Key words: malaria, pyruvate kinase deficiency.

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Familial erythrocytosis: molecular links to red blood cell control

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Familial or hereditary erythrocytosis is a rare disorder of red cell production that can be inherited in either an autosomal dominant or recessive fashion. It is characterized by an absolute increase in red cell mass with elevated hematocrit and hemoglobin levels. In contrast to the acquired myeloproliferative disorder of polycythemia vera, the erythrocytosis is not accompanied by increased numbers of white cells and platelets. Familial erythrocytosis can exhibit a spectrum of erythropoietin (EPO) levels, which reflects the diverse genetic origins of this disorder. Erythrocytosis can be further classified as either primary or secondary depending on whether the defect is intrinsic or extrinsic to the erythroid progenitor cells. In primary erythrocytosis the serum EPO level is subnormal and the erythroid progenitors are hypersenstive to EPO, which suggests the defect lies in the EPO-induced signaling pathway. Secondary erythrocytosis is associated with inappropriately normal or raised serum EPO levels indicating a defect in the control of EPO synthesis by the oxygen-sensing pathway. Both reflect our current understanding of how red blood cell mass is regulated. This perspective focuses on primary and secondary familial erythrocytoses, as currently recognized by Online Mendelian Inheritance in Man (OMIM; available online at URL http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim). For other causes of erythrocytoses, including those arising from high oxygen affinity hemoglobins, the reader is referred to an excellent review by Hodges *et al.*[1]

In adults, EPO is synthesized predominantly in the kidney, although it should be recognized that liver can be a secondary source.[1] *EPO* gene transcription is regulated by a transcriptional mechanism that is more generally employed in the cellular response to changes in oxygen tension, to be described later. EPO then exerts its influence by binding to a specific receptor, the EPO receptor (EPOR), on the cell surface of erythroid progenitor cells, resulting in erythroid proliferation, differentiation and inhibition of apoptosis. The EPOR homodimerizes in the presence of EPO and autophosphorylation of the Janus tyrosine kinase 2 (JAK2) occurs.[2] Once JAK2 is activated, specific EPOR tyrosines are phosphorylated and form docking sites for adaptor molecules such as Grb2, the signal transducer and activator of transcription 5 (STAT5) and phosphatidylinositol 3-kinase (PI3-K) (Figure 1). Activated STAT5 forms dimers and translocates into the nucleus where it induces transcription of genes involved in proliferation and cell survival. PI3-K, via activated Akt, also induces the expression of several anti-apoptotic proteins, such as Bcl-2 and Bcl_x, to prolong cell survival.[2] Furthermore, activation of the Ras/extracellular-signal-regulated kinase mitogen-activated pro-

tein (Ras/Erk MAP) kinase pathway by Lyn kinase and Grb2 sustains cell proliferation. Grb2 can interact with both the adaptor protein APS and the phosphotyrosine phosphatase (SHP-2), which acts as a positive regulator of EPO signaling.

To prevent sustained erythroid proliferation EPO signaling is returned to basal levels within 30 to 60 minutes after stimulation. Several different mechanisms are called into play.[3] There is rapid downregulation of the EPOR at the cell surface and suppression of the EPO signal transduction pathway. Subsequent to JAK2 activation and phosphorylation of the EPOR there is ubiquitinylation of the cytoplasmic domains of the EPOR, which are then degraded by the proteasome.[4] This process efficiently removes the phosphotyrosine binding sites essential for signaling. The remaining extracellular receptor fragment with bound EPO is then internalized and processed by the lysosome.[4] Independent of JAK2 activity, any EPOR once bound to EPO can also be rapidly internalized.[4]

The JAK/STAT pathway is regulated by a negative feedback loop involving the suppressors of cytokine signaling (SOCS) family of proteins. Two members, SOCS3 and cytokine inducible SH2-containing protein (CIS), bind directly to phosphotyrosine residues on the EPOR to compete with STAT5.[5] SOCS proteins can also modulate signaling by targeting proteins of the signaling complex for proteasomal degradation. A particularly important mechanism for downregulation is Src-homology tyrosine phosphatase 1 (SHP-1) recruitment to the phosphorylated tyrosines at 454 and 456 of the EPOR (Figure 1), which induces dephosphorylation of JAK2 to attenuate signaling.[3] The adaptor molecule Lnk becomes tyrosine-phosphorylated upon EPO stimulation and is able to inhibit EPOR phosphorylation and JAK2 activation.[6] Consequently, the JAK/STAT, MAPK and Erk signaling pathways are all inhibited by Lnk.[6]

Since the first report of an EPOR truncation mutation being associated with erythrocytosis in 1993 (a nonsense mutation at the codon for Trp-439; Figure 2),[7] a further ten different truncating mutations, in addition to several point mutations, have been described.[8] Mutations in EPOR constitute a cause of primary erythrocytosis. In this issue of the journal, Al-Sheikh and colleagues report on 36 new cases of primary erythrocytosis.[9] EPOR mutations are described in 15% of cases and three new mutations in the gene encoding this protein were identified. In all of the latter cases the serum EPO level was subnormal, which is consistent with previous cases. All of these 14 different mutations are located in a 176 base pair region (from nucleotides g.5828 to g.6003) in exon 8 of the *EPOR* gene and either form a stop codon immediately or induce a frameshift that ultimately results in a stop codon. Two missense mutations, Asn487Ser and Pro488Ser, of the EPOR have also been detected in erythrocytosis patients, but they do not appear to be associated with the erythrocytosis phenotype.[8] In the case of the nonsense and frameshift mutations, between 57 and 127 amino acids are removed from the cytoplasmic region of the receptor. Several of these mutations have been shown to confer hypersensitivity of erythroid progenitors to EPO and prolonged activation of the JAK/STAT pathway.[8] Discrete portions of the cytoplasmic region of the EPOR are important for the negative regulation of EPO signaling (Figure 1). The binding site for SHP-1 is located at Tyr454 and Try456. All the truncating mutations, including the minimum one of 57 amino acids, result in the loss of these SHP-1 binding sites. This suggests that dephosphorylation of JAK2 by SHP-1 is a key component of the attenuation of EPO signaling. OMIM classifies mutations in EPOR as familial erythrocytosis 1 (ECYT1) (Table 1). The remaining three categories of familial erythrocytosis (ECYT2-4) are secondary to dysregulated EPO production due to defects in the oxygen sensing pathway.

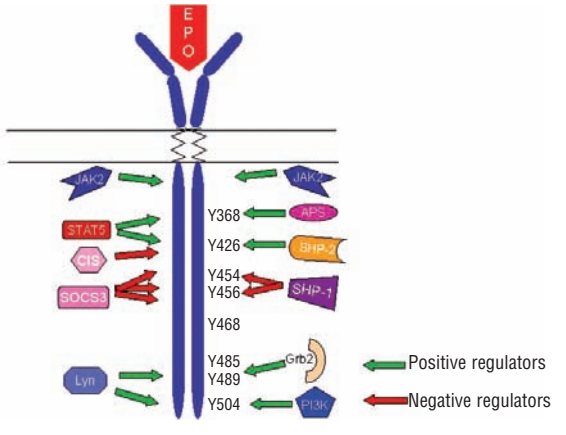

Figure 1. Schematic diagram of the human EPOR showing the phosphorylated tyrosines that form binding sites for the components of the EPO signaling pathway. The EPOR is 508 amino acids long and is numbered to include the 24 amino acids present in the immature receptor.

A central element of the oxygen-sensing pathway that controls the transcriptional response of mammalian cells in response to hypoxia is the enzyme prolyl hydroxylase domain (PHD; also known as HIF prolyl hydroxylase or egg laying defective nine protein; EGLN1) (Figure 3).[10] This enzyme, of which there are three isoforms, PHD1, PHD2, and PHD3, hydroxylates the α subunit of the transcription factor hypoxia inducible factor (HIF). HIF-α, in turn, has three isoforms, HIF-1α, HIF-2α, and HIF-3α.[11] The site-specific prolyl hydroxylation of HIF-α allows recognition by an E3 ubiquitin ligase complex that contains, as its recognition subunit, the von Hippel Lindau (VHL) tumor suppressor protein.[12] Under normoxic conditions, PHD is constitutively active, and the constitutive prolyl hydroxylation of HIF-α targets the latter for degradation by the ubiquitin-proteasome pathway. The prolyl hydroxylation reaction is inherently oxygen-dependent, and, under hypoxic conditions, the activity of PHD, which is also sensitive to hypoxia-induced reactive oxygen species, is diminished, thereby allowing HIF-α to escape degradation. In essence then, HIF-α is unstable under normoxic conditions and stable under hypoxic conditions. HIF-α then heterodimerizes with its obligate partner, the aryl hydrocarbon nuclear translocator, and this heterodimeric HIF complex then

transactivates a multitude of genes involved in hypoxic adaptation. HIF regulation of the *EPO* gene, with its central role in red blood cell regulation, is considered a paradigm of hypoxic gene regulation.

It can be seen that both PHD and VHL are negative regulators of HIF-α. Hence, it is possible to predict that loss of function of either PHD or VHL would lead to elevations of HIF-α and aberrant upregulation of HIF target genes, such as *EPO*. Mutations have indeed been identified in both. Mutations were identified in the *VHL* gene in a population located in the Chuvash republic of the former Soviet Union.[13] This disease, denoted Chuvash polycythemia, is an autosomal recessive disorder due to an Arg to Trp substitution at amino acid 200 of the VHL protein (Figure 2). This mutation results in a partial loss of function, impairing the ability of VHL to recognize hydroxylated HIF-α and therefore impairing its capacity to promote its degradation. The disease is not unique to Chuvashia, as additional patients with this VHL mutation have been identified in other countries, including the United Kingdom and the island of Ischia in Italy.[14, 15] Mutations distinct from the Chuvash mutation have been identified as well, with many, but not all mutations, residing in the vicinity of Arg-200 in the three-dimensional structure of VHL.[16] Interestingly, a number of patients with VHL-associated erythrocytosis are heterozygous for *VHL* mutations, suggesting the possibility of an additional genetic lesion in these particular patients. Collectively, patients with VHL mutations are classified as having familial erythrocytosis 2 (ECYT2) (Table 1). It is possible to regard Chuvash polycythemia as a secondary erythrocytosis due to dysregulation of EPO resulting in elevated EPO levels. However, it has features of a primary erythrocytosis as well, reflected in heightened sensitivity of erythroid precursors to EPO, as seen in both studies of human patients and a mouse model of the disease.[13, 17]

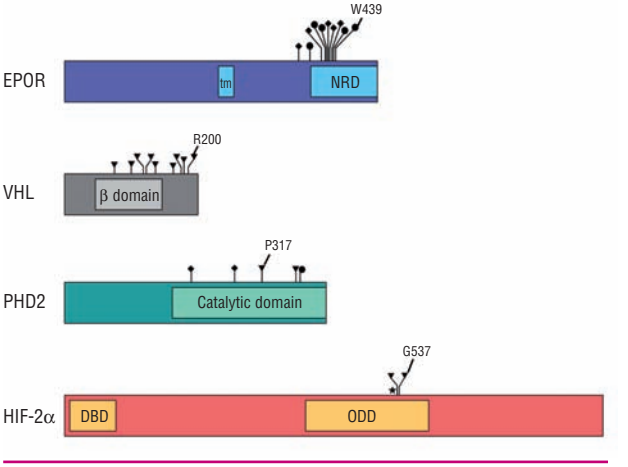

Figure 2. Erythrocytosis-associated mutations in EPOR, VHL, PHD2, and HIF-2α. The locations of the transmembrane (tm) and negative regulatory (NRD) domains of EPOR, the HIF-binding β domain of VHL, the prolyl hydroxylase catalytic domain of PHD2, and the DNA binding (DBD) and oxygen-dependent degradation (ODD) domains of HIF-2α are shown. The positions of erythrocytosis-associated missense (triangles), frameshift (diamonds), and nonsense (circle) mutations are indicated, as are specific residues that are discussed in the text. The asterisk indicates the primary site of hydroxylation in HIF-2α (Pro-531).

A notable feature of the clinical presentation of Chuvash polycythemia is the lack of tumors classically associated with VHL syndrome, namely renal clear cell carcinoma, central nervous system and retinal hemangioblastomas, and pheochromocytomas. Instead, these patients are predisposed to develop vertebral hemangiomas.[18] Moreover, these patients are at risk of peripheral and cerebrovascular thrombotic events and pulmonary hypertension, and they display exaggerated ventilatory and cardiovascular responses to acute hypoxia.[19, 20]

Erythocytosis-associated mutations in the *PHD2* gene identify the encoded protein as being critical to normal EPO regulation in humans, and they are classified as familial erythrocytosis 3 (ECYT3) (Table 1). These first described cases involved a kindred with a heterozygous Pro317Arg missense mutation (Figure 2).[21] The mutation affects a residue in the vicinity of the active site of *PHD2*, and markedly diminishes prolyl hydroxylase activity *in vitro*. A subsequent report described a sporadic case of *PHD2*-associated erythrocytosis that predicts a distinct change, Arg371His, which – while somewhat distant in the primary structure – is quite close in the tertiary structure to Pro317.[22] These two residues might contribute to an active site binding groove for HIF-α in PHD2. Three additional *PHD2*-associated erythrocytosis mutations have subsequently been reported.[23] Interestingly, two of the mutations are frameshift

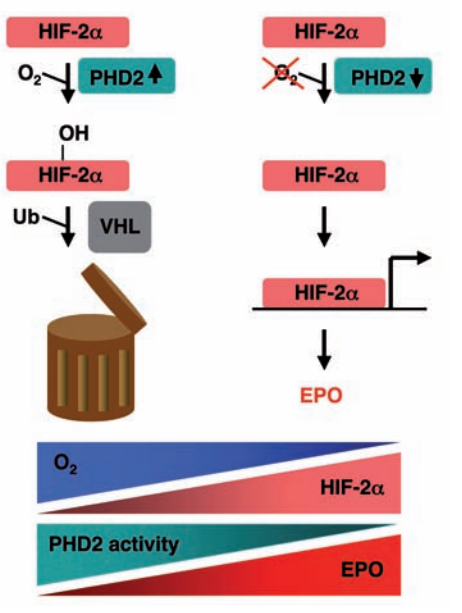

Figure 3. The PHD2:HIF-2α:VHL pathway and *EPO* gene regulation. Under normoxic conditions (left), HIF-2α is constitutively and site-specifically prolyl hydroxylated at Pro-531 by PHD2 in an oxygen-dependent manner. A VHL-containing E3 ubiquitin ligase complex specifically recognizes this hydroxylated HIF-2α, allowing it to covalently attach ubiquitin (Ub) chains to HIF-2α and thus target it for degradation by the proteasome. Under hypoxic conditions (right), prolyl hydroxylation of HIF-2α is inhibited, thereby allowing its stabilization and transactivation of target genes, including the *EPO* gene. Gradients of oxygen, HIF-2α protein, PHD2 enzymatic activity, and EPO protein are shown schematically at the bottom.

Table 1. OMIM classification of familial erythrocytosis.[a]

Abbreviation	*OMIM #*	*Gene*	*Affected exons*	*Mode of Inheritance*[b]	*Serum Epo*	*Clinical features*[c]	*Select references*
ECYT1	133100	*EPOR*	8	AD	Low		(7, 8)
ECYT2	263400	*VHL*	2,3	AR[d]	High	Thromboses, Cerebrovascular accidents, Pulmonary hypertension, Vertebral hemangiomas	(13, 14, 20)
ECYT3	609820	*PHD2*[e]	1,2,3	AD	Normal[f]		(21, 22)
ECYT4	611783	*HIF2A*[g]	12	AD	High	Thromboses	(24, 25)

[a]*As of April 21, 2008;* [b]*AD, autosomal dominant; AR, autosomal recessive;* [c]*these features show variable penetrance. For additional information, the reader is referred to the indicated references;* [d]*some cases are compound heterozygotes. In addition, there are several reported cases of simple heterozygotes, raising the possibility of mutations at an additional locus;* [e]*also known as EGLN1;* [f]*this should be regarded as inappropriately normal, given the elevated hemoglobin observed in these patients;* [g]*also known as EPAS1.*

mutations at amino acid residues 202 or 281 that are predicted to completely lack the C-terminal prolyl hydroxylase domain of PHD2. The third is a nonsense mutation at amino acid 377 that deletes the C-terminal 50 residues of PHD2. In contrast to Chuvash polycythemia, all *PHD2* mutations described to date are heterozygous mutations. Furthermore, patients with *PHD2*-associated erythrycytosis have inappropriately normal EPO levels in contrast to those with *VHL*-associated erythrocytosis, in whom EPO levels are typically elevated.

Recent findings have established a fourth category, familial erythrocytosis 4 (ECYT4) (Table 1). This is due to mutations in the *HIF2A* gene, thereby identifying HIF-2α as the HIF-α isoform critical for EPO regulation in humans. The first kindred described harbored a Gly537Trp mutation, and the affected residue is in close vicinity to the primary site of hydroxylation in HIF-2α, Pro-531 (Figure 2).[24] Indeed, functional studies demonstrated that this mutation impaired both the capacity of HIF-2α to be hydroxylated by PHD2 as well as its capacity to be subsequently recognized by VHL, thereby providing evidence that this is a gain-of-function mutation, in contrast to the *VHL* and *PHD2* mutations, which behave as loss-of-function mutations (in those cases in which it has been examined). Subsequent studies, including a new report by Martini and colleagues in this issue of the journal,[25] have identified patients with both familial and sporadic *HIF2A* mutations.[26,27] Importantly, all patients have mutations in close vicinity to the primary hydroxylation site, with the mutations being either Gly537Trp, Gly537Arg, Met535Ile or Met535Val. Patients with *HIF2A* mutations present at a young age, and as in Chuvash polycythemia, display elevated EPO levels, sometimes markedly so. Moreover and similar to Chuvash polycythemia, some of the patients with *HIF2A*-associated mutations have clinical histories of thromboses. In the case of the *HIF2A*-associated erythrocytoses, whether these are a secondary consequence of the erythrocytosis or related directly to the *HIF2A* mutation (mediated, for example, through a HIF-2α gene target other than *EPO*) is unclear.

Currently, we take the following approach in newly diagnosed cases of idiopathic erythocytosis (in which acquired causes such as polycythemia vera and the presence of an EPO-secreting tumor have been excluded), which conforms to previously published guidelines.[28] EPO levels are measured to indicate whether the erythrocytosis is related to dysregulated EPO production. P50 values are obtained to eliminate the presence of a high affinity hemoglobin variant. A bone marrow aspirate is examined for erythroid hypercelluarity. As can be garnered from the above considerations, clinical history such as age of presentation, affected family members, and history of thrombotic events, can be critical. Finally, our current panel of testing includes screening the *EPOR*, *VHL*, *PHD2*, and *HIF2A* genes for mutations.

The identification of molecular lesions as the basis of erythrocytosis has provided a compelling correlation with, and indeed, physiological validation of proteins implicated in the pathway that senses oxygen and transmits it to signals that eventually propagate erythroid progenitors. In fact, it is interesting to speculate whether all of the major elements in the oxygen sensing pathway have now been accounted for in the current OMIM classification of familial erythrocytosis. It must be recognized, however, that in many cases of this disorder the molecular basis remains elusive and thus these erythrocytoses remain idiopathic. It is, therefore, conceivable that our current classification captures only a subset of all genetic causes of familial polycythemia/erythrocytosis, and continued investigation of erythrocytosis and the oxygen-sensing pathway promises to yield additional insights in this regard.

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Key words: erythocytosis, EPO, EPOR, VHL, PHD2.

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Myelodysplastic syndrome with isolated 5q deletion (5q- syndrome). A clonal stem cell disorder characterized by defective ribosome biogenesis

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In 1974 Herman van den Berghe *et al.*[1] reported a distinct hematologic disorder associated with acquired deletion of the long arm of chromosome 5 [del(5q)]. This novel nosological entity was described in more detail one year later by Sokal, van den Berghe, and coworkers.[2] Patients with del(5q) had macrocytic anemia with oval macrocytes, normal to slightly reduced white blood cell counts, and normal to elevated platelet counts. With respect to the bone marrow, there was erythroid hypoplasia but "*the most striking abnormality concerned the megakaryocytes and especially their nuclei, which were generally small, round or oval, and nonlobulated*".[2] These morphological abnormalities are illustrated in Figure 1. Until that time, the only specific chromosomal abnormality in hematologic disorders was the Philadelphia chromosome associated with chronic myeloid leukemia.[3,4] Sokal *et al.*[2] concluded that del(5q) represented a novel specific chromosomal abnormality associated with refractory anemia, although they had no explanation to connect the abnormal chromosome 5 with the hematologic manifestations.

The 5q- syndrome

Subsequent studies showed that a chromosome 5q deletion can be found in different myeloid disorders, and underscored the need to define the 5q- syndrome properly. Boultwood and Wainscoat[5] proposed the following simple definition of the 5q- syndrome: primary myelodysplastic syndrome (MDS) with del(5q) as the sole karyotypic abnormality and without excess of blasts. In their experience, patients with the 5q- syndrome so defined had macrocytic anemia, a normal or