Erythropoietin and its Receptor: Signaling and Clinical Manifestations

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The proliferation of erythroid progenitor cells and their differentiation into mature erythrocytes are mediated by the glycoprotein hormone erythropoietin. EPO is synthesized predominantly in the kidney and is secreted by renal cortical interstitial cells and proximal tubule cells in response to tissue hypoxia. The EPO receptor spans the membrane of developing erythroid cells and binds circulating EPO. Treatment of anemic patients with EPO increases their hemoglobin level, decreases the need for transfusion, and improves their quality of life. These significant clinical manifestations, as well as the key role of EPO in development of the red blood cell lineage, have prompted extensive studies on the molecular mechanisms underlying the activation of its receptor.

The EPO-R is a 476 amino acid protein containing a transmembrane segment that divides the molecule into extracellular and cytosolic domains of equal length. It belongs to the family of cytokine receptors, as indicated by the lack of endogenous kinase activity and by small regions of homology in its extracellular domain. These homologous regions include four conserved cysteines forming two disulfide bonds, as well as the WSXWS motif near the membrane-spanning segment. The first tyrosine kinase found to interact with the EPO-R was Janus kinase-2, a member of the Janus protein kinase family. These kinases phosphorylate ligand-activated cytokine receptors, generating recognition sites for a wide range of intracellular signaling molecules. This review focuses on the intracellular signaling pathways activated upon binding of EPO to its receptor, hematologic diseases associated with the EPO-R, and clinical treatment with EPO.

Signaling via the EPO-R

Activation of cell-surface EPO-R

Binding of ligand induces dimerization of growth factor receptors, leading to receptor phosphorylation and eliciting of signaling. Similarly, binding of EPO to the EPO-R induces homodimerization and activation of the receptor [Figure 1]. The first line of evidence for this mode of activation of the EPO-R, subsequently confirmed by additional experimental strategies, was provided by the formation of an intermolecular disulfide bond in the membrane proximal region of R129C EPO-R, rendering it constitutively active [1]. Structural analysis suggested that unliganded EPO-R exists as a preformed homodimer in an open scissor-like conformation [2] and that dimerization induced by the transmembrane domain keeps the unliganded EPO-R in an inactive state. Physiologic amounts of EPO, by binding to the receptor, can then switch the receptor to an activated state [3].

A central signaling pathway activated by the EPO-R requires the participation of JAK2. EPO-mediated receptor dimerization brings two receptor-associated JAK2 molecules into close proximity [4]. This enables them to transphosphorylate and activate each other, after prior phosphorylation of some or all of the eight Tyr residues in the intracellular domain of EPO-R. Gene targeting studies have demonstrated that JAK2 plays a pivotal role in signal transduction via cytokine receptors, which are required for definitive erythropoiesis [5]. Tyr phosphorylation of STAT proteins (**s**ignal **t**ransducers and **a**ctivators of **t**ranscription) precedes JAK2 activation and links growth factor receptors to gene transcription.

Tyr phosphorylation of the EPO-R generates binding sites for ***s**rc* **h**omology 2 (SH2) domains, thus eliciting cellular responses via several signaling pathways [6–8]. Binding of EPO to the receptor activates three different STAT proteins – STAT1, STAT3, and STAT5. STAT5 binds to P-Tyr 343 and P-Tyr 401 of the EPO-R [9] and is phosphorylated by JAK2. Tyr-phosphorylated STAT5 dissociates from the receptor and forms dimers, which are translocated to the nucleus to activate target genes [Figure 1]. STAT5 is essential for the high rate of erythropoiesis during fetal development, and promotes the anti-apoptotic effect of EPO-R by inducing Bcl-xL expression [10], as demonstrated in STAT5 -/- mice. Activation of STAT1 and STAT3, on the other hand, occurs via P-Tyr 432 of the human EPO-R [11]. Specificity of EPO-R STAT signaling is not essential for red blood cell development, as demonstrated by EPO-R mutagenesis targeted to STAT5 binding sites for which STAT3 binding sites have been substituted [12]. STAT proteins regulate various target genes – e.g., **c**ytokine-**i**nducible **S**H2 containing proteins (CISs) [Figure 1]. The range of genes activated by each STAT protein and the specific mechanism(s) by which they are activated are important areas for additional research.

Besides activating JAK2, EPO activates the protein Tyr kinases

EPO = erythropoietin
EPO-R = EPO receptor

JAK2 = Janus kinase-2

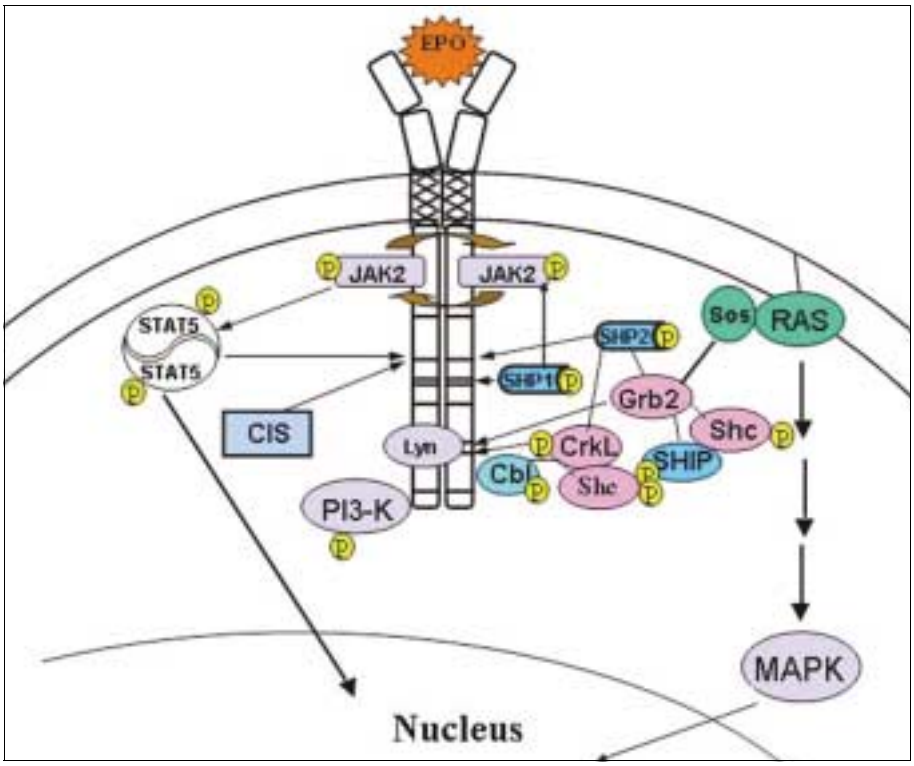

Figure 1. Model for signal transduction via the EPO-R. Binding of EPO to the EPO-R induces dimerization of the receptor and initiates a cascade of phosphorylation events. Horizontal lines mark Tyr-residues of the EPO-R cutosolic domain. The major signaling pathways are depicted.

Table 1. Major proteins that participate in signaling via EPO-R, color-coded on the basis of Fig. 1 and classified by their role in the process

	Protein	Positive regulator	Negative regulator	Adaptor
Kinases	JAK2- Janus kinase 2	X		
	STAT	X		
	PI kinase	X		
	MAP kinase	X		
	Lyn	X		
Cytokine inducible SH2-containing protein	CIS		X	
Phosphatase	SHP1 and 2		X	
Ubiquitin ligase	Cbl		X	
Adaptors	Shc			X
	Grb			X
	CrkL			X
GTPase switch protein and GEF	Ras and Sos	X		

Lyn [Figure 1], Syc, and Fes. The specificity of these Tyr kinases in EPO signaling is demonstrated by the fact that STAT1 and STAT3 are activated by Fes but not by Lyn [11]. Lyn associates with the EPO-R and participates in EPO-mediated differentiation of erythroid progenitor cells [13]. Since Lyn -/- mice do not exhibit obvious hematologic defects, the exact role of Lyn and other *src* kinases in EPO signaling is not clear. It is possible that they function in fine-tuning of the EPO response. Binding of EPO to the receptor also activates the GTPase switch protein Ras; such activation is largely facilitated by two cytosolic proteins, Grb2 and Sos [Figure 1]. An SH2 domain in the adaptor protein Grb2 binds to P-Tyr EPO-R, either directly, or indirectly via protein Tyr phosphatase SHP2 or Shc [14]. Shc is Tyr-phosphorylated by JAK2 in an EPO-dependent manner, promoting complex formation between EPO-R, Grb2, and the guanine nucleotide exchange factor Sos [15]. As a result Sos translocates to the plasma membrane, where it activates Ras, leading to the activation of the downstream raf-1 and MAP kinases [Figure 1]. The key participation of Ras in EPO-mediated signal transduction was demonstrated by the increased proliferation and differentiation of CD34 (3+) cord blood cells upon transduction of Ras and EPO-R cDNAs [16]. Stimulation of hematopoietic cells by EPO induces recruitment of the cytosolic adaptor CrkL to the EPO-R and its physical association with Shc, SHP2, and Cbl [17]. Receptor-associated Lyn probably phosphorylates CrkL, leading to the activation of Ras/Erk signaling pathways [18].

Several lines of evidence point to the participation of phosphatidylinositol 3-kinase in signal transduction via the EPO-R: a) Antisense oligonucleotide of p85alpha (the regulatory subunit of PI3-kinase) or LY294002 (a selective inhibitor of PI3-kinase) independently inhibits the formation of EPO-dependent colonies, a process in which Src activation has been implicated [8]. b) A constitutively active form of STAT5 interacts with p85, activating the PI3-kinase pathway. Other activated proteins include the serine/threonine kinase Akt (protein kinase B), which directly phosphorylates FKHRL1, a member of the transcription factor Forkhead family [19], and p70 S6 kinase, required for cell cycle progression.

Future analysis of the molecular basis of cytokine action should determine how JAK/STAT signals are integrated with signals from other downstream pathways such as those originating from Ras/Raf/MAPK, Akt, and p70 S6 kinase.

Negative regulators of EPO-R signaling

EPO-R signaling is terminated as a result of the interaction of certain proteins with the EPO-R or with signaling molecules. Among these proteins a major player is SHP1, which is recruited via its SH2 domains to P-Tyr 429 of the EPO-R, leading to dephosphorylation of JAK2 and down-regulation of positive signals [20] [Figure 1]. Another group of negative regulatory

PI3 = phosphatidylinositol 3

proteins is the CIS protein family, also known as SOCS (**s**uppressor **o**f **c**ytokine **s**ignaling) or SSI (**S**TAT-induced **S**TAT **i**nhibitor), whose members are activated via the JAK/STAT pathway and have been implicated in regulating the signal transduction of a variety of cytokines including EPO [7]. CIS1 binds P-Tyr 401 of the EPO-R, which serves as one of the major binding sites for STAT5, thereby negatively regulating STAT5 activation [21]. A second member of CIS family was cloned independently by three groups and was termed JAB (**JA**K-**b**inding protein), SOCS1, or SSI1 [22]. Mutation analysis and biochemical characterization revealed that JAB binds specifically to Tyr at position 1007 in the activation loop of JAK2, whose phosphorylation is required for kinase activation [23]. The SH2 domain and a C-terminal 40 amino acid region, designated the CIS homology domain, are highly conserved in this family, whereas the N-terminal regions of these proteins share little similarity [22]. While both CIS3 and JAB inhibit EPO-mediated proliferation and STAT5 activation [24], the *in vitro* affinity of CIS3 for JAK2 is significantly lower than that of JAB [7], further illustrating the complexity of negative regulation of cytokine signaling by CIS proteins.

The complexity of stimulatory pathways activated by EPO suggests that these inhibitory pathways are probably only the tip of the iceberg. Better understanding of the inhibitory mechanisms of EPO-mediated signaling will facilitate the design of specific pharmacologic compounds capable of intervening in cytokine signaling.

EPO-R in clinical disorders

Polycythemia

Familial and congenital polycythemia are clinical disorders characterized by an absolute increase in red blood cell mass. Low serum EPO characterizes primary familial and congenital polycythemia, whereas secondary familial polycythemia is associated with high serum EPO and intact EPO-R signaling.

The extracellular domain of the EPO-R binds circulating EPO, and the intracellular domain controls signal transduction. The latter domain also contains a C-terminal regulatory region that acts as a brake on red cell production. Some cases of PFCP were found to be associated with dominantly inherited heterogeneous EPO-R mutations [25] that were characterized by nonsense mutations with consequent truncations of the C-terminal end of the EPO-R. This region includes Tyr 429, which in its phosphorylated form is required for binding of SHP1, the negative regulator of Tyr phosphorylation and signal transduction [20]. Loss of the EPO-R SHP1 binding site prevents dephosphorylation of JAK2 and prolongs activation of STAT5, leading to hypersensitivity to EPO *in vitro* [26]. Support for this finding comes from the observations that cells co-transfected with wild-type EPO-R and truncated EPO-R display hypersensitivity to EPO, and moreover that they demonstrate the dominant effect of the truncated EPO-R, in correlation with the dominant inheritance observed in affected families [27].

Studies *in vivo*, using a mouse model, showed that heterozygosity of the truncated mutant EPO-R gene causes polycythemia [28]. In cases where no EPO-R mutation is found and serum EPO is low, mutations of other genes involved in the signaling pathway of the EPO-R (such as JAK2, STAT5, and SHP1) should be considered.

Another cause of erythrocytosis with low serum EPO levels is polycythemia vera, a hematologic malignancy that leads to excessive proliferation of erythroid, myeloid, and megakaryocytic elements within the bone marrow [26]. Despite extensive research, the involvement of EPO-R in the pathogenesis of polycythemia vera is still controversial.

Neoplastic disorders

The EPO-R, like other growth factor receptors such as granulocyte colony-stimulating factor-R and thrombopoietin-R, is involved in several neoplastic disorders. In addition to its expression in erythroleukemia, EPO-R is expressed in approximately 60% of patients with acute myeloid leukemia [29]. Leukemic cells were found to be responsive to EPO *in vitro* in 16% of the cases in which EPO-R was expressed [30]. The expression of EPO-R has been observed in all subtypes of acute myeloid leukemia according to the FAB (French, American, British) classification. In patients displaying both EPO-R expression and response to EPO, the duration of complete remission is shorter than in those without EPO-R [29]. Furthermore, patients harboring leukemia cells displaying more than 230 EPO-binding sites per cell tend to have less favorable cytogenetics than patients with fewer EPO-R sites per cell [30]. Expression of EPO-R was also observed in 29% of patients with acute lymphoblastic leukemia [29].

Solid malignancies

EPO and EPO-R expression are also observed in solid malignancies such as breast carcinoma samples and tumor vasculature compared to benign changes or normal tissues. Expression of EPO and of EPO-R is highest in hypoxic tumor regions, where functional EPO signaling is observed [31]. EPO-R expression is also observed in human renal carcinoma cells [32], which display EPO-mediated proliferation. The effect of EPO treatment on tumor proliferation should be taken in account when using EPO to treat such patients.

Recombinant human EPO in clinical practice

Renal failure

The cloning of the EPO gene led to production of the recombinant hormone, allowing its introduction into clinical practice. The first to benefit from recombinant human erythropoietin were, as expected, patients with end-stage renal failure [33], in whom the diseased kidneys do not produce endogenous EPO. Indicators of successful therapy are increased levels of hemoglobin and hematocrit, reduction in the blood transfusion requirements, and a significant improvement in the quality of life and performance status. The successful results in over 90% of these patients have encouraged investigators to treat other types of anemia with rHuEPO.

PFCP = primary familial and congenital polycythemia

rHuEPO = recombinant human EPO

Cancer-associated anemia

Cancer patients often suffer from anemia. Patients with various malignancies respond to anemia with an increase in EPO production, which however is still inadequate and less than the expected normal amount in relation to the degree of anemia ("relative EPO deficiency") [34]. This finding constitutes the scientific basis for numerous clinical trials of the use of rHuEPO to treat patients suffering from various types of neoplasms with anemia [35]. The overall response rate has been about 60%. Patients with multiple myeloma or head and neck cancer achieve a success rate of about 70–80%, whereas the response rate of patients with myelodysplastic syndromes is around 20%. More experimental designs are required to estimate the benefits of rHuEPO in the wide range of cancer patients undergoing treatment.

Anemias in hematologic and other disorders

rHuEPO was also shown to be effective in the treatment of other types of anemia, such as that seen in patients with AIDS, and the anemia that accompanies chronic diseases such as systemic lupus erythematosus, rheumatoid arthritis, and inflammatory bowel disease [36]. EPO can ameliorate anemia of infancy, hemoglobinopathies, and Gaucher's disease. EPO has become an important component of the bone marrow transplantation procedure, as a measure to increase progenitor cell recruitment prior to transplantation, as well as after the procedure when it promotes proliferation of the erythroid series [37].

Perisurgical treatment

Additional clinical indications for rHuEPO include its perisurgical use. Several studies have shown that injection of EPO before and immediately after elective surgery, especially cardiac and orthopedic operations, may minimize the need for blood transfusions [for review see 38].

Novel non-erythroid effects

Newly emerging data suggest that EPO may exert non-erythroid effects, such as neuroprotection [39] and anti-cancer effects [40]. The molecular mechanisms underlying these effects, as well as their biologic and clinical significance, remain to be determined.

Mode of treatment

Currently EPO is administered parenterally only, with minimal adverse effects. The common side effects are local irritation and flu-like symptoms, which often respond to symptomatic treatment. Raised blood pressure and thrombotic events due to an uncontrolled rise in hematocrit are rare today, thanks to careful follow-up. Onset of the clinical effect of EPO is observed after treatment for 4–6 weeks.

The usual dose is about 150 U/kg three times weekly for anemia of cancer, and might need to be doubled in some patients. Patients with end-stage kidney disease may require a lower dose. Often a loading dose is given initially, with a lower maintenance dose prescribed later on.

Concluding Remarks

A wealth of data has accumulated on the signaling pathways activated by the EPO-R, yet some key issues remain unresolved. These include structural requirements for complex formation between EPO-R and downstream effectors as well as the *in vivo* interactions between the diversity of downstream effectors, some of which have yet to be discovered. The emerging diverse effects of rHuEPO coupled with its extensive clinical application will most likely yield exciting avenues for future research.

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If all the year were playing holidays,
To sport would be as tedious as to work

Shakespeare, ***Henry IV***

Capsule

A wake-up call

Intercellular signaling among bacteria is well documented, but autocrine signaling is less well known. In a pair of papers, Mukamolova et al. describe a protein called resuscitation-promoting factor (Rpf) that is secreted by Gram-positive bacteria. Rpf awakens dormant *Micrococcus* luteus cells that have become non-culturable after reaching stationary phase. Not merely a rescuer, Rpf also acts like a cytokine to maintain the growth of actively replicating cells; if Rpf is washed away, bacterial growth halts. Several bacterial species, including the pathogen *Mycobacterium tuberculosis*, encode rpf-like genes. Of particular note is the fact that *M. tuberculosis* is known to persist in a latent state in individuals, with disease reappearing if the host becomes immunocompromised. If latency involves regulation via Rpf, this protein and its as yet unidentified receptor could be attractive drug or vaccine targets.

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