



CRITICAL REVIEW

Erythropoietin Expression and Regulation: *Piecing Together Known Mechanisms and Emerging Insights*

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ABSTRACT

Erythropoietin (EPO) is a circulating glycoprotein hormone essential for red blood cell production. The history of EPO stretches from early observations of hypoxia in the mid-19th century to its gene cloning and the clinical use of recombinant forms. Structurally, EPO's extensive glycosylation shapes stability, receptor binding, and therapeutic potential, inspiring engineered analogs with distinct pharmacokinetics. Developmentally, EPO expression shifts from embryonic neural crest and fetal hepatocytes to renal interstitial fibroblasts after birth. *EPO* gene regulation integrates hypoxia-inducible factors, transcriptional repressors, enhancers, with HIF-2 α as the principal activator, and post-translational mechanisms. Recent findings reveal genetic variants within the *EPO* gene in patients with erythrocytosis. Isoelectric focusing profiles of EPO in these patients was similar to the hepatic-derived EPO profiles in premature newborns, highlighting a dynamic and context-dependent regulation. These findings suggest that reactivation of *EPO* expression in the liver could be therapeutically valuable, given that hepatic-derived EPO exhibits enhanced activity. Clinically, erythropoiesis-stimulating agents transformed anemia management but raised safety concerns, leading to refined guidelines. The recent introduction of hypoxia-inducible factor prolyl hydroxylase inhibitors represents a new strategy that restores endogenous EPO production and coordinates iron metabolism through transient HIF stabilization. Outstanding challenges include the absence of faithful human EPO-producing cell models and incomplete understanding of the full molecular mechanisms controlling *EPO* expression and production. Combining insights from developmental biology, genetics, and epigenomics may open new avenues for therapies targeting disorders of erythropoiesis and oxygen homeostasis.

1 | A Brief Overview of the History of EPO

Erythropoietin (EPO) is a multi-functional cytokine hormone that is best known to maintain red blood cell mass by promoting the proliferation, differentiation, and survival of erythroid progenitor cells in the bone marrow [1].

The evolution of our knowledge about EPO has navigated significant milestones, constructing the bedrock of our present insights on its production, regulation and function. This journey began in the mid-19th century, around the 1860s,

when Denis Jourdanet (1815–1892), a French physician, highlighted the role of hypoxia at high altitude. Jourdanet recognized that some of his patients experienced symptoms such as rapid pulse, dizziness, and fainting spells that resembled those of anemia at sea level. He invented the terms “anoxémie” and “anémie barométrique” to describe these conditions and attributed these observations to low oxygen levels in the blood due to reduced barometric pressure at high altitudes [2]. In 1906, Paul Carnot (1869–1957) and Camille-Clothilde Deflandre (1871–1946) proposed that red blood cell production is subjected to humoral regulation. They reported that serum

from blood-depleted rabbits stimulated erythropoiesis when injected into normal animals, and they named the circulating factor “h mopo  t  ne” (hemopoietin) [3, 4]. Although their claim of a rapid 20%–40% increase in circulating red blood cells within 1–2 days was almost certainly an overestimation and might reflect transient hemoconcentration or splenic release rather than *de novo* erythropoiesis [5], their work laid the foundation for the discovery of erythropoietin. Few years later, different researchers confirmed the existence of this protein and reported evidence that a factor in anemic or hypoxic plasma could stimulate red blood cell production and increase hematocrit [6–9]. In 1948, the term “erythropoietin” was introduced by Eva Bonsdorff and Eeva Jalavisto from Helsinki [10]. In 1950, Reissman provided evidence of the presence of a humoral mechanism by experiments in parabiotic rats and demonstrated that when one partner is made hypoxic, erythropoiesis is stimulated in the normoxic rats [11]. A few years later, Leon O. Jacobson, Eugene Goldwasser, Walter Fried, and Louis F. Plzak established that the kidney is the site of EPO production [12]. They monitored that nephrectomised rats failed to elevate plasma Epo level on hypoxic stress [13].

In 1977, Takaji Miyake, Charles Kung, and Eugene Goldwasser purified human EPO from 2550 L of urine from patients with aplastic anemia and determined its amino acid sequence [14]. In 1985, two independent groups of scientists: the group Fu-Kuen Lin [15] and the group of Kenneth Jacobs [16], cloned the native human *EPO* gene. By 1986, industrial production of recombinant human erythropoietin (rhEPO) was under way. The first reports from clinical studies appeared in late 1986 and 1987, confirming that the rhEPO could correct anemia in patients with kidney failure. The first rhEPO was approved by the FDA for the treatment of renal anemia in 1989, marking a substantial milestone in its therapeutic application [17].

2 | Post-Translational EPO Modifications

At the structural level, the 193 amino acid long premature polypeptide is post-translationally cleaved, releasing a 27 amino acids N-terminal signal peptide and giving rise to the mature form of human EPO, which consists of 165 amino acids (UniProt P01588). EPO is an acidic sialo-glycoprotein of about 30.4 kDa, with three N-linked carbohydrates at asparagine residues 24, 38 and 83 and one O-linked carbohydrate at serine 126 [18]. The three N-glycans contribute to around 12 kDa of the glycoprotein's total mass [19]. Glycosylation influences the stability, solubility, and bioactivity of EPO [20]. The attached carbohydrates can modulate EPO's receptor binding affinity, serum half-life, and clearance rate from circulation. Circulating EPO exhibits several different glycoforms that differ in their electrical charges as distinguished by isoelectric focusing (IEF) analysis [21, 22]. Considerable efforts have been conducted to synthesize rhEPO using different expression systems [21, 23–25], including mammalian CHO and HEK cells. The glycan composition of rhEPO is influenced by factors such as the host cell line, transfected plasmid, culture conditions, and purification methods. Even minor variations in these parameters can impact the quality, efficacy, and safety of the final biopharmaceutical product [26]. However, achieving the endogenous glycosylation pattern of human EPO remains a major challenge for the pharmaceutical industries.

Several rhEPO have been developed with distinct glycosylation profiles and pharmacokinetic properties. For instance, epoetin alfa and beta, both produced in CHO cells, have similar amino acid sequences but slight differences in glycosylation that affect the half-life (~19 h for epoetin alfa and ~24 h for epoetin beta when administered subcutaneously) [27]. Epoetin delta, which is produced in a human cell line, has more authentic human glycosylation patterns, potentially reducing immunogenicity [28]. Darbepoetin alfa is an engineered analog with two additional N-linked glycosylation sites, resulting in increased sialic acid content and a longer half-life (48–72 h), allowing for less frequent dosing [29]. Pegylated epoetin beta (Mircera) is conjugated with polyethylene glycol, which extends the half-life to approximately 134 h [30, 31]. These structural and glycosylation modifications influence pharmacokinetics and receptor interactions, which are critical for the development of next-generation EPO therapeutics with customized efficacy and safety profiles.

3 | Tissue-Specific Sites of EPO Expression and Production

Epo [32, 33] and its mRNA [34–36] were, for the first time, extracted from the kidneys of hypoxic rodents, where Epo was mainly detected in the renal cortex rather than the medulla [32]. In situ hybridization analysis identified that renal EPO producing (REP) cells are localized in the interstitial space between tubular cells of the outer medulla and cortex [37–40]. These REP cells, a specific subset of interstitial fibroblasts originating from the neural crest cells [41], are primarily situated in the renal cortex and at the corticomedullary boundary [42, 43] and are responsible for EPO production. A recent study developed by Roland Wenger [44] team elaborated that REP cells express markers common to fibroblasts (e.g., PDGFR , CD73, and Col1A1), pericytes, neurons and mesenchymal stem cell-like cells. This suggests a high degree of plasticity and potential heterogeneity in the REP cell population, which may contribute to variable EPO responsiveness in physiological and pathological conditions. It is demonstrated that a drop in blood oxygen concentration triggers a transient transcriptional burst in EPO expression in the REP cells [44–46]. However, studies that define the factors that selectively mark active REP cells under hypoxia from inactive cells still lag behind.

The expression of the *EPO* gene is developmentally controlled and occurs in an inducible and tissue-specific manner. Mouse experiments revealed that embryonic neural tissues in the mid-gestation embryo transiently express Epo. *Epo* mRNA was first detected in the neural crest cells (NCCs) at E8.5, then a gradual decrease was detected during development to reach undetectable levels at E11.5. Whereas *Epo* mRNA expression in the fetal liver achieved a gradual increase over this period [42, 47–48]. This transient expression is believed to be essential to stimulate primitive erythropoiesis in the yolk sac, thus facilitating oxygen transport in mid-stage embryos. During the fetal period, hepatocytes of the liver become the primary site of *EPO* expression and definitive erythropoiesis [49]. Later, by the last third of gestation in mammalian development till birth, a gradual liver-to-kidney switch occurs through which the interstitial peritubular cells in the renal cortex become the main site of EPO production. In adults, the kidney contributes to approximately 90%

of circulating EPO, while the remaining 10% originates from the hepatocytes and hepatic stellate cells of the liver [22, 50]. Moreover, several other organs and tissues, including the bone marrow, central nervous system, intestine, spleen, osteoblasts, testis, and uterus support EPO expression in small amounts [51–54]. Recently, a group of researchers identified a population of kidney stromal cells, termed Norn cells, as the major site of EPO production in mice [55], and demonstrated that these cells are molecularly distinct from fibroblasts and pericytes.

The studies by Storti et al. [56] and Orlando et al. [57] underscore the complexity of *EPO* gene regulation and stress the implication of the 5' and 3' hypoxia-response elements (HRE) in this regulation under different cellular contexts. While the 3' HRE deletion in hepatic cells was sufficient to perturb the hypoxic *EPO* expression, the combined deletion of the 5' and 3' HREs was needed to abrogate *EPO* expression in neuroblastoma cell lines. Their work highlights that the hypoxia-dependent and tissue-specific expression of the *EPO* gene relies on a complex interaction between multiple HREs, HIFs (hypoxia-inducible factors), and other regulatory factors at the *EPO* locus [57].

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4 | *EPO* Regulation

4.1 | *EPO* Regulation by the Hypoxia-Inducible Transcription Factors (HIFs)

EPO production is greatly modulated by a decisive parameter which is the renal tissue oxygen tension (pO_2), that in turn depends on both arterial oxygen content (CaO_2) and arterial oxygen tension (PaO_2) [58]. The regulation of the *EPO* gene is also tightly associated with the HIF pathway, representing a vital molecular mechanism in response to oxygen levels, through which hypoxia-induced binding of HIF to its binding site at the 5' end of the *EPO* gene initiates *EPO* transcriptional activation [59, 60].

HIF is a heterodimeric transcription factor consisting of an oxygen-sensitive alpha subunit (HIF-1 α , HIF-2 α and HIF-3 α) and a constitutively expressed beta subunit (HIF- β). Under normoxic conditions, Prolyl Hydroxylase Domain proteins 2 (PHD1, PHD2, and PHD3, also known as the *EGLN* family) hydroxylate the labile α subunit of the heterodimeric transcription factor (HIF), thereby targeting it for degradation by the von Hippel–Lindau (VHL) tumor suppressor protein [61–64]. VHL is a critical substrate recognition adaptor protein which functions as part of an E3 ubiquitin ligase complex that also comprises elongin B, elongin C, cullin-2 and ring box 1 (rbx1) [65]. Under hypoxic conditions, this ubiquitin-proteasome degradation is attenuated, HIF- α is stabilized [1, 66], binds to HIF- β (also known as ARNT), which subsequently bind to the HREs in the regulatory regions of the *EPO* gene and initiates its transcriptional activation. Consequently, EPO stimulates erythropoiesis by promoting the survival, proliferation and differentiation of the bone

marrow erythroid progenitor cells and thereby enhancing the blood oxygen-carrying capacity [67].

The distinguished discovery of how mammalian cells sense and adapt to oxygen availability laid the foundation for the 2019 Nobel Prize award in Physiology or Medicine to William G. Kaelin Jr., Sir Peter J. Ratcliffe and Gregg L. Semenza [68, 69]. Semenza's pioneering research demonstrated that HIF, as a DNA-binding transcription factor, triggers *EPO* gene expression. Subsequent investigations evidenced that HIF, across various cell types, also activates the expression of other hypoxia-response genes that are involved in the cellular response processes to hypoxic challenges. These processes include angiogenesis, iron metabolism, glucose uptake, cell survival, and apoptosis, as well as erythropoiesis [70]. On the other hand, the groundbreaking finding of William G. Kaelin Jr. emerged from his studies on the VHL disease, which is a rare, inherited genetic disorder characterized by the development of tumors in multiple organs, such as familial hemangioblastoma, pheochromocytoma, renal cell carcinoma (RCC), and pancreatic neuroendocrine tumors. His research revealed the function of VHL protein as a tumor suppressor, and established that *VHL* gene deletion increases the expression of multiple hypoxia-induced genes [71, 72]. Few years later, Sir Peter J. Ratcliffe demonstrated the critical role for the VHL tumor suppressor in HIF- α regulation and deep-rooted the physical association between VHL and HIF- α subunit by co-immunoprecipitation experiments [73]. Later research showed that once recognized by VHL, HIF- α is ubiquitinated and directed to the proteasome for degradation [74]. Subsequently, Ratcliffe [75] and Kaelin [76] both identified that hydroxylation of HIF-1 α at specific proline residues is a critical step for its recognition by VHL and subsequent degradation. This hydroxylation of HIF- α subunits was later found to be hydroxylated by a group of prolyl hydroxylase enzymes. These enzymes are non-heme and Fe²⁺-dependent and belong to the 2-oxoglutarate-dependent-oxygenase superfamily [77–79]. Beyond its tumor-suppressive function, germline loss-of-function mutations in *VHL* cause congenital erythrocytosis (ECYT2 [OMIM: 263400]), by stabilizing HIF- α under normoxic conditions, resulting in constitutive activation of hypoxia-inducible genes, including the *EPO* gene. The molecular basis of VHL-related congenital erythrocytosis was first elucidated in the autonomous Russian Republic of Chuvashia, where the disorder is endemic. A homozygous c.598C>T p.Arg200Trp (R200W) mutation in the *VHL* gene mutation was identified as the cause of Chuvash polycythemia, a form of congenital erythrocytosis characterized by elevated serum erythropoietin levels and increased red blood cell mass [80]. Subsequent studies have identified additional mutations occurring either in the homozygous or compound heterozygous state [81, 82]. Affected patients typically do not develop tumors (except for rare cases of hemangioblastomas) as the associated polycythemia results from mild loss-of-function of *VHL* (hypomorphic mutations) [82, 83]. It has also been shown that the kidney is the main site of increased EPO production in these patients [84].

In humans, there are two paralogs of the HIF- β subunit: ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator) and ARNT2 [85]. Either HIF-1 α or HIF-2 α can heterodimerize with any of the HIF- β subunits, thereby assembling into functional HIF transcription factor complexes. Across various species, under

hypoxic conditions, the HIF- α /HIF- β complex stimulates target gene expression necessary to restore oxygen homeostasis [86–89].

Research data from different cell models and transgenic mice have documented the contribution of HIF-1 α and HIF-2 α to the stimulation of *EPO* expression [90–93]. However, several studies have evidently established that the two isoforms are not redundant. For instance, it was demonstrated that *Hif-1 α* deficiency contributes to impaired erythropoiesis during embryogenesis [94]. In vivo studies in conditional liver knockout mice showed that the hypoxic induction of hepatic *Epo* expression is mediated by Hif-2 α , but not Hif-1 α ⁹⁵. Moreover, hepatic Hif-2 α seemed to be vital for erythropoiesis during infancy before the liver-to-kidney switch in EPO production is accomplished [95]. In adult mice, acute postnatal global deletion of *Hif-2 α* , but not *Hif-1 α* , resulted in anemia, persuasively illustrating that HIF-2 α is the main regulator of *Epo* expression under physiologic and stress conditions [92].

5 | Transcriptional Regulation of *EPO*

EPO gene regulation is a complex and finely adjusted process that involves various transcription factors and elements. In vitro studies have shown that certain transcription factors, such as the human GATA proteins, play a crucial role in modulating *EPO* gene expression. For instance, in vitro experimentation established that human GATA transcription factors (GATA-1, 2, and 3) negatively regulate *EPO* expression through binding to the GATA element in the *EPO* promoter [96]. Furthermore,

Obara et al. provided in vivo evidence for essential GATA-based repression for the inducible and cell type–specific expression of the *Epo* gene. By creating a single nucleotide mutation in the GATA motif within the *Epo* proximal promoter in a BAC transgenic line, constitutive ectopic expression was observed of transgenic GFP in renal distal tubules, collecting ducts, and certain populations of epithelial cells in other tissues [97]. In contrast, C. Dame monitored that the inhibition of GATA-4 by RNA interference caused a significant reduction in *EPO* transcription in Hep3B cells, illustrating the importance of GATA-4 for the transcriptional activation of the *EPO* gene [98]. Furthermore, several studies highlighted differential regulatory elements for kidney and liver *EPO* production, with the liver-inducible element located within the 0.7kb 3' flanking sequence and the kidney-inducible element positioned between 9.5kb and 14kb in the 5' flanking sequence [99–101]. Active repressive elements lie within 6kb upstream (5') of the gene, known as the Negative Regulatory Element (NRE), while a specific Negative Regulatory Liver Element (NRLE) is located between 2.2 and 7kb downstream (3') of *EPO* gene [102] (Figure 1). Both contribute to repressing *EPO* transcription under normoxic conditions, ensuring tissue-specific and oxygen-sensitive gene expression. Besides, *EPO* expression is believed to be regulated by other transcription factors such as the hepatic nuclear factor 4 (HNF-4), Wilms tumor suppressor (WT1), retinoic acid receptor- α (RXR- α), Specificity protein 1 (Sp-1), SMAD3 transcription factors and early growth response Egr [59, 103–108]. It has been demonstrated these factors cooperate to stabilize the multifactorial complex interacting with the 3' enhancer region of the *EPO* gene to favorize promoter-enhancer contact and guide *EPO* expression. Within the 3' enhancer, a critical DR-2 (direct repeat

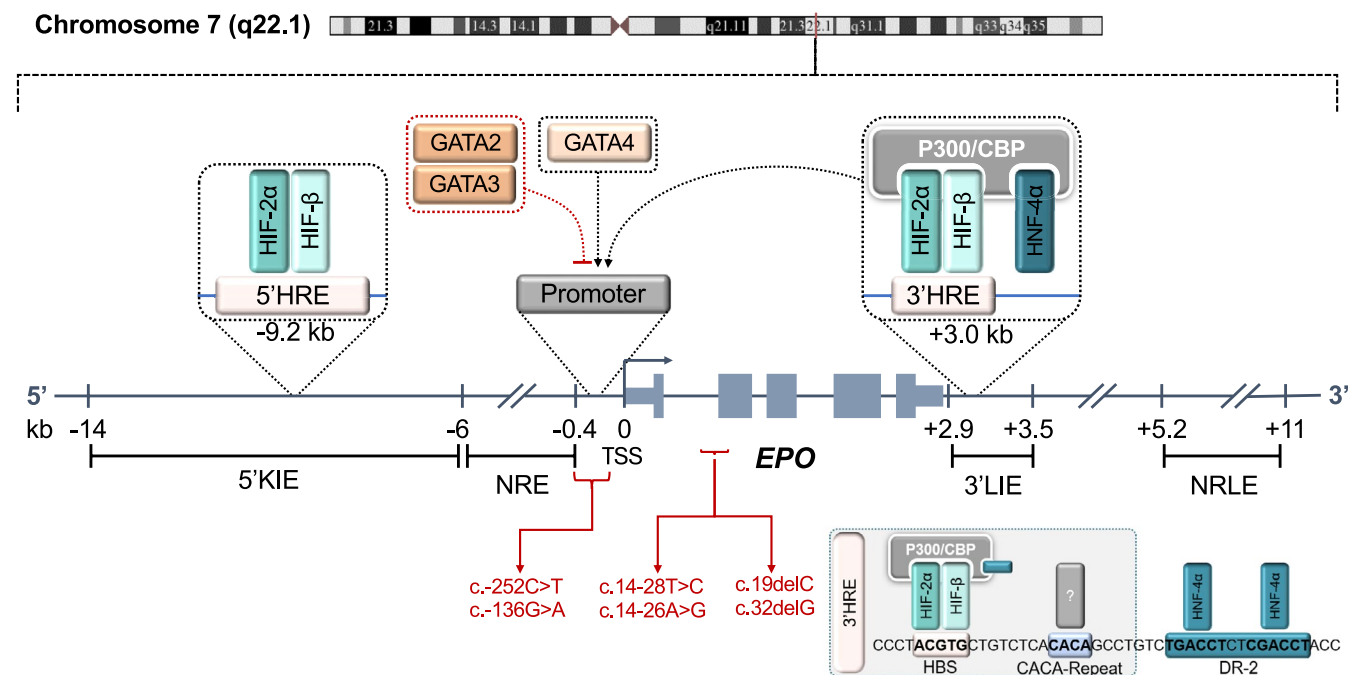


FIGURE 1 | Schematic representation of the *EPO* gene, its important regulatory sequences, and the genetic variants of the *EPO* gene associated with erythrocytosis. Under hypoxic conditions, stabilized HIF-2 α dimerizes with the HIF- β subunit and translocates to the nucleus where it binds to the hypoxia response element (HRE). Detailed view of the 3' HRE with a HIF- α binding site (HBS) and an adjacent CACA-repeat (HIF ancillary sequence), both required for full hypoxic activation. Nearby lies a nuclear receptor half-site (DR-2), which serves as a binding site for hepatic nuclear factor 4 (HNF4). We thank Dr. med. Thomas Sommerhalder for generously providing the initial template used to design Figure 1. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

with two nucleotide spacer) motif serves as a binding site for nuclear receptors, particularly HNF-4 α and RXR- α . The DR-2 site is essential for hypoxic responsiveness and plays a central role in mediating the tissue-specific regulation of *EPO* transcription [102]. The HIFs and HNF-4 cooperate and form a complex with the transcriptional co-activators (p300) and cAMP response element-binding protein (CREB)-binding protein (CBP) for the activation of hypoxia-induced and tissue-specific *EPO* transcription [104, 109]. Recent analysis proposed that GATA6, TCF21, and C/EBP δ might be candidate transcription factors that regulate Norn cells (a population of kidney stromal cells) identity and Epo secretion [55].

6 | Post-Transcriptional Regulation of EPO

It has been shown that Epo is regulated at the post-transcriptional level primarily through the action of the erythropoietin mRNA-binding protein (ERBP). The stability of *Epo* mRNA was revealed to be influenced by the binding of ERBP to specific regions within the mRNA, particularly to a pyrimidine-rich 120-bp region in the 3' untranslated region (3'-UTR), thus its half-life [110–112]. Moreover, *EPO* mRNA stability has been shown to depend on the activity of p38 α MAP kinase [113]. This was underlined by the finding that p38 α -deficient embryos were anemic and demonstrated defective definitive erythropoiesis, which was triggered by a marked decrease in *Epo* gene expression. On the other hand, it was suggested that tissue-specific retention of introns 2 and 4 in *EPO* is another physiological mechanism of post-transcriptional regulation, particularly in response to ischemia [114]. This tissue specificity in intron retention might be due to variable splicing machinery or epigenetic factors that seem to be crucial for regulating *EPO* expression adaptively in response to physiological demands, such as oxygen availability and nutrient sensing.

7 | Epigenetic Modulation of *EPO* Gene Expression

Beyond being regulated by HIF, epigenetic changes such as histone acetylation and methylation also play a concrete role in regulating the expression of *EPO*. Steinmann et al. [115] showed that *EPO* activation under hypoxic conditions was accompanied by chromatin modification through which the promoter and enhancer regions were subjected to increased histone H3 acetylation and decreased histone H3 lysine 9 trimethylation [115]. Yin and Blanchard showed that a CpG island is found in the promoter and 5'-UTR of the human *EPO* gene, which possessed a differential methylation level that was inversely correlating with the expression level [116]. This might explain the tissue-specific expression of *EPO*. Notably, R ssler et al. [117] demonstrated that HIF binding to its consensus DNA recognition site in the *EPO* 3'HRE is CpG DNA methylation sensitive. In another study, Wang and colleagues well-established that the histone acetyl transferase (HAT) co-activators p300 is required for hypoxia-induced acetylation at lysine 5, 8, 12, and/or 16 of histone 4 and thus acts as a central coactivator in hypoxia-induced transcription of the *EPO* gene [118]. More recently, the histone H3K9-specific demethylase

JMJD1A (also known as KDM3A) has been identified as a new epigenetic regulator of *EPO* expression under hypoxic conditions [119]. By forming a co-activator complex with HIF-2 α , JMJD1A can bind *EPO* HRE and demethylase the transcription suppression marker H3K9me2 leading to augmented *EPO* gene transcription. The fact that these epigenetic alterations are reversible provides a viable way to modulate the expression of *EPO*. Especially, such investigations can bring up possible targets for new pharmacological strategies and epigenetic therapies intended to treat human blood diseases including erythrocytosis and anemia.

8 | The Dynamic Regulation of Erythropoietin Expression: Developmental Transitions and Pathological Reactivations

Recent discoveries, including our own contributions, started to reshape the classical understanding of *EPO* regulation and expression. In a 2013 study, L nnberg et al. [120] identified a hepatic form of EPO in patients with end-stage renal disease undergoing hemodialysis. Notably, in some patients, liver-derived EPO constituted 90–100% of total EPO levels in urine, indicating the liver's substantial EPO production capacity. This finding suggests that, under certain conditions, the liver can compensate for renal EPO production, potentially offering an alternative source of endogenous EPO in cases of renal insufficiency [121]. A recent publication by our group [84] highlighted that a hepatic-like EPO exhibits an atypical glycosylation pattern and enhanced biological activity, where we reported its association with pathological erythrocytosis independent of renal function. This work detected similar EPO patterns in patients with hereditary erythrocytosis due to regulatory *EPO* gene variants, as well as in individuals with liver disease-associated erythrocytosis and premature newborns. These findings strongly suggest the presence of a “non-renal” form of EPO, distinct from renal-derived EPO (Figure 2). These observations challenge the traditional view that the kidney is the only organ that produces EPO postnatally and emphasize the importance of alternative sources in both physiological and disease states. Taken together, these studies suggest a more dynamic and context-dependent regulation of *EPO* expression than previously thought and open new avenues for research and therapeutic intervention.

9 | Genetic Blueprints to Better Understanding of *EPO* Regulation

Inherited or acquired dysregulation in EPO production, regulation or activity has been linked to multiple human diseases characterized by either too few or too many red blood cells [122]. Identifying all the genetic determinants such as single nucleotide polymorphisms (SNPs) that might impact EPO is critical to alleviate our understanding of EPO's implication in these diseases. A study by Beverborg et al. identified the *HBS1L-MYB* locus as a regulator of erythropoiesis, and more prominently of EPO levels [123]. The intergenic locus between the *HBS1L* (GTP-binding elongation factor) and *MYB* (myeloblastosis oncogene) genes had previously been found to be associated with deregulated fetal hemoglobin (HbF) [124]. In 2691 randomly

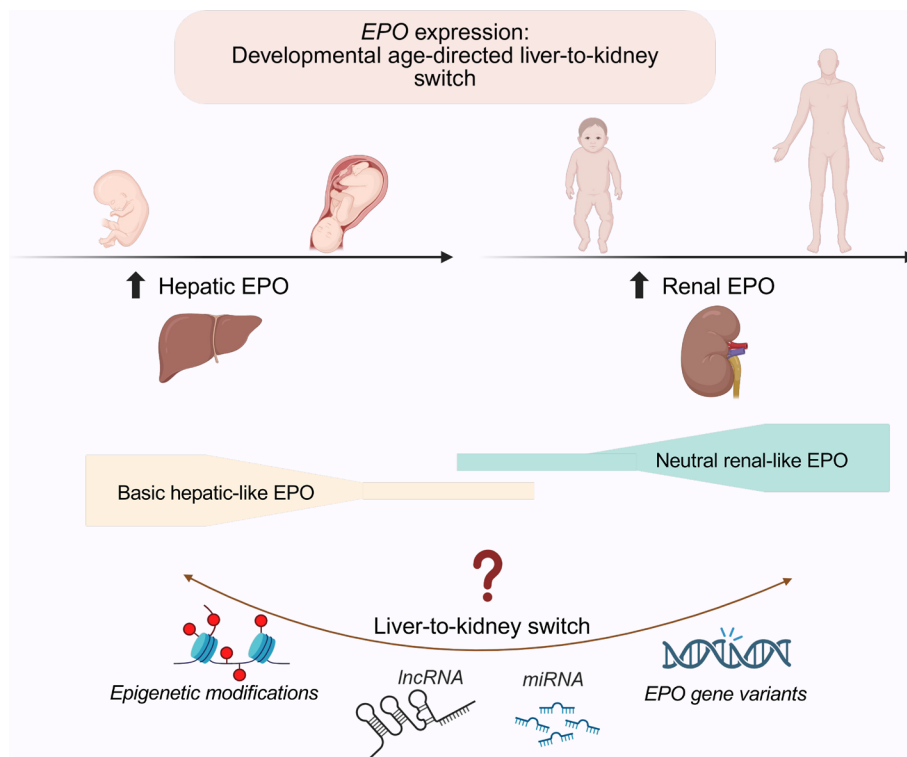


FIGURE 2 | Developmental regulation and tissue-specific expression of Erythropoietin (EPO). This schematic illustrates the ontogenic transition of EPO production from the fetal liver to the adult kidney. The top panel shows the shift in EPO isoform glycosylation from basic hepatic-like profiles to neutral renal-like profiles. The timeline depicts increased hepatic EPO expression during fetal life, followed by a postnatal switch to renal EPO production. The lower section highlights possible mechanisms controlling or influencing this transition, including epigenetic modifications, long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and EPO gene variants. Created with [BioRender.com](https://www.biorender.com). [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/ajh.1302/2006)]

selected non-anemic subjects, 3477282 imputed autosomal SNPs were used in a genome wide association (GWA) analysis. The researchers determined 108 SNPs located near the *HBS1L-MYB* locus and significantly associated with higher EPO levels (significance threshold $p < 5.0 \times 10^{-8}$). Among these, SNP rs7776054 exhibited the strongest association with EPO levels, emphasizing its likelihood of explaining 3.1% of the total phenotypic variation in EPO levels [123]. This association between the *HBS1L-MYB* locus and EPO levels was further confirmed by the team of Roland Wenger [125], where the authors further identified an association with the *MAP2K5-SKOR1-PIAS1* locus and circulatory EPO levels. Through a candidate-based approach, the researchers selected 33 genes known to impact the expression of the *EPO* gene, and conducted an association analysis using the PASCAL algorithm. Their analysis retrieved that the *OS9* gene possessed the highest association with circulating EPO levels. Remarkably, one *OS9* gene variant (chr12:58109559G>A) has previously been reported in a single case with erythrocytosis [126]. On the other hand, a significant correlation between EPO serum levels and *ACE* gene polymorphisms was illustrated in a cohort of 70 chronic kidney disease (CKD) patients (40 males and 30 females) [127]. Patients with *ACE* DD and *ACE* ID genotypes (rs1799752) displayed a substantial increase in their serum EPO levels. In contrast, an additional study underlined an insignificant difference in the plasma EPO levels in patients with *ACE* G2350A (rs4343) II, ID, and DD [128]. Another GWAS examined ten cytokines extracted from neonatal blood birth spots [129] and reported that

the *CRP* locus is potentially associated with circulating EPO. Establishing the mechanistic links between these genes/loci and *EPO* gene expression as well as circulating levels have yet to be elucidated.

10 | EPO and Erythropoiesis

Erythropoiesis, a vital process whereby mature red blood cells are produced from hematopoietic stem and progenitor cells, takes place in the bone marrow. Erythropoietic homeostasis is sensibly orchestrated where its regulation depends upon both systemic and local cues. EPO plays a crucial role in the regulation of erythropoiesis especially when oxygen availability in the body's tissues is compromised [130]. When oxygen levels drop, the renal tubular interstitial cells sense relative hypoxia and respond by secreting EPO into the bloodstream via a classic endocrine mechanism. Subsequently, EPO travels to the bone marrow, where it binds to its receptor (EPOR) on the erythroid progenitor cells. EPOR, a member of the cytokine class I receptor superfamily [131], forms a homodimer upon EPO binding, which initiates signaling through the JAK2/STAT5, MAPK, and PI3K/AKT kinases [132]. EPO/EPOR signaling promotes erythroid survival, proliferation, and differentiation through the activation of the JAK2/STAT3/STAT5 signaling pathway. Interestingly, due to the high affinity of EPOR, even trace amounts of EPO present in human serum are regulated by a classic negative feedback loop [133]. This delicate balance helps maintain erythropoiesis homeostasis.

11 | Genetic Variants in the *EPO* Gene That Are Associated With Erythrocytosis

The expansion of the erythrocyte count in the peripheral blood, including raised red blood cell numbers, hematocrit and hemoglobin contents is defined as erythrocytosis. Several genetic variants, in genes belonging to diverse pathways including erythropoiesis and oxygen transport and sensing, have been identified to cause erythrocytosis. Erythrocytosis mechanism can be primary or secondary; primary erythrocytosis results from intrinsic abnormal proliferation of the erythroid progenitor cells [134], typically associated with low serum EPO (e.g., *JAK2* mutations in polycythemia vera or germline *EPOR* mutations). In contrast, secondary erythrocytosis is a heterogeneous group of disorders characterized by increased red blood cell mass due to conditions that cause tissue hypoxia which result in inappropriate EPO production [81, 135–136]. This latter includes both acquired causes (chronic hypoxia, tumors) and hereditary causes due to mutations in oxygen-sensing pathway genes such as *VHL* [80], *EPAS1/HIF2A* [60, 137], or *EGLN1/PHD2* [138, 139]. In 2013, the first report of an *EPO* gene mutation associated with familial erythrocytosis was reported [140], and the gene has since been included in the OMIM database as a new genetic cause of the disease [126, 141–143]. Seven genetic variants of the *EPO* gene have been documented to be associated with erythrocytosis or elevated hematocrit levels, where these variants may play a role in the regulation and expression of *EPO* [144]. Khabour et al. reported two *EPO* variants, i.e., rs1617640 and rs551238 (c.*772G>T), located in the 3'-UTR regions of *EPO* where they were found at a significantly higher frequency among individuals with elevated hematocrit levels [145]. On top of that, two other variants, with unknown functional implication and unknown association with erythrocytosis have been retrieved through genomic database screening. The two SNPs, i.e., rs796952255 (c.*689G>A) and rs539229161 (c.*719C>T), are located within the regulatory regions of *EPO* (more precisely in the 3' enhancer sequence); the first SNP is located in HIF-binding site (HBS) and the second is located within HNF4A binding region (DR-2) [144]. Taylor and colleagues have identified a strong candidate pathogenic variant within the 5'-UTR region of *EPO* gene at a highly conserved base (chr7:100318468G>A). This variant (c.-136G>A) was found in 8 affected members belonging to two independent families with erythrocytosis [143]. The group of Radek Skoda identified a heterozygous single-base deletion mutation (c.32delG, referred to as Δ G) in exon 2 of the *EPO* gene, acting as a gain-of-function variant, and associating with familial erythrocytosis. Elegantly, the authors manifested the presence of an alternative promoter (named P2) in intron 1, in addition to the original *EPO* promoter (P1), from which non-coding alternative *EPO* mRNA transcripts originate. The researchers highlighted that this mutation creates a frameshift which truncates EPO signal peptide and converts the non-coding mRNA transcribed from P2 into an mRNA that produces functional EPO protein. The authors highlighted that P2 transcripts were more abundant in the liver than in the kidney and appeared to be the predominant *EPO* mRNA species in the liver, suggesting that hepatic P2 transcripts might be the main source of EPO in affected individuals [142].

In this context, a recent work by our group [84] identified, in six unrelated families, three novel regulatory mutations in the *EPO*

gene. These mutations; the c.-252C>T variant located in the main promoter and the c.14-28T>C and c.14-26A>G variants located in intron 1 in the proximity of exon 2; seemed to affect crucial non-coding elements controlling *EPO* expression. Through *EPO*-promoter/enhancer-driven luciferase reporter gene assays, mutants exhibited significantly higher activity under hypoxic conditions compared to the wild-type, thus implying enhanced responsiveness to low oxygen levels. Furthermore, we generated induced pluripotent stem cells (iPSCs) from patients carrying the variants and differentiated them into hepatocyte-like cells, which naturally produce EPO. These cells showed markedly increased *EPO* mRNA expression under hypoxic conditions compared to controls, confirming the variants' effect on endogenous gene regulation in a relevant cell type.

So far, for some of these variants, profound functional examinations would be compulsory to definitively prove causality for these variants. The latter would be critical to unleash the impact of these variants on *EPO* gene regulation and expression on one hand and on erythropoiesis and associated clinical conditions, primarily erythrocytosis on the other hand. Above all, it would be imperative to dissect the pathophysiological mechanisms of *EPO* variants and decipher whether these mutations impact the origin of EPO, more specifically kidney and liver, as some of these mutations might target tissue-specific regulatory elements of the *EPO* gene.

12 | Pharmacologic Modulation of the HIF-*EPO* Pathway: Evolution From Erythropoiesis Stimulating Agents (ESAs) to Prolyl Hydroxylase Inhibitors (HIF-PHI)

The development of EPO-based treatments began in 1985, following the identification of the *EPO* gene and its in vitro production. The use of rhEPOs therefore began in the late 1980s, initially in chronic renal failure, and was subsequently extended to pediatrics, surgery, and, by the early 1990s, oncology. Although most early studies showed an improvement in the quality of life of patients treated with Erythropoiesis-Stimulating Agents (ESAs), particularly through increased hemoglobin levels and reduced dependence on blood transfusions, numerous adverse effects were gradually reported. These included severe thromboembolic events and decreased survival in cancer patients, which led to the implementation of strict guidelines for ESAs use. These recommendations are periodically reviewed by the American Society of Clinical Oncology and the American Society of Hematology [146].

Currently, ESAs may be prescribed to patients with chemotherapy-associated anemia whose treatment is not intended to be curative and whose hemoglobin has declined below 100 g/L. On the contrary, with the exception of patients with lower-risk myelodysplastic syndromes (MDS), ESA treatment is not recommended for cancer patients with non-chemotherapy-associated anemia. Importantly, iron supplementation may improve hemoglobin response and reduce transfusions requirement in patients receiving ESA therapy, whether or not iron deficiency is present [146].

In routine hematology practice, ESAs are mainly used in lower-risk MDS [147], as well as in primary and secondary

myelofibrosis, although their use in the latter setting remains off-label [148].

More recently, the pharmacologic landscape of EPO regulation has expanded with the introduction of hypoxia-inducible factor prolyl hydroxylase inhibitors (HIF-PHIs). These orally administered medications, such as roxadustat, daprodustat, vadadustat, and molidustat, act by transiently inhibiting prolyl hydroxylase domain (PHD) enzymes, thereby stabilizing HIF- α subunits and promoting endogenous EPO transcription, iron absorption and utilization, and erythropoiesis [149, 150]. HIF-PHIs have demonstrated efficacy in treating anemia associated with CKD, both in dialysis-dependent and non-dialysis-dependent settings, and are increasingly being evaluated for other hypoxia-related conditions [150, 151]. Their dual effect on EPO and iron metabolism offers a physiological alternative to exogenous EPO administration. It has been shown that inhibition of PHD2 alone is sufficient to activate the expression of EPO in the kidney, whereas inhibition of all three PHD isoforms is required to induce EPO expression in the liver [152]. This differential sensitivity highlights distinct regulatory requirements between renal and hepatic EPO production and warrants re-examination in light of a potential gain-of-function of hepatic EPO [84]. However, because HIF activation exerts various pleiotropic effects extending well beyond erythropoiesis, long-term safety data, particularly concerning tumor progression, vascular remodeling, and cardiovascular risks, remain to be fully elucidated.

13 | Current Challenges and Future Perspectives

Most insights into EPO expression, regulation and function have been generated from mouse models and few cell lines, i.e., the human hepatocarcinoma cell lines (Hep3B, HepG2) and the neuroblastoma cell lines (Kelly, SHY5Y) [50, 57, 153]. There remains a limited understanding of the cellular and molecular factors that govern the regulation of the EPO gene in vivo. Indeed, the complete network of co-regulators and co-factors that modulate EPO promoter activity and orchestrate EPO transcription in the kidney as well as in fetal or adult hepatocytes has not been fully delineated. Several efforts have been made to generate EPO-producing cells that mimic the human renal cells and hepatocytes. However, these attempts failed to produce a system capable of establishing renal and hepatic cell lines with O₂-regulated EPO expression. Moreover, replicating, in vitro, the precise glycosylation pattern found in naturally occurring EPO has always posed a significant hurdle [21]. In addition, other experimental challenges are always encountered mainly due to the low to undetectable basal EPO transcript levels, except for under severe hypoxic conditions. Once more, the need for a human-derived EPO-producing cell model that is able to simultaneously replicate the maturity of the human adult cells, recapitulate hypoxia-inducible expression and produce the correctly glycosylated EPO protein remains an urgent demand.

Whereas DNA methylation and histone modifications have been shown to have a role in controlling EPO expression [115, 118], little is known about the precise epigenetic alterations and their dynamic changes in response to hypoxia and other physiological and pathological conditions. The detailed mapping of the chromatin states at the EPO gene locus is likely crucial for fine-tuning

EPO expression under various physiological and pathological circumstances. Non-coding RNAs, with a focus on microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), have grabbed the attention of the research communities for their imperative role in controlling gene expression, either at the transcriptional or post-transcriptional levels [154]. Many miRNAs and lncRNAs exhibit tissue-specific and developmental-stage-specific expression [154–156]. Furthermore, several of them have been reported to display hypoxia-inducible regulation [157]. Therefore, it remains to be explored if these molecules may be involved in regulating EPO gene transcription, mRNA stability, or even translation in response to changing oxygen levels (Figure 2). Advanced sequencing techniques could map tissue-specific enhancer activity for the EPO gene under various physiological conditions in normal and mutant contexts and enrich our comprehension of the pathophysiological mechanisms driven by EPO variants.

All in all, addressing these issues will ameliorate our understanding of EPO gene regulation, thus potentially revealing new therapeutic targets for diseases related to erythropoiesis and oxygen homeostasis.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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