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Biology of erythropoietin

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Abstract

Erythropoietin (Epo) controls the proliferation, differentiation and survival of the erythroid progenitors. This cytokine was cloned in 1985 and rapidly became used for treatment of anemia of renal failure, opening the way to the first clinical trials of a hematopoietic growth factor. The clonage of one chain of the Epo receptor followed in 1989, thereby opening the research on intracellular signal transduction induced by Epo. Epo is synthesized mainly by the kidney and the liver and sequences required for tissue-specific expression have been localized in the Epo gene. A 3'enhancer is responsible for hypoxia-inducible Epo gene expression. HIF-1 α and β proteins bind to this enhancer. Gene regulation by hypoxia is widespread in many cells and involves numerous genes in addition to the Epo gene. The Epo receptor belongs to the cytokine receptor family and includes a p66 chain which is dimerized upon Epo activation; two accessory proteins defined by cross-linking remain to be characterized. Epo binding induces the stimulation of Jak2 tyrosine kinase. Jak2 activation leads to the tyrosine phosphorylation of several proteins including the Epo receptor itself. As a result, different intracellular pathways are activated: Ras/MAP kinase, phosphatidylinositol 3-kinase and STAT transcription factors. However, the exact mechanisms by which the proliferation and/or the differentiation of erythroid cells are regulated after Epo stimulation are not known. Furthermore, target disruption of both Epo and Epo receptor showed that Epo was not involved in the commitment of the erythroid lineage and seemed to act mainly as a survival factor.

Key words: erythropoietin, erythropoietin receptor, signal transduction, proliferation, differentiation

Erythropoietin (Epo), a 34-kDa glycoprotein hormone was the first hematopoietic growth factor to be cloned. The role of Epo is to control red blood cell production through the promotion of survival, proliferation and differentiation of the erythroid progenitors in the bone marrow. Because the main function of red cells is to transport oxygen from the lungs to the peripheral tissues, the regulation of Epo production is an important feature of the control of tissue oxygenation. Accordingly, Epo is the only hematopoietic growth factor whose production is regulated by hypoxia. Numerous reviews have been published these last years on Epo biology.[1-9] Therefore, our aim is to emphasize on some aspects of Epo biology which seem to us of particular interest.

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Tissue-specific Epo gene expression

In a pioneering work published in 1977, Epo was successfully purified by Miyake *et al.*[10] from urine of aplastic patients. Tryptic fragments of this urinary Epo were then obtained and their amino acid sequences permitted the synthesis of Epo DNA probes for the isolation and cloning of the human Epo gene.[11,12] The use of recombinant Epo in the treatment of anemia of chronic renal failure followed shortly thereafter.[13]

The clonage of the Epo gene also allowed insights to be gained into the molecular biology of Epo. In the fetal stage, the liver is the major site of Epo synthesis,[14] however, the Epo gene also appears to be strongly expressed in the mammalian mesonephric kidney early in gestation.[15] The renal synthesis of Epo was first demonstrated by Jacobson *et al.*[16] Studies on mice have shown that Epo gene transcription was stimulated by hypoxia or cobalt treatment,[17] and there was a clear correlation between induction of anemia and increase of Epo mRNA content in the kidney.[18] It was further shown, by in situ hybridization experiments, that Epo mRNA was produced by interstitial cells of the kidney cortex.[19,20] Epo mRNA was also detected in interstitial cells within cyst walls of polycystic kidneys.[21]

This specialized population of interstitial cells was shown to be labelled by immunohistochemical staining with antibodies to 5' ectonucleotidase,[22] and thereby to belong to a fibroblast-like cell population of the renal interstitium.

Similar results were obtained in transgenic mice containing the SV40 large tumor antigen (SV40 T-antigen) placed behind the Epo gene regulatory sequences; immunohistochemical detection of T-antigen was found in the same fibroblast-like renal interstitial cells.[23] Unfortunately, the use of an onco-

gene like SV40 T-antigen did not induce any formation of tumor in the kidney, nor the establishment of transformed cell lines from this interstitial cell population in the kidney. In the absence of such cell lines, more information is still necessary to fully understand the mechanism of Epo synthesis in the kidney. In addition, in renal adenocarcinomas associated with polycythemia, the tumoral cells themselves which derive from the epithelial tubular cells are producing Epo.[24] A possible explanation would be that a cellular cooperation in the kidney cortex is required for Epo production. Interestingly enough, Epo mRNA could be obtained from isolated perfused rat kidneys but never from anatomically disrupted renal preparations.[25]

The liver accounts for 20% of the Epo production. Hepatocytes surrounding central veins were responsible for most of the Epo production in the liver,[26] whereas other Epo-producing cells were shown to belong to the Ito cells which share many similarities with the fibroblast-like interstitial cells of the kidney.[27]

In addition to these two main sites of secretion, low levels of Epo mRNA have been detected in lung, testes and spleen when animals were subjected to hypoxia.[28,29] Epo is also produced in the brain by astrocytes,[30] accordingly Epo receptors have been detected in mouse brain[31] and in cell lines with neuronal properties.[32] These data suggest that Epo could play a neurotrophic role in the brain and that the hypoxic induction of brain Epo could protect neurons from ischemia-induced cell death.[33]

Regulation of Epo production

Epo production is regulated by hypoxia that leads to an increase of the level of gene transcription;[34] there are no preformed stores of Epo. Control of Epo gene expression involves complex interactions between DNA and nuclear proteins. To gain insights into tissue-specific Epo gene expression, Semenza *et al.* developed several constructs of human Epo gene containing various lengths of *cis* regulatory regions for production of transgenic mice. The pattern of human Epo gene expression in these transgenic mice led the authors to describe different DNA sequences located in *cis* of the Epo gene and required for tissue-specificity and hypoxia-inducible gene expression.[35] Sequences required for expression in the kidney have been localized to a region located 9.5 to 14 kb from the 5' end of the human Epo gene.[36] A negative regulatory element which represses Epo gene expression in non-Epo producing cells is located in a region 0.4 to 6 kb from the Epo transcription start site.[37] A 50 bp hypoxia-inducible enhancer has been defined approximately 120 bp from the 3' end of the polyadenylation site, and is responsible for hypoxia-inducible Epo gene expression.[38-40] Mice transgenic for a construct containing the Epo gene and this 3' enhancer harbored hypoxia-inducible Epo gene expression in the liver.

The 3' enhancer contains three different segments.[41] A conserved sequence located near the 5' end of the enhancer is the binding site for a new transcription factor designated hypoxia-inducible factor 1 (HIF-1).[42,43] The middle segment is less conserved between species, but seems to play a role in the inducibility of both the human and the murine Epo enhancers.[44] The third part corresponds to 3' DNA sequences which are binding sites for hepatocyte nuclear factor 4 (HNF-4). Proteins that bind to this enhancer interact synergistically to stimulate Epo gene transcription, and HNF-4 can augment transcriptional activation mediated by the Epo enhancer in hypoxic cells.[45] Furthermore, the C-terminal portion of HIF-1 specifically binds to P300 and overexpression of P300 enhances hypoxic induction.[46] Thus, it is likely that hypoxia induces the formation of a large complex of proteins directly or indirectly bound to the enhancer, which in turn transduces a signal to the Epo promoter, thereby permitting gene transcription[47] (Figure 1).

The identification of HIF-1 as a DNA transcriptional complex has been a critical step to understanding the enhancer function. Affinity purification showed that HIF-1 is composed of two subunits.[41,48] Molecular cloning of HIF-1 by Semenza *et al.*[49] showed that the DNA binding complex was composed of two basic-loop-helix PAS proteins called HIF-1α and HIF-1β.

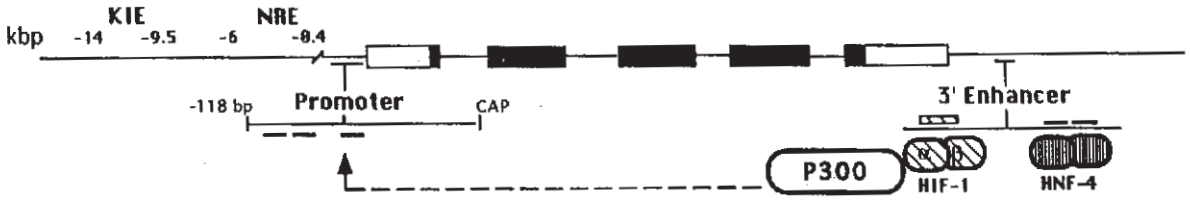

Figure 1. Cis elements and Trans-acting factors involved in Epo gene regulation. The 5 exons of the Epo gene are represented, coding portions are solid areas. KIE: kidney inducible elements; NRE: negative regulatory elements. *From H.F. Bunn and R.O. Poyton.*[56]

HIF-1β had previously been identified as the aryl hydrocarbon nuclear receptor translocator (ARNT), a molecule involved in the xenobiotic response.[50] In contrast, HIF-1α was a new member of this family of PAS proteins. In hypoxic conditions, the levels of the mRNAs encoding either HIF-1α or HIF-1β were not altered, suggesting that the activity of the HIF-1α-ARNT complex is regulated by a post-transcriptional mechanism and a conformational change after recruitment of the ARNT transcription factor.[51]. Furthermore, another step of regulation of HIF-1α involves the ubiquitin-proteasome system in its proteolytic destruction in normoxia, while it accumulates rapidly following exposure to hypoxia.[52]

The mechanism of regulation by hypoxia was first studied in hepatoma cells Hep3B or HepG2 which produced Epo. It was further shown that identical responses could be obtained in a large array of non-Epo producing cells and that the system of gene regulation by oxygen was widespread from mammalian to insect cells.[53,54] Many genes have now been identified as targets of HIF-1 function; these include in addition to Epo, vascular endothelial growth factor (VEGF), several glycolytic enzymes, glucose-transporter 1, inducible nitric oxide synthase, heme oxygenase and transferrin.[55] These recent data strengthen the idea that cellular response to hypoxia is an important physiological process and that a similar mechanism for oxygen sensing and signal transduction must be shared by many tissues and cells.[56] However, oxygen-sensing mechanisms are still not completely understood. According to the model of Hep3B cells, a single cell type apparently can sense hypoxia and respond by increasing Epo RNA levels.[5] It was proposed that the oxygen sensor is a heme protein that changes its conformation depending on the binding of oxygen to its heme moiety. The iron atom of heme can be replaced by cobalt, thereby mimicking the hypoxic state.[57] This explanation remains very plausible but the exact mechanism of activation of transcription factors by hypoxia remains, however, to be determined.

Structure of the Epo receptor

The number of Epo receptors (EpoR) at the cell surface of normal or transformed erythroid cells is low: around one thousand per cell (reviewed in ref. #58). The receptors are mainly expressed at the colony-forming unit erythroid (CFU-E) stage, receptor expression then decreases with erythroid maturation.[59]

One chain of the EpoR was cloned by an expression strategy from murine erythroleukemia cells.[60] This 66 kDa protein confers Epo-binding ability to transfected cell lines, both of hematopoietic and non hematopoietic lineages. In addition to this cloned chain, cross-linking of Epo to the cell surface of erythroid cells detects the association of Epo with two accessory molecules, one of 85 kDa and one of 100 kDa. These proteins are associated with p66 but are not recognized by anti-p66 antibodies.[61] Moreover, in the presence of truncated forms of p66, the apparent molecular masses of p85 and p100 are unchanged, thereby demonstrating that these proteins are indeed different from p66.[62,63] Isolation of these proteins is an important challenge to fully understand the structure of the Epo receptor.

The p66 cloned chain of the EpoR is a 507 amino-acid type I membrane spanning protein and belongs to the cytokine receptor family.[64] In the extracellular domain, a WSXWS sequence and two pairs of cysteines are hallmarks of this receptor family. These two structures seem to be required for the correct folding and cell surface expression of the molecule.[65] Epo appears to activate the EpoR by dimerization of the p66 protein.[66] The first EpoR molecule binds Epo with a high affinity (Kd around 1 nM) whereas the second EpoR molecule binds to the complex with a lower affinity (Kd around 2 μM). It is possible that these 2:1 complexes are further stabilized by the accessory proteins described above, and/or by interactions in the intracellular domains of these clustered EpoRs.[66] The active sites of Epo have been mapped using mutation and deletion experiments.[67-69] Two sites have been identified, each is believed to associate with one molecule of p66 EpoR.[68] A model of the complex between Epo and EpoR has been proposed,[70] which is reminiscent of the structure of the growth hormone and its receptor obtained by crystallization studies.[71]

Besides Epo binding, EpoR can be activated by other mechanisms. The gp55 envelope of the murine Friend virus is able to bind and to interact directly with the EpoR which becomes constitutively activated.[72] Interestingly, some of these gp55 proteins are dimerized by disulfide bonds at the cell surface, thus leading to the dimerization of the associated EpoR.[73] Moreover, a constitutive activation of the EpoR has been obtained by mutation in the extracellular domain of the Arg 129 residue into a Cys.[74] The presence of a Cys residue allows the formation of a disulfide bond between two EpoR molecules and thus dimerization of the receptor. This mutation of the EpoR is also tumorigenic.[75] Bivalent anti-p66 antibodies have been reported to activate the EpoR, probably by inducing the dimerization of this receptor.[76] Finally, small synthetic peptides that do not share any sequence homology with the Epo molecule are also able to mimic the biologic effects of Epo when they are dimerized but with a lower affinity.[77,78] Despite an increase of potency after covalent dimerization, clinical replacement of Epo by these peptides does not seem realistic considering the difference in biological activity from native Epo.[79] Thus, all the mechanisms that lead to an activation of the Epo receptor are also responsible for its dimerization. The participation of the p85 and p100 accessory proteins in this stoichiometry remains to be determined.

Signalling induced by Epo

Like all the receptors of the hemopoietin receptor family, the EpoR does not possess endogenous tyrosine kinase activity. Despite this fact, Epo stimulates the rapid tyrosine phosphorylation of a number of proteins (Figure 2). The first step of intracellular signalling is activation of Jak2 tyrosine kinase which is constitutively associated with the EpoR.[80] Jak2 is known to associate with the EpoR in a region close to the transmembrane that involves the Box 1 motif, and deletion of this part of the EpoR totally inhibits Epo-induced cellular proliferation.[81] Lyn tyrosine kinase has recently been reported to associate with the EpoR in the J2E1 cell line, and to play a role in its Epo-induced erythroid differentiation.[82] It is not known if Lyn plays a similar role in other erythroid cells.

Among the proteins phosphorylated on tyrosine residues in response to Epo is the EpoR itself.[83-86] Most, if not all, of the eight tyrosines which are located in the cytoplasmic domain of the EpoR are phosphorylated after Epo stimulation. These phosphorylated tyrosines are in turn docking sites for various intracellular proteins containing *src* homology 2 (SH2) domains. After binding, these proteins can be subsequently tyrosine phosphorylated and activated. Therefore, the stimulation by Epo leads to localization close to the receptor and the plasma membrane of different activated molecules which participate in downstream signal transduction (Figure 3).

The Ras/MAP kinase pathway is activated by Epo.[87,88] This pathway is involved in cell proliferation in response to Epo, and raf-1 antisense oligonucleotides have been shown to inhibit such cell proliferation.[89] On the other hand, other investigators found that Ras/MAPK activation was not required for Epo-induced proliferation.[88] The presence of fetal calf serum which directly activates the Ras/MAPK pathway could be an explanation for this discrepancy.[9]

A direct association between PI 3-kinase and the EpoR has been shown.[90-93] This involves the SH2 domains of the p85 subunit of the PI 3-kinase and the last tyrosine of the EpoR. An alternative pathway for the activation of PI 3-kinase has been recently described which involves tyrosine phosphorylation of the adaptor protein IRS2 and its subsequent association with PI 3-kinase; this mode of activation therefore does not require the interaction of PI 3-kinase with the EpoR tyrosines.[94] An interesting pathway downstream of PI 3-kinase, leading to the sequential activation of SHIP[95,96] and AKT[97] has been recently described. The Ser/Thr kinase AKT appears to play a major role in the inhibition of apoptosis after stimulation by cytokines such as IL-3 or IGF-1.[98] It remains to be determined whether similar AKT activation and protection from apoptosis exists after Epo stimulation.

The STAT (*Signal Transducer and Activator of Transcription*) pathway also plays a major role in cytokine-induced signalling.[99] Epo activates both STAT5A and STAT5B.[100-102] The two first tyrosines of the intracellular domain of the EpoR (Tyr 343 and Tyr 401) are responsible for STAT5 fixation and activation.[103-107] Despite a large number of publications, the precise role of STAT5 in the signalling induced by Epo is the subject of controversy. Whereas a correlation between STAT5 activation and cell proliferation was described by some groups,[104,105,108] others did not obtain such results.[106,107] Furthermore, a correlation between STAT5 activation and Epo-mediated erythroid differentiation was observed in some reports,[109,110] while the opposite was shown by others.[108] One possible explanation is the use of different erythroleukemic cell lines which may respond with various intracellular pathways to the same cytokine. Alternatively, there is some redundancy in function between the different STAT proteins, especially STAT5 and STAT6. It is therefore difficult to elucidate their exact mode of action, even after gene disruption.

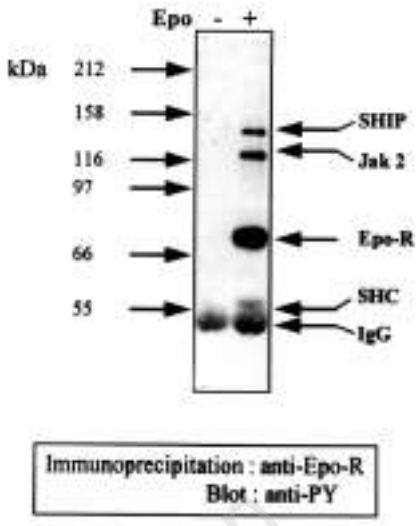

Figure 2. Tyrosine-phosphorylated proteins associated with the Epo-R in UT-7 cells. UT-7 cells were starved from Epo overnight,and stimulated for 10 minutes with 10 U/mL Epo or not. 5×10^6 UT-7 cells immunoprecipitated with anti EpoR antibodies were analyzed in each lane.

Two tyrosine phosphatases, SHP-1 and SHP-2, also play a role in Epo-induced signalling. SHP-2 is phosphorylated on tyrosine in response to Epo and associates with the second tyrosine residue of the EpoR (Tyr 401); SHP-2 seems to play a positive role in stimulating cell proliferation.[111] In contrast, SHP-1 plays a negative role in Epo-induced signal transduction; its association with Tyr 429 of the EpoR leads to the dephosphorylation of Jak2.[112] Interestingly, De La Chapelle *et al*.[113] described a familial erythrocytosis in which a truncated EpoR was found in the polycythemic members of the family; these truncated receptors were shown to be hypersensitive to Epo probably because they lacked the SHP-1 binding site.

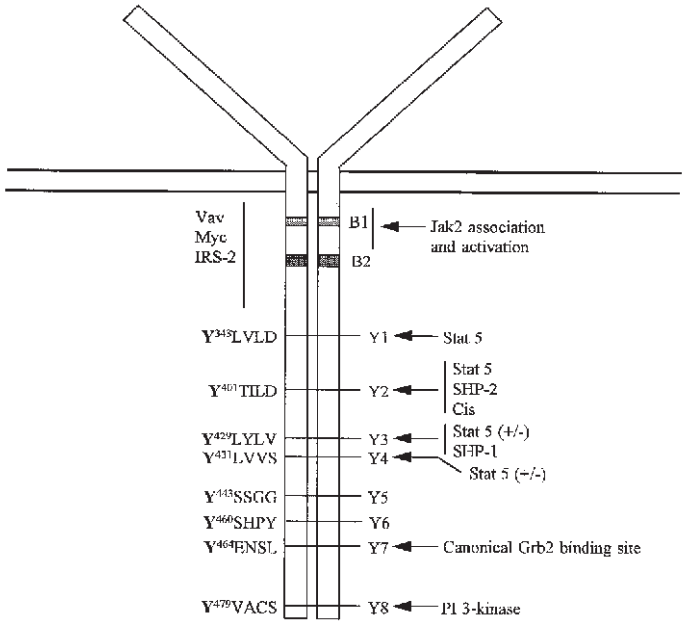

Figure 3. Schematic representation of the intracellular part of the Epo-R. Upon stimulation by Epo, the p66 chain of the Epo-R dimerizes. Jak2 tyrosine kinase, associated to the Epo-R close to the transmembrane region, is activated and phosphorylates most of the 8 tyrosine residues which become docking sites for signal transduction proteins.

The role of tyrosine phosphorylation of the EpoR in Epo-induced signalling is debated. An EpoR completely devoid of tyrosine residues is still able to transduce a proliferative signal; some reports found that these EpoR were less sensitive to Epo,[62,104,105,114] whereas this decrease in sensitivity was not mentioned by others.[103,107] Expression of β globin can be obtained in Ba/F3 cells after transfection of mutant EpoRs that do not contain any tyrosine residues.[62,114] However, in normal erythroid progenitors, the last tyrosine of the EpoR cytoplasmic domain (Tyr 479) seems to be required for erythroid colony formation.[115,116] This Tyr 479 is sufficient to obtain erythroid differentiation of progenitors from fetal liver;[116] similar properties were recently reported by Longmore *et al.* in an *in vivo* model.[117]

Another cytokine receptor belonging to the tyrosine kinase receptor family, the stem-cell-factor receptor or c-kit, seems to interact with the EpoR. It was shown that this receptor associated with the extended box2 region of the EpoR and could activate and phosphorylate the EpoR, thus enhancing erythroid cell proliferation and differentiation.[118] In addition, this cooperation between c-kit and EpoR has been found to be essential for normal erythroid differentiation of progenitors derived from fetal liver.[115] The exact mechanism of the interaction between these two receptors is, however, not clear and they seem to act through distinct intracellular signals.[119]

Role of Epo in erythropoiesis

Cultures of hematopoietic progenitors in semi-solid media have shown that the main targets of Epo are the late erythroid progenitors, especially the colony forming unit-erythroid (CFU-E). Indeed, studies on knock out mice lacking Epo or the EpoR have shown that Epo is crucial *in vivo* for the proliferation and survival of CFU-E and their irreversible terminal differentiation, whereas it is not required for generation of BFU-E and their differentiation to CFU-E.[120-122] Thus, Epo does not appear to be involved in the commitment of the erythroid lineage and seems to act mainly as a survival factor, allowing both the maintenance of cell proliferation and the induction of expression of erythroid specific proteins.[123] A recent report is in agreement with the idea that the EpoR would function mainly to transduce anti-apoptotic signals; in this work, erythroid progenitors from murine fetal liver were able to differentiate fully into erythroblasts after infection with prolactin receptors and further stimulation with prolactin instead of Epo.[124] These prolactin receptors belong to the same cytokine receptor family as the EpoR and it seems that activation of this class of receptors during the stem cell maturation process is sufficient to trigger lineage differentiation, according to the stochastic model. We obtained similar results after infection of human $CD34^+$ cells derived from cord blood with a prolactin receptor (unpublished data). HCD57 cells are murine erythroid cells which respond to Epo and undergo apoptosis after Epo deprivation. However, HCD57 cells infected with retroviral vectors encoding Bcl-2 or Bcl-x_L remain viable in the absence of Epo, thereby confirming the anti-apoptotic role of Epo.[125]

The main indication of Epo treatment is for correction of anemia of renal failure. In some cases, this treatment has been extended to anemia of chronic diseases. In these disorders, cytokines involved in the inflammatory response inhibit both Epo synthesis and erythroid colony formation *in vitro*.[126, 127] It was

recently shown that INFγ downregulates SCF and EpoR at the surface of the erythroid progenitors, thus leading to reduction of the survival and growth of these cells and eventually to apoptosis of the progenitors.[128] Further studies showed that interferon-γ induced the concomitant expression of Fas and Fas ligand at the surface of the erythroid progenitors, thereby leading to apoptotic cell death.[129] More work is needed to understand whether physiologic interactions between the intracellular signals induced by Epo and the Fas system play a role in erythroid cell survival.

Conclusions

Considerable progress in understanding the physiology of Epo has been made, especially in the mechanism of hypoxia-inducible gene regulation, and in the identification of different proteins involved in Epo-induced signal transduction. Basic researches are following two different directions. First, insights have been gained into the regulation of the oxygen-signalling pathway. HIF-1 is a crucial component for hypoxia-induced regulation of many genes and this process is widespread in a various array of cells. Second, multiple pathways were depicted in the cytokine-induced signalling cascade, but none was described as being specifically triggered by Epo. It is still not understood whether Epo is necessary for cell proliferation, or differentiation or only for cell survival. Recent reports seem to favor the hypothesis that there is some redundancy in the cytokines for the survival and proliferation of the hematopoietic cells. Further work is needed to determine the specific mode of action of Epo in the erythroid lineage.

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CL was primarily responsible for the conception of this review article and the writing of the paper. PM contributed to the analysis of the literature and writing of the paper.

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References

1. Jelkmann W, Metzen E. Erythropoietin in the control of red cell production. Ann Anat 1996; 178:391-403.
2. Wang GL, Semenza GL. Molecular basis of hypoxia-induced erythropoietin expression. Curr Opin Hematol 1996; 3:156-62.
3. Ratcliffe PJ. Molecular biology of erythropoietin [clinical conference]. Kidney Int 1993; 44:887-904.
4. Sawyer ST, Penta K. Erythropoietin cell biology. Hematol Oncol Clin North Am 1994; 8:895-911.
5. Porter DL, Goldberg MA. Physiology of erythropoietin production. Semin Hematol 1994; 31:112-21.
6. Fisher JW. Erythropoietin: physiologic and pharmacologic aspects. Proc Soc Exp Biol Med 1997; 216: 358-69.
7. Fried W. Erythropoietin. Annu Rev Nutr 1995; 15:353-77.
8. Lacombe C, Mayeux P. L'érythropoïétine. Médecine/sciences 1995; 11:947-55.
9. Damen JE, Krystal G. Early events in erythropoietin-induced signaling. Exp Hematol 1996; 24:1455-9.
10. Miyake T, Kung CK, Goldwasser E. Purification of human erythropoietin. J Biol Chem 1977; 252:5558-64.
11. Lin FK, Suggs S, Lin CH, et al. Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci USA 1985; 82:7580-4.
12. Jacobs K, Shoemaker C, Rudersdorf R, et al. Isolation and characterization of genomic cDNA clones of human erythropoietin. Nature 1985; 313:806-10.
13. Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. N Engl J Med 1987; 316:73-8.
14. Zanjani ED, Ascensao JL, McGlave PB, Banisadre M, Ash RC. Studies on the liver to kidney switch of erythropoietin production. J Clin Invest 1981; 67:1183-8.
15. Wintour EM, Butkus A, Earnest L, Pompolo S. The erythropoietin gene is expressed strongly in the mammalian mesonephric kidney. Blood 1996; 88:3349-53.
16. Jacobson LO, Goldwasser E, Fried W, Plzak L. Role of the kidney in erythropoiesis. Nature 1957; 179:633-4.
17. Beru N, McDonald J, Lacombe C, Goldwasser E. Expression of the erythropoietin gene. Mol Cell Biol 1986; 6:2571-5.
18. Bondurant MC, Koury MJ. Anemia induces accumulation of erythropoietin mRNA in the kidney and liver. Mol Cell Biol 1986; 6:2731-3.
19. Koury ST, Bondurant MC, Koury MJ. Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. Blood 1988; 71:524-7.
20. Lacombe C, Da Silva JL, Bruneval P, et al. Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. J Clin Invest 1988; 81:620-3.
21. Eckardt KU, Mollmann M, Neumann R, et al. Erythropoietin in polycystic kidneys. J Clin Invest 1989; 84:1160-6.
22. Bachmann S, Le Hir M, Eckardt KU. Co-localization of erythropoietin mRNA and ecto-5'-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. J Histochem Cytochem 1993; 41:335-41.
23. Maxwell PH, Osmond MK, Pugh CW, et al. Identification of the renal erythropoietin-producing cells using transgenic mice. Kidney Int 1993; 44:1149-62.
24. Da Silva JL, Lacombe C, Bruneval P, et al. Tumor cells are the site of erythropoietin synthesis in human renal cancers associated with polycythemia. Blood 1990; 75:577-82.
25. Ratcliffe PJ, Jones RW, Phillips RE, Nicholls LG, Bell JI. Oxygen-dependent modulation of erythropoietin mRNA levels in isolated rat kidneys studied by RNase protection. J Exp Med 1990; 172:657-60.
26. Koury ST, Bondurant MC, Koury MJ, Semenza GL.

Localization of cells producing erythropoietin in murine liver by in situ hybridization. Blood 1991; 77: 2497-503.
27. Maxwell PH, Ferguson DJ, Osmond MK, et al. Expression of a homologously recombined erythopoietin-SV40 T antigen fusion gene in mouse liver: evidence for erythropoietin production by Ito cells. Blood 1994; 84:1823-30.
28. Fandrey J, Bunn HF. In vivo and in vitro regulation of erythropoietin mRNA: measurement by competitive polymerase chain reaction. Blood 1993; 81:617-23.
29. Tan CC, Eckardt KU, Ratcliffe PJ. Organ distribution of erythropoietin messenger RNA in normal and uremic rats. Kidney Int 1991; 40:69-76.
30. Masuda S, Okano M, Yamagishi K, Nagao M, Ueda M, Sasaki R. A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes. J Biol Chem 1994; 269:19488-93.
31. Digicaylioglu M, Bichet S, Marti HH, et al. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci USA 1995; 92:3717-20.
32. Masuda S, Nagao M, Takahata K, et al. Functional erythropoietin receptor of the cells with neural characteristics. Comparison with receptor properties of erythroid cells. J Biol Chem 1993; 268:11208-16.
33. Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience 1997; 76:105-16.
34. Schuster SJ, Badiavas EV, Costa-Giomi P, Weinmann R, Erslev AJ, Caro J. Stimulation of erythropoietin gene transcription during hypoxia and cobalt exposure. Blood 1989; 73:13-6.
35. Semenza GL, Traystman MD, Gearhart JD, Antonarakis SE. Polycythemia in transgenic mice expressing the human erythropoietin gene. Proc Natl Acad Sci USA 1989; 86:2301-5.
36. Semenza GL, Koury ST, Nejfelt MK, Gearhart JD, Antonarakis SE. Cell-type-specific and hypoxia-inducible expression of the human erythropoietin gene in transgenic mice. Proc Natl Acad Sci USA 1991; 88:8725-9.
37. Semenza GL, Dureza RC, Traystman MD, Gearhart JD, Antonarakis SE. Human erythropoietin gene expression in transgenic mice: multiple transcription initiation sites and cis-acting regulatory elements. Mol Cell Biol 1990; 10:930-8.
38. Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci USA 1991; 88:5680-4.
39. Beck I, Ramirez S, Weinmann R, Caro J. Enhancer element at the 3'-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. J Biol Chem 1991; 266:15563-6.
40. Blanchard KL, Acquaviva AM, Galson DL, Bunn HF. Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol Cell Biol 1992; 12:5373-85.
41. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992; 12:5447-54.
42. Beck I, Weinmann R, Caro J. Characterization of hypoxia-responsive enhancer in the human erythropoietin gene shows presence of hypoxia-inducible 120-Kd nuclear DNA-binding protein in erythropoietin-producing and nonproducing cells. Blood 1993; 82: 704-11.
43. Wang GL, Semenza GL. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J Biol Chem 1993; 268:21513-8.
44. Pugh CW, Ebert BL, Ebrahim O, Ratcliffe PJ. Characterisation of functional domains within the mouse erythropoietin 3' enhancer conveying oxygen-regulated responses in different cell lines. Biochim Biophys Acta 1994; 1217:297-306.
45. Galson DL, Tsuchiya T, Tendler DS, et al. The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. Mol Cell Biol 1995; 15: 2135-44.
46. Arany Z, Huang LE, Eckner R, et al. An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci USA 1996; 93:12969-73.
47. Huang LE, Ho V, Arany Z, et al. Erythropoietin gene regulation depends on heme-dependent oxygen sensing and assembly of interacting transcription factors. Kidney Int 1997; 51:548-52.
48. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995; 270:1230-7.
49. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 1995; 92:5510-4.
50. Hoffman EC, Reyes H, Chu FF, et al. Cloning of a factor required for activity of the Ah (dioxin) receptor. Science 1991; 252:954-8.
51. Kallio PJ, Pongratz I, Gradin K, McGuire J, Poellinger L. Activation of hypoxia-inducible factor 1alpha: post-transcriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proc Natl Acad Sci USA 1997; 94:5667-72.
52. Salceda S, Caro J. Hypoxia-inducible factor 1 alpha (HIF-1 alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997; 272:22642-7.
53. Maxwell PH, Pugh CW, Ratcliffe PJ. Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. Proc Natl Acad Sci USA 1993; 90:2423-7.
54. Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci USA 1993; 90:4304-8.
55. Wenger RH, Gassmann M. Oxygen(es) and the hypoxia-inducible factor-1. Biol Chem 1997; 378:609-16.
56. Bunn HF, Poyton RO. Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev 1996; 76:839-85.
57. Goldberg MA, Dunning SP, Bunn HF. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science 1988; 242:1412-5.
58. D'Andrea AD, Zon LI. Erythropoietin receptor: subunit structure and activation. J Clin Invest 1990; 86: 681-7.
59. Mayeux P, Billat C, Jacquot R. The erythropoietin receptor of rat erythroid progenitor cell: Characterization and affinity cross-linkage. J Biol Chem 1987; 262:13985-90.
60. D'Andrea AD, Lodish HF, Wong GG. Expression cloning of the murine erythropoietin receptor. Cell 1989; 57:277-85.
61. Mayeux P, Lacombe C, Casadevall N, Chretien S, Dusanter I, Gisselbrecht S. Structure of the murine erythropoietin receptor complex. Characterization of the erythropoietin cross-linked proteins. J Biol Chem 1991; 266:23380-5.
62. Gobert S, Porteu F, Pallu S, et al. Tyrosine phosphorylation of the erythropoietin receptor: role for differ-

entiation and mitogenic signal transduction. Blood 1995; 86:598-606.
63. Winkelmann JC, Ward J, Mayeux P, Lacombe C, Schimmenti L, Jenkins RB. A translocated erythropoietin receptor gene in a human erythroleukemia cell line (TF-1) expresses an abnormal transcript and a truncated protein. Blood 1995; 85:179-85.
64. Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 1990; 87:6934-8.
65. Hilton DJ, Watowich SS, Katz L, Lodish HF. Saturation mutagenesis of the WSXWS motif of the erythropoietin receptor. J Biol Chem 1996; 271:4699-4708.
66. Philo JS, Aoki KH, Arakawa T, Nahri LO, Wen J. Dimerization of the extracellular domain of the erythropoietin (Epo) receptor by Epo: one high-affinity and one low-affinity interaction. Biochemistry 1996; 35:1681-91.
67. Boissel JP, Lee WR, Presnell SR, Cohen FE, Bunn HF. Erythropoietin structure-function relationships. Mutant proteins that test a model of tertiary structure. J Biol Chem 1993; 268:15983-93.
68. Elliott S, Lorenzini T, Chang D, Barzilay J, Delorme E. Mapping of the active site of recombinant human erythropoietin. Blood 1997; 89:493-502.
69. Wen D, Boissel JP, Showers M, Ruch BC, Bunn HF. Erythropoietin structure-function relationships: Identification of functionally important domains. J Biol Chem 1994; 269:22839-46.
70. Caravella JA, Lyne PD, Richards WG. A partial model of the erythropoietin receptor complex. Proteins, structure, function. Genetics 1996; 24:394-401.
71. De Vos AM, Ultsch M, Kossiakoff AA. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 1992; 255: 306-12.
72. Li J-P, D'Andrea AD, Lodish HF, Baltimore D. Activation of cell growth by binding of friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature 1990; 343:762-4.
73. Casadevall N, Lacombe C, Muller O, Gisselbrecht S, Mayeux P. Multimeric structure of the membrane erythropoietin receptor of murine erythroleukemia cells (Friend cells): cross-linking of erythropoietin with the spleen focus-forming virus envelope protein. J Biol Chem 1991; 266:6952-6.
74. Yoshimura A, Longmore G, Lodish HF. Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. Nature 1990; 348:647-9.
75. Longmore GD, Lodish HF. An activating mutation in the murine erythropoietin receptor induces erythroleukemia in mice: a cytokine receptor superfamily oncogene. Cell 1991; 67:1089-102.
76. Schneider H, Chaovapong W, Matthews DJ, et al. Homodimerization of the erythropoietin receptor by a divalent monoclonal antibody triggers cell proliferation and differentiation of erythroid precursors. Blood 1997; 89:473-82.
77. Livnah O, Stura EA, Johnson DL, et al. Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 A. Science 1996; 273:464-71.
78. Wrighton NC, Farrell FX, Chang R, et al. Small peptides as potent mimetics of the protein hormone erythropoietin. Science 1996; 273:458-64.
79. Wrighton NC, Balasubramanian P, Barbone FP, et al. Increased potency of an erythropoietin peptide mimetic through covalent dimerization. Nature Biotechnol 1997; 15:1261-5.
80. Witthuhn B, Quelle FW, Silvennoinen O, et al. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following Epo stimulation. Cell 1993; 74:227-36.
81. Hilton CJ, Berridge MV. Conserved region of the cytoplasmic domain is not essential for erythropoietin-dependent growth. Growth Factors 1995; 121:263-76.
82. Tilbrook PA, Ingley E, Williams JH, Hibbs ML, Klinken SP. Lyn tyrosine kinase is essential for erythropoietin-induced differentiation of J2E erythroid cells. EMBO J 1997; 16:1610-9.
83. Damen J, Mui ALF, Hughes P, Humphries KR, Krystal G. Erythropoietin-induced tyrosine phosphorylation in a high erythropoietin-receptor expressing lymphoid cell line. Blood 1992; 80:1923-32.
84. Dusanter-Fourt I, Casadevall N, Lacombe C, et al. Erythropoietin induces the tyrosine phosphorylation of its own receptor in human erythropoietin-responsive cells. J Biol Chem 1992; 267:10670-5.
85. Miura O, D'Andrea A, Kabat D, Ihle JN. Induction of tyrosine phosphorylation by the erythropoietin receptor correlates with mitogenesis. Mol Cell Biol 1991; 11:4895-902.
86. Yoshimura A, Lodish HF. In vitro phosphorylation of the erythropoietin receptor and an associated protein, pp130. Mol Cell Biol 1992; 12:706-15.
87. Gobert S, Duprez V, Lacombe C, Gisselbrecht S, Mayeux P. Erythropoietin activates three forms of MAP kinase in UT7 erythroleukemia cells. Eur J Biochem 1995; 234:75-83.
88. Miura Y, Muira O, Ihle JN, Aoki N. Activation of the mitogen-activated protein kinase pathway by the erythropoietin receptor. J Biol Chem 1994; 269:29962-9.
89. Carroll MP, Spivak JL, McMahon M, Weich N, Rapp UR, May WS. Erythropoietin induces Raf-1 activation and Raf-1 is required for erythropoietin-mediated proliferation. J Biol Chem 1991; 266:14964-9.
90. Damen JE, Mui ALF, Puil L, Pawson T, Krystal G. Phosphatidylinositol 3-kinase associates, via its Src homology 2 domains, with the activated erythropoietin receptor. Blood 1993; 81:3204-10.
91. He TC, Zhuang H, Jiang N, Waterfield MD, Wojchowski DM. Association of the p85 regulatory subunit of phosphatidylinositol 3-kinase with an essential erythropoietin receptor subdomain. Blood 1993; 82: 3530-8.
92. Mayeux P, Dusanter-Fourt I, Muller O, et al. Erythropoietin induces the association of phosphatidylinositol 3' kinase with a tyrosine phosphorylated complex containing the erythropoietin receptor. Eur J Biochem 1993; 216:821-8.
93. Miura O, Nakamura N, Ihle JN, Aoki N. Erythropoietin-dependent Association of Phosphatidylinositol 3-Kinase with Tyrosine-phosphorylated Erythropoietin Receptor. J Biol Chem 1994; 269:614-20.
94. Verdier F, Chrétien S, Billat C, Gisselbrecht S, Lacombe C, Mayeux P. Erythropoietin induces the tyrosine phosphorylation of insulin receptor substrate-2: An alternate pathway for erythropoietin-induced phosphatidylinositol 3-kinase activation. J Biol Chem 1997; 272:26173-8.
95. Lioubin MN, Algate PA, Tsai S, Carlberg K, Aebersold R, Rohrschneider LR. p150 SHIP, a signal specific transduction molecule with inositol phosphate-5-phosphatase activity. Genes Develop 1996; 10: 1084-95.
96. Damen JA, Liu L, Rosten P, et al. The 145-kDa protein induced to associate with Shc with multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase. Proc Natl Acad Sci USA 1996; 93:1689-93.
97. Franke TF, Kaplan DR, Cantley LC. PI3K: downstream

AKTion blocks apoptosis. Cell 1997; 88:435-7.
98. del Peso L, Gonzalez-García M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 1997; 278:687-9.
99. Ihle JN. Cytokine receptor signalling. Nature 1995; 377:591-4.
100. Gouilleux F, Pallard C, Dusanter-Fourt I, et al. Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-STAT5 DNA binding activity. EMBO J 1995; 14: 2005-13.
101. Pallard C, Fabrice G, Martine C, Groner B, Gisselbrecht S, Dusanter-Fourt I. Interleukin-3, erythropoietin, and prolactin activate a STAT5 like factor in lymphoid cells. J Biol Chem 1995; 270:15942-5.
102. Wakao H, Harada N, Kitamura T, Mui ALF, Miyajima A. Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways. EMBO J 1995; 14:2527-35.
103. Chin H, Nakamura N, Kamiyama R, Miyasaka N, Ihle JN, Miura O. Physical and functional interactions between Stat5 and the tyrosine-phosphorylated receptors for erythropoietin and interleukin-3. Blood 1996; 88:4415-25.
104. Damen JE, Wakao H, Miyajima A, et al. Tyrosine 343 in the erythropoietin-receptor positively regulates erythropoietin-induced cell proliferation and STAT5 activation. EMBO J 1995; 14:5557-68.
105. Gobert S, Chrétien S, Gouilleux F, et al. Identification of tyrosine residues within the intracellular domain of the erythropoietin receptor crucial for STAT5 activation. EMBO J 1996; 15:2434-41.
106. Klingmuller U, Bergelson S, Hsiao JG, Lodish HC. Multiple tyrosine residues in the cytosolic domain of the erythropoietin receptor promote activation of STAT5. Proc Natl Acad Sci USA 1996; 93:8324-8.
107. Quelle FW, Wang D, Nosaka T, et al. Erythropoietin induces the activation of STAT5 through association with specific tyrosines on the receptor that are not required for a mitogenic response. Mol Cell Biol 1996; 16:1622-31.
108. Chrétien S, Varlet P, Verdier F, et al. Erythropoietin-induced differentiation of the human erythroleukemia cell line TF-1 correlates with impaired STAT5 activation. EMBO J 1996:4174-81.
109. Iwatsuki K, Endo T, Misawa H, et al. STAT5 activation correlates with erythropoietin receptor-mediated erythroid differentiation of an erythroleukemia cell line. J Biol Chem 1997; 272:8149-52.
110. Wakao H, Chida D, Damen JE, Krystal G, Miyajima A. A possible involvement of Stat5 in erythropoietin-induced hemoglobin synthesis. Biochem Biophys Res Commun 1997; 234:198-205.
111. Tauchi T, Damen JE, Toyama K, Feng GS, Broxmeyer HE, Krystal G. Tyrosine 426 within the activated erythropoietin receptor binds Syp, reduces the erythropoietin required for Syp tyrosine phosphorylation and promotes mitogenesis. Blood 1996; 87:4495-501.
112. Klingmuller U, Lorenz U, Cantley LC, Neel BC, Lodish HC. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell 1995; 80:729-38.
113. De La Chapelle A, Traskelin A, Juvonen E. Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. Proc Natl Acad Sci USA 1993; 90:4495-9.
114. Krosl J, Damen J, Krystal G, Humphries RK. Interleukin-3 (IL-3) inhibits erythropoietin-induced differentiation in Ba/F3 cells via the IL-3 receptor alpha subunit. J Biol Chem 1996; 271:27432-7.
115. Wu H, Klingmuller U, Acurio A, Hsiao JG, Lodish HF. Functional interaction of erythropoietin and stem cell factor receptors is essential for erythroid colony formation. Proc Natl Acad Sci USA 1997; 94:1806-10.
116. Klingmuller U, Wu H, Hsiao JG, et al. Identification of a novel pathway important for proliferation and differentiation of primary erythroid progenitors. Proc Natl Acad Sci USA 1997; 94:3016-21.
117. Longmore GD, You Y, Molden J, et al. Redundant and selective roles for erythropoietin receptor tyrosines in erythropoiesis in vivo. Blood 1998; 91:870-878.
118. Wu H, Klingmuller U, Besmer P, Lodish HF. Interaction of the erythropoietin and stem-cell-factor receptors. Nature 1995; 377:242-6.
119. Jacobs-Helber SM, Penta K, Sun Z, Lawson A, Sawyer ST. Distinct signaling from stem cell factor and erythropoietin in HCD57 cells. J Biol Chem 1997; 272: 6850-3.
120. Kieran MW, Perkins AC, Orkin SH, Zon LI. Thrombopoietin rescues in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. Proc Natl Acad Sci USA 1996; 93:9126-31.
121. Lin C-S, Lim SK, D'Agati V, Costantini F. Differential effects of an erythropoietin receptor gene disruption on primitive and definitive eythropoiesis. Genes Develop 1996; 10:154-64.
122. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell 1995; 83:59-67.
123. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed cell death in erythroid progenitor cells. Science 1990; 248:378-80.
124. Socolovsky M, Dusanter-Fourt I, Lodish HF. The prolactin receptor and severely truncated erythropoietin receptors support differentiation of erythroid progenitors. J Biol Chem 1997; 272:14009-12.
125. Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna JL. Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. Blood 1996; 88:1576-82.
126. Faquin WC, Schneider TJ, Goldberg MA. Effect of inflammatory cytokines on hypoxia-induced erythropoietin production. Blood 1992; 79:1987-94.
127. Means RT, Jr., Krantz SB. Progress in understanding the pathogenesis of the anemia of chronic disease. Blood 1992; 80:1639-47.
128. Taniguchi S, Dai CH, Price JO, Krantz SB. Interferon gamma downregulates stem cell factor and erythropoietin receptors but not insulin-like growth factor-I receptors in human erythroid colony-forming cells. Blood 1997; 90:2244-52.
129. Dai C-H, Price JO, Brunner T, Krantz SB. Fas ligand is present in human erythroid colony-forming cells and interacts with Fas indiced by Interferon g to produce erythroid cell apoptosis. Blood 1998; 91:2135-1242.