Erythrocytosis: the HIF pathway in control

Kristin Franke, Max Gassmann, and Ben Wielockx

Review Article

Organisms living under aerobic conditions need oxygen for the metabolic conversion of nutrition into energy. With the appearance of increasingly complex animals, a specialized transport system (erythrocytes) arose during evolution to provide oxygen to virtually every single cell in the body. Moreover, in case of low environmental partial pressure of oxygen, the number of erythrocytes automatically increases to preserve sustained oxygen delivery. This process relies predominantly on the cytokine erythropoietin (Epo) and its transcription factor hypoxia inducible factor (HIF), whereas the von Hippel-Lindau (VHL) ubiquitin ligase as well as the oxygen-sensitive prolyl hydroxylases (PHDs) represent essential regulators of this oxygen-sensing system. Deregulation of particular members of this pathway (e.g., PHD2, HIF2α, VHL) lead to disorders in blood homeostasis as a result of insufficient (anemia) or excessive (erythrocytosis) red blood cell production. (Blood. 2013;122(7):1122-1128)

Introduction

High altitude is accompanied by low atmospheric oxygen pressure, which sequentially leads to insufficient oxygen uptake and reduced tissue oxygenation. In general, inadequate oxygen supply is detrimental and might lead to the death of cells, tissues, or, ultimately, even the organism. To avoid this, complex cardiovascular, respiratory, and hematologic mechanisms have evolved, and one such long-term adaptation process is the elevation of erythrocyte numbers to boost the blood’s oxygen transport capacity.

As early as the 19th century, scientists recognized the correlation between low atmospheric oxygen pressure and elevated red blood cell numbers in humans and animals. Several decades later, it became evident that low oxygen does not directly act on hematopoietic cells but induces the production of a soluble factor called erythropoietin (Epo). In 1977, Epo was purified from the urine of anemic patients and in 1985 the corresponding EPO gene was isolated and cloned. Approximately 2 decades ago, the transcription factor hypoxia inducible factor (HIF) was first identified in hematopoietic cells as the regulator of Epo through its binding to a hypoxia-responsive element (HRE) present in the 3' enhancer region of the EPO gene. In subsequent work, HIF was found to be expressed widespread in mammalian cells and even in lower animals that do not produce Epo or red blood cells. In the past decadum, much knowledge has been acquired regarding the role of HIFs during red blood cell production (erythropoiesis). Accordingly, this review aims to discuss recent findings on these essential proteins in humans and mice, and the detrimental impact of their deregulation.

Epo: the driving force of erythropoiesis

Epo, a glycoprotein hormone, is the principal stimulator of erythropoiesis and is induced under hypoxic conditions. In 1957, the kidney was first identified as the primary Epo-producing organ in adult mammals, whereas the liver is the major source of Epo during embryogenesis (e.g., hepatocytes, Ito cells). In the kidney, a specialized Epo-producing cell (REPC) was identified in the cortex and outer medulla that was initially described as an interstitial fibroblastlike cell with neuronal characteristics. Interestingly, and in contrast to other organs, the kidney is able to increase the total amount of REPCs in an oxygen-dependent manner rather than increasing Epo expression per cell. Neurons and glial cells in the central nervous system represent an additional source of Epo; it has been suggested that the glycohormone functions in a paracrine fashion as a protective, ventilatory, or cognition-enhancing factor.

Erythropoiesis is a complex multistep process during which erythroid progenitors enucleate and develop into mature red blood cells. Upon Epo binding to its receptor, the EpoR signaling through the Janus kinase 2 (JAK2) activates multiple pathways including Stat5, phosphoinositide-3 kinase/Akt, and p42/44 mitogen-activated protein kinase. This reduces apoptosis and promotes expansion and differentiation of the progenitors. In adult mammals, erythropoiesis is mainly carried out by the bone marrow. However, in response to stress (e.g., anemia, bone marrow transplant, certain diseases), erythropoiesis may extend to extramedullary sites, such as the spleen and liver, thereby increasing erythrocyte output. As has been shown in mice, stress erythropoiesis is characterized by massive self-renewal of burst-forming unit–erythroid cells and is regulated by additional extrinsic factors such as the stress hormone cortisol, stem cell factor, and the bone morphogenetic protein 4. In humans, analogous pathways have not yet been identified, and the molecular basis is also not well-described. However, recently, erythroblastic island macrophages have been reported to facilitate human stress and pathological erythropoiesis.
HIFs (mainly HIF1α and HIF2α) becomes stabilized nearly instantaneously during low oxygen conditions and translocates to the nucleus, where it dimerizes with the constitutively expressed HIFβ subunit and promotes transcription of genes containing a HRE. In human cells, pan-genomic analyses of HIF binding to DNA have now revealed the existence of direct transcriptional targets of HIF in a given cell line. More than a decade ago, the groups of Ratcliffe and Kaelin discovered that both HIF1α and HIF2α are regulated at the posttranscriptional level by the HIF prolyl-hydroxylase domain enzymes (PHDs). These oxygen sensors hydroxylate the α-subunits and prime them for poly-ubiquitination by the von Hippel-Lindau (VHL) tumor suppressor complex, which ultimately leads to proteolytic degradation (Figure 1). To date, 4 PHDs have been identified in mammals, of which PHD2 (gene name: Egl nine homolog 1 (Egln1)) has been described as the key limiting enzyme targeting HIFα for degradation under normoxic and mild hypoxic conditions.

HIF1α exhibits a ubiquitous expression pattern, whereas HIF2α is found in a limited number of cell types including endothelial cells, cardiomyocytes, hepatocytes, glial cells, and interstitial cells of the kidney. Both isoforms have overlapping sets of target genes but can also play nonredundant roles, depending on the cell type and oxygen concentrations. Accordingly, HIF1α has been suggested to represent the response to acute hypoxia, whereas HIF2α is the predominant subunit to chronic exposure to low oxygen that occurs at high altitudes. In addition, several studies have demonstrated that both HIF isoforms can even display opposing roles in vivo, for example, in renal cell carcinoma growth and metastasis formation. Glycolysis enzymes like phosphoglycerate kinase 1 and lactate dehydrogenase A are predominantly HIF1α-dependent. In contrast, HIF2α has been described to induce matrix metalloproteinase 9 and the transcription factor oct-4, which is involved in stem cell function and the elevation of hemoglobin gene expression in humans (Figure 1). Until recently, it was unclear which of the HIF and PHD isoforms regulated erythropoiesis, and the expression of Epo in particular. Only with knowledge gained from patients with erythrocytosis and transgenic mice did it become evident that the HIF2α isoform and not HIF1α is the key player in EPO gene expression and erythropoiesis-enhancing processes (eg, iron absorption and transport).

**Mutations in HIF pathway proteins can lead to erythrocytosis in humans**

Erythrocytosis is an aberrant increase in red blood cell numbers and comprises a heterogeneous group of disorders. A general distinction is made between the hypersensitivity of the erythroid progenitors to Epo (primary erythrocytosis) and the excessive activation of EPO gene transcription (secondary erythrocytosis). The most common example of primary erythrocytosis is polycythemia vera. Here, erythroid progenitors carry a gain-of-function mutation in the JAK2 gene, which leads to constitutive activation of the EPO signaling pathway at the EPO-R level. On the other hand, patients bearing point mutations in specific members of the HIF pathway can develop secondary erythrocytosis (Table 1).
VHL

In 1997, the first type of erythrocytosis related to the HIF pathway was discovered by Prchal and colleagues. They described 103 individuals from 81 families living in the Chuvash region in Russia with erythrocytosis. Several patients were studied in detail and displayed markedly increased hematocrit levels accompanied by significantly higher levels of Epo. However, molecular analysis failed to demonstrate mutations in the EPO-R or previously described erythrocyte alterations (eg, high oxygen affinity hemoglobin). Subsequent genetic studies revealed a homozygous mutation in the VHL gene (C598T leading to the R200W amino acid change) in all affected individuals. This resulted in reduced affinity of VHL for the hydroxylated HIFs subunit and subsequent increase of Epo and red blood cells. Recently, the underlying molecular mechanism was discovered: the R200W VHL mutation alters the affinity hemoglobin). Subsequent genetic studies revealed a homozygous mutation in the VHL gene (C598T leading to the R200W amino acid change) in all affected individuals. This resulted in reduced affinity of VHL for the hydroxylated HIFs subunit and subsequent increase of Epo and red blood cells. Recently, the underlying molecular mechanism was discovered: the R200W VHL mutation alters the affinity of VHL for the hydroxylated HIF2α subunit and subsequent increase of Epo and red blood cells.

Table 1. HIF pathway–related mutations that have resulted in erythrocytosis and/or tumor development in humans

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of mutation</th>
<th>Mutation</th>
<th>Erythrocytosis</th>
<th>Tumor type</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>Germline (Homo)</td>
<td>R200W</td>
<td>+</td>
<td>—</td>
<td>45,55</td>
</tr>
<tr>
<td></td>
<td>(Homo)</td>
<td>H191D P138L</td>
<td>+</td>
<td>—</td>
<td>57-59</td>
</tr>
<tr>
<td></td>
<td>Germline (Hetero)</td>
<td></td>
<td></td>
<td>—</td>
<td>55-56</td>
</tr>
<tr>
<td></td>
<td>somatic</td>
<td></td>
<td></td>
<td>—</td>
<td>60</td>
</tr>
<tr>
<td>PHD2</td>
<td>Germline (Hetero)</td>
<td></td>
<td></td>
<td>—</td>
<td>55,61,64,66,67</td>
</tr>
<tr>
<td></td>
<td>(Homo)</td>
<td>H374R</td>
<td>+</td>
<td>Paraganglioma</td>
<td>68</td>
</tr>
<tr>
<td>HIF2α</td>
<td>Germline (Hetero)</td>
<td></td>
<td></td>
<td>—</td>
<td>55,69,70</td>
</tr>
<tr>
<td></td>
<td>(Homo)</td>
<td>F374Y</td>
<td>+</td>
<td>Pheochromocytoma/paraganglioma</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>somatic</td>
<td>A530V A530T</td>
<td>+</td>
<td>Paragangliomas/somatostatinoma</td>
<td>74</td>
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</table>

PHD2

Since 2006, several patients and families with heterozygous loss-of-function mutations in the PHD2 gene have been described. The first mutations discovered were the P317R and the P371H variants, which affect the catalytic rate and substrate binding of PHD2, leading to partial inhibition of HIF hydroxylation. A few of the reported PHD2 mutations, apart from erythrocytosis, also led to other pathologic conditions such as superficial thrombophlebitis, sagittal sinus thrombosis, and hypertension. However, the number of such patients is currently still too small to draw firm conclusions. In only 1 case has PHD2 also been described to be associated with tumor formation—in particular to a recurrent paraganglioma. This patient with this tumor is a heterozygous carrier of a PHD2 germline mutation (H374R), which affects one of the 3 conserved amino acids that coordinate Fe2+ binding, therefore contributing to the functionality of the enzyme. Interestingly, sequence analysis of the removed tumor mass showed that not one but both PHD2 alleles were mutated in the tumor cells (loss of heterozygosity). Functional analysis of the described PHD2 variants revealed that only the H374R variant has a detrimental effect, and all other studied PHD2 mutations show only weak deficiency in HIFα regulation. Such functional differences may permit PHD2 to act as a tumor suppressor in patients.

HIF2α

A new form of familial erythrocytosis was discovered in a family where the phenotype was associated with a heterozygous missense mutation in the HIF2α gene (EPAS1). The mutation is predicted to produce a G537W change in the amino acid sequence of HIF2α, which is very close to the primary site of hydroxylation (Pro-531). The resulting impairment of the hydroxylation of HIF2α and its subsequent VHL binding leads to an aberrant stabilization of this transcription factor during normoxia. Further studies have revealed numerous other HIF2α alterations, all near the primary hydroxylation site, typically leading to elevated Epo levels and erythrocytosis in classical patients with Chuvash erythrocytosis. Conversely, the well-known autosomal dominant cancer predisposition to VHL, with >1500 known VHL mutations, does not lead to erythrocytosis and is caused by inheritance of a single mutated allele of VHL.
the affected patients.\textsuperscript{55,70} In addition, numerous single nucleotide polymorphisms in the HIF\textsubscript{2}\textalpha gene are found in Tibetans and are associated with only a moderate increase in hemoglobin concentrations. This adaptation to high altitude strengthens the link between HIF2\textalpha and erythrocytosis.\textsuperscript{71,72} Contrarily, mutations of the HIF1\textalpha isoform have not been associated with altered red blood cell production.

Interestingly, mutations of the HIF2\textalpha gene have not only been shown to lead to erythrocytosis but have also been recently described to cause neoplasia. In particular, 1 patient carrying an inherited gain-of-function mutation in HIF2\textalpha (F374Y) displayed erythrocytosis, with additional recurrent multiple paragangliomas.\textsuperscript{73} In addition, 2 erythrocytosis patients with paragangliomas, one who had an additional somatostatinoma, have also been described to carry somatic HIF2\textalpha mutations (A530T and A530V), which increase the half-life of the HIF2\textalpha subunit and enhance HIF downstream signaling.\textsuperscript{74} The mutation was found in DNA from the tumor cells only and not in other cell types nor in the patients’ parents, which argues for a causative postzygotic event.\textsuperscript{74} Screening of patients with chromaffin-cell tumors (i.e., paragangliomas, pheochromocytomas) led to the discovery of numerous other somatic HIF2\textalpha mutations that are only partially accompanied by erythrocytosis.\textsuperscript{75-78} This predicts a direct oncogenic role for HIF2\textalpha, independent of its impact on red blood cell production.

Taken together, patients bearing a polymorphism in VHL, PHD2, or HIF2\textalpha collectively highlight the importance of the HIF signaling pathway in red blood cell homeostasis. Both somatic and germline mutations in HIF pathway members have been shown to lead to erythrocytosis. In some cases, erythrocytosis was accompanied by neuroendocrine tumors whose molecular basis remains to be unraveled (Table 1).

### Genetically modified mice reveal important players in erythropoiesis

Only a limited amount of erythrocytosis-associated mutations in the HIF pathway proteins in humans have been described thus far, most of them only very recently. To unravel the effective role of the different HIF pathway proteins during erythropoiesis, various genetically modified mice have been developed in the past 15 years (Table 2).

### HIF\textalpha\text{s}, PHDs, and VHL

Although HIF1\textalpha was initially discovered as the isoform that activates EPO transcription,\textsuperscript{7} it was only after both systemic and cell type–specific HIF1\textalpha and HIF2\textalpha knockout mice were made that the distinct role of both these transcription factors in erythropoiesis became clear. HIF1\textalpha knockout mice (HIF1\textalpha\textsuperscript{−/−}) are only viable up to E11.5, and these embryos show major defects of the cardiovascular system and the neural tube.\textsuperscript{79,80} However, the lack of HIF1\textalpha does not lead to complete abolishment of erythropoiesis, but rather to multiple disturbances in the adaptive responses to hypoxia. Conversely, HIF2\textalpha-deficient mice revealed that the observed pancytopenia is caused by abnormally low plasma Epo levels and impaired renal Epo induction.\textsuperscript{81} Ablation of this subunit after birth resulted in anemia accompanied by decreased circulatory Epo.\textsuperscript{82} Interestingly, even heterozygous-deficient mice (HIF2\textalpha\textsuperscript{+/-}) show a mild form of anemia (unpublished data). The group of Haase and colleagues was able to demonstrate that the regulation of erythropoiesis is essentially driven by renal HIF2\textalpha.\textsuperscript{83} Indeed, specific deletion of HIF2\textalpha in the kidney resulted in Epo-dependent anemia, which was only partially compensated by hepatic HIF2\textalpha.\textsuperscript{84} Moreover, although both HIF isoforms are expressed in the kidney, only HIF2\textalpha is found in the peritubular interstitial cells,\textsuperscript{32,74} and co-localized with EPO mRNA in these cells.\textsuperscript{84} At the molecular level, it was shown that HIF2\textalpha is actually the major isoform binding the 3’ enhancer of the EPO gene in its native form, whereas HIF1\textalpha primarily binds to the isolated HRE, as initially described.\textsuperscript{4,5,85} Moreover, the existence of additional transcription factors that bind to sites outside the actual HRE, which promote the preferential binding of HIF2\textalpha, has been proposed as well.\textsuperscript{85} Recently, Lee and coworkers presented a new mouse line bearing a G536W missense mutation in HIF2\textalpha that corresponds to the first such human mutation identified (G537W). Remarkably, not only did these mice show elevated hematocrit and pulmonary hypertension, but these findings attest that missense mutations in HIF2\textalpha can indeed cause erythrocytosis.\textsuperscript{86}

The HIF\textalpha subunits are regulated by different PHDs—the oxygen sensors. However, it is only after the mutant mouse lines were created that the functional differences among the family members became clear. Systemic deletion of PHD2, leads to embryonic lethality caused by placental and heart defects, whereas PHD1 and PHD3-specific knockout mice do not show any apparent abnormalities.\textsuperscript{87} Inducible PHD2-deficient mice on the other hand, develop severe erythrocytosis and show decreased life expectancy.\textsuperscript{88,89} Mice that are systemically deficient for either PHD1 or PHD3 do not display increased hematocrit values, and only mice lacking both of these isoforms simultaneously develop a moderate form of erythrocytosis. In the latter mice, plasma EPO and renal EPO expression is decreased, whereas hepatic EPO mRNA is induced.\textsuperscript{89} Thus, PHD1 and PHD3 appear to have only minor roles in the regulation of EPO expression, although their additional loss in the background of PHD2 deficiency can ameliorate the erythrocytosis phenotype.\textsuperscript{90,91} Our research group recently developed a conditional PHD2-deficient mouse line displaying severe but nonlethal erythrocytosis.\textsuperscript{92} Using different genetic approaches (PHD2/HIF\textalpha double-deficient mice) we could show that the Epo-dependent red blood cell increase is driven by HIF2\textalpha, which is in line with other observations made in familial erythrocytosis.\textsuperscript{69,93} Conversely, we found that HIF1\textalpha actually serves as

<table>
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<tr>
<th>Table 2. Available genetically modified mice illustrating the impact of a deregulated HIF system on murine erythropoiesis</th>
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<tbody>
<tr>
<td><strong>Gene</strong></td>
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<tr>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>VHL</td>
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<tr>
<td>PHD2</td>
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<tr>
<td>HIF2\textalpha</td>
</tr>
<tr>
<td>Liver-specific KO</td>
</tr>
<tr>
<td>Kidney-specific KO</td>
</tr>
<tr>
<td>Heterozygosity</td>
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<tr>
<td>G536W mutation</td>
</tr>
</tbody>
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KO, knockout.
*In anemic mice and during early postnatal development.
a protective factor in these PHD2-deficient mice via the local induction of PHD3.92

Mice carrying a homozygous deletion of the VHL gene die in utero because of a defect in placental vasculogenesis.34 A liver-specific VHL deletion led to hepatic vascular tumors and erythrocytosis, which was accompanied by increased Epo levels.93 The increase in erythrocytes was not reversible by additional hepatic HIF1α deletion,97 but only by deletion of HIF2α.98 Mice with an astrocyte-specific deletion of VHL not only exhibited a significant increase in cerebral EPO mRNA, but also a significant induction of plasma Epo and erythrocytosis.16 The additional deletion of HIF1α did not correct this increase in red blood cell count but rather made the phenotype more severe and shortened the survival time of these double-deficient mice. On the other hand, elimination of HIF2α along with VHL normalized the red blood cell count and most of the cerebral EPO transcript.95 Recently, ablation of VHL in osteoblasts led to HIF2α-dependent EPO induction in these cells, accompanied by erythrocytosis and enhanced bone formation.97 In 2007, a mouse line carrying the homozygous R200W mutation (leading to Chuvash erythrocytosis in humans) was created. Interestingly, this point mutation resulted in moderate erythrocytosis accompanied by splenic erythropoiesis.98 Embryonic stem cells carrying this mutation exhibited normoxic stabilization of HIF2α, which was accompanied by upregulation of HIF2α targets like vascular endothelial growth factor.

Conclusion

Deregulation of EPO transcription caused by mutations in HIF pathway proteins is an important underlying cause of erythrocytosis in patients. Moreover, these mutations can also result in other pathologic conditions like tumor development. Recently, various point mutations in the HIF2α/EPAS1, VHL, and PHD2 genes have been identified, and additional studies have led to new insights into the HIF pathway. Complementary to these mutations, many genetically modified mice have provided a powerful tool to study the effect and location of HIF pathway members in relation to erythropoiesis and its additional risk factors. Furthermore, it might be of great interest to develop new mouse models for erythrocytosis and related diseases including mice that carry specific point mutations found in humans (as mentioned previously for the R200W VHL and very recently the G537W HIF2α mutation).

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Authorship

Contribution: K.F. and B.W. wrote the manuscript; and M.G. provided helpful discussions and helped write the manuscript.

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