responses, safety and tolerability of AKB-6548; 60 patients on HD; dosing for 16 weeks
BAY85-3934/Molidustat (Bayer)	Non-dialysis-dependent CKD: NCT02021370 (Dialogue 1)	Phase II safety and efficacy fixed-dose correction study; 120 EPO-naive patients; duration 16 weeks.
	Non-dialysis-dependent CKD: NCT02021409 (Dialogue 2)	Phase II safety and efficacy study; 120 patients; duration 16 weeks; active comparator darbepoetin alfa
	Non-dialysis-dependent CKD: NCT02055482 (Dialogue 3)	Phase II long-term safety and efficacy extension study recruiting patients from NCT02021370 and NCT02021409; 240 patients; duration ≤36 months
	Dialysis-dependent CKD: NCT01975818 (Dialogue 4)	Phase II safety and efficacy study; 148 patients on HD who switched from ESA; duration 16 weeks
	Dialysis-dependent CKD: NCT02064426 (Dialogue 5)	Phase II safety and efficacy long-term extension study; 148 patients on HD from parent study NCT01975818; duration ≤36 months
DS-1093 (Daiichi Sankyo Inc.)	Healthy males: NCT02142400	Phase I study to assess pharmacokinetics, pharmacodynamics, safety and tolerability
	Non-dialysis and HD-dependent CKD: NCT02299661	Pilot pharmacokinetic and pharmacodynamic study
FG-4592/ASP1517/Roxadustat (FibroGen in collaboration with Astellas Pharma and AstraZeneca)	Nondialysis-dependent CKD: NCT01964196 (intermittent)	Phase II safety and efficacy study of switching from thrice weekly to once weekly dosing
	Nondialysis-dependent CKD: NCT01750190	Phase III safety and efficacy study; 450 patients; duration 52 weeks
	Nondialysis-dependent CKD: NCT01887600 (ALPS)	Phase III safety and efficacy study; 600 patients; treatment period 52-104 weeks
	Nondialysis-dependent CKD: NCT02021318 (Dolomites)	Phase III safety and efficacy study; 570 patients; treatment period 104 weeks; active comparator darbepoetin alfa
	Nondialysis-dependent CKD: NCT02174627	Phase III safety, efficacy and cardiovascular outcome study; 2,600 patients; event-driven treatment period ≤2 years
	Nondialysis and dialysis-dependent CKD: NCT01630889 (by invitation)	Phase II and III observational study to evaluate the long-term efficacy and safety of FG-4592; 150 patients with history of previous completion of a FG-4592 study; duration ≤2 years
	Dialysis-dependent CKD: NCT02174731	Phase III safety, efficacy and cardiovascular outcome study; 1,425 patients on HD or PD; event-driven up to 2 years; active comparator epoetin alfa

Compound (sponsor)	Trials registered with ClinicalTrials.gov*	Study characteristics
	Dialysis-dependent CKD: NCT02273726 (active, not yet recruiting)	Phase III safety and efficacy study; 1,200 patients on HD or PD; duration 52 weeks; active comparator epoetin alfa
	Dialysis-dependent CKD: NCT02278341 (Pyrenees)	Phase III safety and efficacy study; 750 patients on PD or HD; duration 104 weeks; active comparator epoetin alfa or darbepoetin alfa
	Dialysis-dependent CKD: NCT02052310 (Himalayas)	Phase III safety and efficacy study; 750 patients on newly initiated HD or newly initiated PD; maximum duration 3 years; active comparator epoetin alfa
GSK1278863 (GlaxoSmithKline)	Healthy individuals: NCT02293148	Pharmacokinetic study to evaluate effects on cardiac repolarization
	Nondialysis-dependent CKD: NCT01977573	Phase II safety and efficacy study; dosing for 24 weeks
	Dialysis-dependent CKD: NCT02243306	Pharmacokinetic study in patients on PD
	Dialysis-dependent CKD: NCT01977482 (not yet recruiting)	Phase II safety and efficacy study in patients who switch from rhEPO; 176 patients; dosing for 24 weeks; active comparator placebo for 4 weeks followed by rhEPO
	Dialysis-dependent CKD: NCT02075463	Phase II safety and efficacy study; 20 patients on HD who are chronically hypo-responsive to rhEPO therapy; duration 16 weeks.
JTZ-951 (Akros Pharma Inc.)	Dialysis-dependent CKD: NCT01978587, NCT01971164 (both studies completed)	Phase I safety and tolerability study, pharmacokinetic and pharmacodynamic study; patients with ESRD and anaemia on HD

*Trials are currently active and/or enrolling unless noted otherwise; updated 2 January 2015. Additional information can be found in company websites.[204-206] Abbreviations: CKD, chronic kidney disease; EPO, erythropoietin; ESA, erythropoietin-stimulating agent; ESRD, end-stage renal disease; HD, haemodialysis; HIF, hypoxia-inducible factor; PD, peritoneal dialysis; rhEPO, recombinant human EPO.