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# Pathogenesis of polycythemia vera

José Luis Fernandez-Luna, Maite Silva, Carlos Richard,* Cristina Sanz, Adalberto Benito

*Servicio de Inmunologia and *Servicio de Hematologia, Hospital Universitario Marques de Valdecilla, INSALUD, Santander, Spain*

**Abstract**

***Background and Objective.*** **Polycythemia vera (PV) is an acquired clonal myeloproliferative disorder characterized by increased production of mature red cells. We still lack a molecular target responsible for this disorder; however, recent investigations have focused on a number of molecules involved in signal transduction pathways mediated by erythropoietin (Epo) and other growth factors. Here we review the implication of these molecules in the pathogenesis of PV.**

***Information sources.*** **The material reviewed in this work includes articles published in journals covered by Medline. We also include data obtained in our laboratory regarding to the significance of apoptosis inhibitory proteins in erythroid development.**

***State of the Art and Perspectives.*** **Overproduction of erythroid cells in PV is particular in that it occurs in the absence of a recognizable physiologic stimulus, since circulating serum levels of Epo are normal or lower than normal. Genetic analysis as well as *in vitro* studies, have established an essential role for Epo in the survival and maturation of committed erythroid progenitors. Epo initiates its cellular response by binding to the Epo receptor (EpoR) expressed on the surface of immature erythroblasts. Following ligand binding, EpoR is known to activate a cytoplasmic protein tyrosine kinase, Jak2 which triggers a signal transduction cascade that leads to the development of early erythroid progenitors into mature erythroblast cells. Although the mechanism underlying the increased erythroid production in PV is not well understood, a number of causes have recently came for which may provide insights not only for the pathogenesis of PV but also for a fundamental biological process: the mechanism whereby a multipotential stem cell gives rise to a particular cell lineage.**


Key words: polycythemia vera, pathogenesis, erythropoietin, IGF-I

In polycythemia vera (PV), genetic alterations are thought to change the behavior of a hematopoietic stem cell, which eventually produces most peripheral blood cells and generates increased numbers of erythroid cells, often with a concomitant rise in neutrophils and/or megakaryocytes (Table 1).[1] Because a multipotential progenitor cell is involved in PV, clinical manifestations of the various myeloproliferative disorders often overlap; however, in each disorder one cell lineage is primarily involved. Several mechanisms have been suggested for the preferential involvement of a different cell lineage in each myeloproliferative disorder.[2-4] It can be argued that different abnormal clones may respond differently to normal hematopoietic growth factors.[2] A second mechanism involves two lesions, one occurring in the multipotential stem cell and a second in the committed progenitor cell compartment.[3] Finally, an attractive hypothesis proposes that hematopoietic differentiation is a stochastic process and consequently it is not directed by hematopoietic growth factors.[4,5] Accordingly, a mutation that occurs in a cell which randomly develops high erythroid potential will result in PV.[4] This model is consistent with the results described by Fairbairn *et al.*,[5] with IL-3-dependent FDCP-Mix myeloid progenitor cells. These cells transduced with the anti-apoptotic gene *bcl-2* acquired the ability to undergo myeloerythroid differentiation in the absence of IL-3, arguing that the growth factor promotes proliferation and survival, but it is not directly implicated in the induction of differentiation.

This review will focus on recent advances in the functional characterization of growth factors and their receptors, signal transducer molecules and apoptosis inhibitory proteins, which may shed light on the molecular basis of PV.

## *Erythropoietin receptor: a first putative target gene*

Erythropoietin (Epo), a growth factor synthesized by the kidney, is the major regulator of mammalian erythropoiesis. Unlike many other hematopoietic growth factors, Epo is very lineage-restricted in its action. Human Epo was the first hematopoietic growth factor to be identified and characterized.[6,7] It exerts its effects by binding to specific receptors that are present on the surface of immature erythroid progenitors.[8] Analysis of mutant mice has

*Correspondence: Dr. José L. Fernandez-Luna, Servicio de Inmunologia, Hospital Universitario Marques de Valdecilla, INSALUD, 39008 Santander, Spain.*
*Phone: international +34-42-203453 • Fax: international +34-42-202655 • E-mail: hospif01@sarenet.es*

**Table 1. Main biological features of polycythemia vera.**

- acquired clonal disorder
- abnormalities are predominantly erythroid
- erythrocytosis
- in vitro growth of erythroid colonies without Epo
- no mutations detected in the Epo receptor gene
- erythroid progenitors are hypersensitive to IGF-I

clearly established an essential role for Epo and its receptor (EpoR) in the development of erythroid progenitors *in vivo*.[9] In mice embryos which were Epo- or EpoR deficient, definitive erythropoiesis is completely impaired and liver tissue contains increased numbers of nucleated erythroid cells undergoing apoptosis. However, neither Epo nor the EpoR are required for erythroid lineage commitment or for proliferation and differentiation of burst-forming unit-erythroid (BFU-E) to colony-forming unit-erythroid (CFU-E) progenitors, because both differentiation stages are present in mice carrying null mutations in the Epo or EpoR genes.[9] Thus, genetic analysis as well as *in vitro* studies[10-12] have established an essential role for Epo in the survival and maturation of CFU-E progenitors and early erythroblasts. Beyond the late basophilic erythroblast stage, it is also known that the level of the EpoR is downregulated, and the cells are no longer dependent on Epo for their maturation.[13] The cloning of the EpoR[14] has allowed very rapid progress in our understanding of the early intracellular events that may be triggered by Epo in erythroid progenitors. Many efforts are centered in characterizing the molecules involved in Epo-mediated signaling pathways and determining whether various hematologic disorders can be attributed to aberrations in these pathways. Since PV is a condition in which the defect is intrinsic to the cells and not driven by an increased Epo production, the hypothesis that PV is caused by a mutation of the EpoR gene was studied first.

Yoshimura *et al.*, isolated mutant EpoRs from an IL-3-dependent hematopoietic cell line Ba/F3, infected with retroviruses expressing the receptor cDNA.[15] They searched for spontaneous mutations in the EpoR gene and after selection by gradually decreasing in the concentration of Epo, they identified two classes of activating mutations. One is a single point mutation in the exoplasmic domain that enables the cells to grow in the absence of growth factor. The other is a truncation of the C-terminal 42 amino acids in the cytoplasmic domain of the EpoR. This mutation makes the receptor hypersensitive to Epo, but is insufficient for inducing growth factor-independent survival. The C-terminal, serine-rich region of the EpoR (approximately 40 amino acids) has been identified as a negative-control domain. Truncation of this domain allows the Ba/F3 hematopoietic cells to grow maximally in 1/10 the concentration of Epo required for growth of cells expressing the wild-type EpoR.[16]

Despite the encouraging results obtained with cell lines that expressed the EpoR carrying different mutations, genetic alterations in the EpoR have not been detected in PV.[17,18] Recently, a new model which explains the molecular mechanism involved in the abnormal growth of erythroid cells in PV has came forth. This model is based on previous data published by Nakamura *et al.*, who described that in addition to the full-length EpoR mRNA, another mRNA species containing unspliced intron VII is expressed in early hematopoietic progenitors.[19,20] This unspliced mRNA encodes an EpoR with a truncated cytoplasmic region that functions as a dominant negative protein. When Ba/F3 cells are transfected with both full-length and truncated EpoR cDNAs, the response to Epo decreases. Based on these data, Chiba *et al.*, found that the expression of mRNA coding for the truncated form of EpoR is markedly decreased in hematopoietic cells of 5 patients with polycythemia vera.[21] They argued that a deregulation of the splicing system in multipotent hematopoietic progenitors could explain the decrease in truncated EpoR mRNA and consequently the erythrocytosis characteristic of PV. However, for this model Epo is needed to trigger the intracellular signals that lead to the development of erythroid progenitors and consequently does not explain the Epo-independent growth of erythroid colonies in semisolid culture media,[22] which is used as a diagnostic tool to distinguish PV from secondary erythrocytosis.[23]

Despite the absence of mutations in PV, a number of genetic alterations of the EpoR gene have been described in some cases of hereditary polycythemia. De La Chapelle *et al.*, described a large family in which inheritance of erythrocytosis is present.[24] Using sequence repeat polymorphism in the 5' region of the EpoR gene, they found a total linkage disequilibrium between one allele and the disease phenotype which indicates that the disease may be caused by a mutation in the EpoR gene. Since then, mutations consisting in C-terminal truncation,[25] frameshift mutation,[26] and short internal deletion[27] have been described.

In conclusion, it seems likely that a target molecule(s) other than the EpoR must account for the hematopoietic abnormalities observed in PV.

### *In search of other growth factors*

Interleukin-3 (IL-3), granulocyte-macrophage growth factor (GM-CSF), and stem cell factor (SCF) are hematopoietic growth factors that enhance the proliferation of erythroid as well as myeloid and megakaryocytic cell lineages. As such,

their effects on hematopoietic progenitor cells in PV have been analyzed.

Several authors have described that both peripheral blood mononuclear cells and purified BFU-E progenitors obtained from patients with PV are hypersensitive to human IL-3 and to GM-CSF.[28-30] In addition, PV colony-forming unit-granulocyte-macrophage (CFU-GM) and CFU-megakaryocyte also show a marked hypersensitivity to IL-3 and to GM-CSF.[30] However, another group has found that the spontaneous megakaryocyte colonies derived from blood and bone marrow of patients with myeloproliferative disorders, including PV, cannot be neutralized by antibodies against IL-3 and GM-CSF either alone or in combination.[31] This suggests that the megakaryocyte progenitor growth in these disorders under *in vitro* conditions is independent of IL-3 and GM-CSF.

Dose-response experiments in serum-free cultures using purified BFU-E from PV patients also shows an enhanced sensitivity of these erythroid progenitors to SCF, compared to normal BFU-E.[32] Flow cytometry and binding studies with radiolabeled SCF indicates that the enhanced sensitivity of PV erythroid progenitors to SCF does not appear to be related to changes in receptor number, binding affinity or internalization and that it must reside in a further internal cellular alteration.[32,33] These data seem to be consistent with the impaired growth of PV erythroid progenitors in the presence of antisense oligodeoxynucleotides that blocks the SCF receptor (*c-kit*) mRNA expression.[34] However, it has also been described that PV erythroid progenitors cultured with SCF and Epo give rise to a number of colonies similar to that obtained with normal erythroid progenitors under the same culture conditions.[35] Furthermore, when SCF is not present in the culture, even low levels of Epo and IL-3 are sufficient to obtain differences in colony formation between PV patients and normal subjects.

Another cause that may account for the abnormal behavior of hematopoietic progenitors in PV is the insulin-like growth factor-I (IGF-I) ligand-receptor system, which has been widely studied by Axelrad *et al*. These authors developed a truly serum-free system for the culture of erythroid progenitors, which omits undefined biological material containing erythroid activators and inhibitors.[36] Using this culture medium, they showed that the PV progenitors have a markedly increased sensitivity to IGF-I,[37] a growth factor that has been demonstrated to stimulate erythroid colony formation.[38,39] However, studies of IGF-I-deficient mice exhibit delayed bone development and reduced body weight among other abnormalities, but no alteration of erythropoiesis.[40,41] Nevertheless, it is conceivable that an increased IGF-I-mediated signaling could lead to the PV phenotype. In line with this theory, it has been described that in the absence of

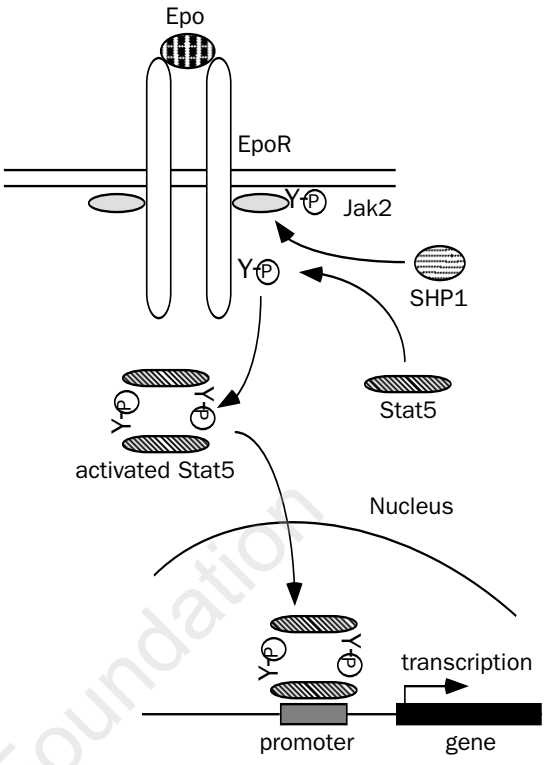


**Figure 1. Schematic representation of the Epo-mediated signaling pathway (see text for details).**

exogenous IGF-I, there is a basal level of tyrosine phosphorylation of the IGF-I receptor and interestingly, this level of phosphorylation is much greater in PV than in normal peripheral blood mononuclear cells.[42] In addition, a pronounced increase in tyrosine phosphorylation in PV mononuclear cells is observed when IGF-I is added.

Most of the IGF-I circulates bound to specific high-affinity binding proteins (IGFBP-1 to 6), which regulate the availability and activity of IGF-I in extracellular fluids.[43] IGFBP-3 is known to inhibit the biological activity of IGF-I,[44,45] whereas IGFBP-1 is believed to act as a functional regulator of IGF-I, either enhancing or attenuating its activity.[45,46] Recently, Axelrad's group studied the circulating levels of IGF-I and its key binding proteins, IGFBP-1 and IGFBP-3 in PV patients.[47] They found that the levels of IGFBP-1 are more than four times higher in PV patients than in normal controls or patients with secondary erythrocytosis, whereas the concentrations of IGFBP-3 and IGF-I are similar in both patient and control populations. Furthermore, IGFBP-1 appeared to be able to stimulate erythroid burst formation by markedly increasing progenitor cell sensitivity to IGF-I.[47] Therefore, the sustained high circulating level of IGFBP-1 provides an attrac-

tive mechanism to account for the constitutively excessive erythropoiesis characteristic of PV.

### *The puzzle of Epo-mediated intracellular signaling*

Many members of the cytokine receptor superfamily initiate intracellular signaling by inducing the rapid phosphorylation of target proteins. The EpoR belongs to this superfamily, and it is known that in response to Epo binding a number of proteins are transiently tyrosine phosphorylated. These include among others, the SHIP inositol 5-phosphatase;[48] the EpoR associated Janus kinase 2 (Jak2);[49] the signal transducer and activator of transcription 5 (Stat5);[50] the EpoR itself;[51] and p70, which most likely represents the SH2-containing phosphatase 1 (SHP1).[52]

From these and other findings, a model for early Epo-inducing signaling events can be summarized as follows:[53] homodimerization of the receptor in response to Epo binding transiently activates the tyrosine kinase Jak2, which in turn phosphorylates some tyrosines within the intracellular domain of the EpoR. This phosphorylation creates sites for binding of SH2 domain(s)-containing proteins, such as SHP1 and Stat5. Once associated with the receptor, Stat5 become phosphorylated on a C-terminal tyrosine residue by Jak2, and then dissociates from the EpoR and dimerizes.[54] This homodimer is the active form of Stat5, which finally translocates to the nucleus and binds to DNA through a consensus promoter sequence inducing the transcription of a number of genes (Figure 1). In addition, the tyrosine phosphatase SHP1 associates with Jak2, and this interaction results in induction of the enzymatic activity of the phosphatase, suggesting that SHP1 is an important functional regulator of Jak2.[55] Furthermore, two recently characterized novel genes, CIS and JAB, have been shown to regulate the Jak-Stat pathway of cytokine receptors.[56,57] CIS is a feedback modulator of Stat5; its expression is induced by Stat5 and it negatively modulates Stat5 activation; whereas JAB interacts with members of the Jak family markedly reducing their tyrosine kinase activity and consequently suppressing the tyrosine-phosphorylation and activation of Stats. Recently, it has been suggested that the Jak/Stat signaling pathway is necessary to prevent apoptosis of erythroid progenitors in response to Epo, but not to account for the proliferation of these cells.[58]

Taking all these data into consideration, one can foresee a complex network of intracellular signal transducers that lead to proliferation, differentiation and survival of hematopoietic progenitors. In line with this theory, molecules such as phosphoinositide 3-kinase (PI3K),[59] mitogen-activated protein kinase (MAPK),[60] phospholipase Cγ (PLCγ),[61] and the *c-vav* proto-oncogene product, *Vav*[62] have been implicated in the Epo-mediated signaling.

The question now is which one of these signal transducers presents an alteration in its expression pattern or its activity that might account for the increased erythropoiesis in PV patients. Three recent papers have focused special attention to the tyrosine phosphatase activity in PV hematopoietic cells. Dai *et al.*, showed that culture of normal erythroid progenitors with an inhibitor of tyrosine phosphatases, results in an increased number of erythroid colonies and enhanced protein tyrosine phosphorylation. In contrast, little enhancement is detected with PV cells. Since a balance between kinase and phosphatase activities is a key parameter for cell proliferation, these authors suggested that PV patients may have a deficient phosphatase activity that potentiates phosphorylation and consequently allows an increased cell proliferation.[63] On the contrary, Sui *et al.* found that the total protein tyrosine phosphatase activity in PV erythroid cells is

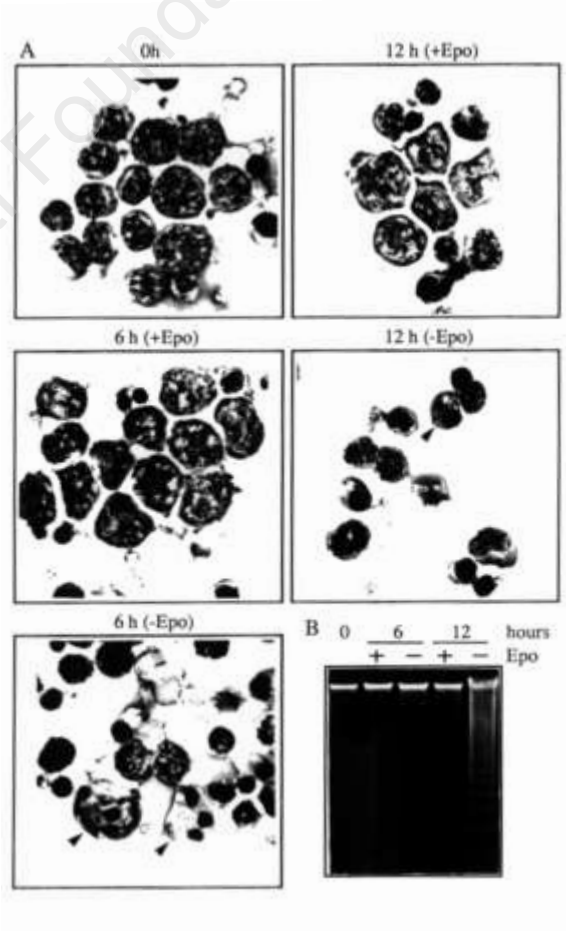


**Figure 2. Mouse erythroblasts cultured with or without Epo. After 6h in the absence of Epo, immature erythroblasts undergo apoptotic cell death and following 12h of Epo deprivation, virtually all surviving cells were mature Epo-independent erythroblasts. Arrowheads indicate apoptotic cells.**

2- to 3-fold higher than that in normal cells. They suggested that the increased activity might be due to a potentially new membrane or membrane-associated phosphatase.[64] Finally, Asimakopoulos *et al.* published a detailed study on the structural and transcriptional characteristics of the SHP1 phosphatase gene in PV patients.[65] As was described above, SHP1 is major regulator of the Jak2 kinase and in consequence regulates the Epo/EpoR-signaling.[55] In addition, mice deficient in SHP1 or carrying a deletion in the catalytic domain of this phosphatase, displayed a number of hematopoietic abnormalities including hypersensitivity of erythroid progenitors to Epo.[66,67] Although these data make SHP1 an attractive candidate gene, no quantitative or qualitative abnormalities are found by Southern blot analysis, methylation studies and genomic sequencing of the entire coding region and splice junctions of the SHP1 gene.[65] Furthermore, the levels of SHP1 protein in hematopoietic cells from PV patients are comparable to those from normal controls. To better understand these apparently conflicting results, it is interesting to note that only does this last work takes into consideration the number of clonal cells in the studied samples, assessed by a PCR-based clonality assay.[68] Accurate detection of any molecular alteration in PV depends on the majority of studied cells being clonal to avoid that normal cells may interfere the results.

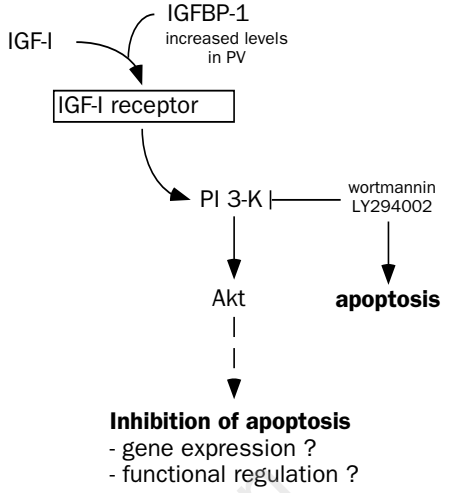


**Figure 3. Scheme of the IGF-I/IGFBP-1-induced intracellular pathway that leads to inhibition of apoptosis. Wortmannin and LY294002 are phosphatidylinositol 3-kinase (PI3K) inhibitors and induce apoptosis by blocking this survival pathway. Akt kinase can be stimulated by binding of the PI3K product, phosphatidylinositol (PI) 3,4-bisphosphate.**

### *Apoptosis regulatory genes: is this the clue?*

Apoptosis, or programmed cell death, has emerged as an important homeostatic mechanism within several hematopoietic lineages. This process is mainly regulated by hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF), GM-CSF, IL-3, IL-6, SCF and Epo, among others (see ref. #69 for review).

In the absence of Epo, erythroid progenitors die and their genomic DNA is degraded into oligonucleosomal fragments, a feature of apoptotic cell death[10,11] (Figure 2). A model has been proposed whereby the concentration of circulating Epo controls the number of erythroid progenitors that survive by repressing apoptosis:[70] During normal erythropoiesis, Epo levels are insufficient for the survival of a majority of the Epo-dependent erythroid progenitors. Thus a small fraction of this progenitor population gives rise to a normal production of red blood cells (RBC). When Epo levels are increased because of anemia or hypoxia, many progenitors that would ordinarily die will survive and consequently increase RBC production. On the contrary, when Epo levels decrease as a result of hypertransfusion or renal failure, the progenitor population decrease below normal values and therefore the production of RBC is reduced.

What is the mechanism by which Epo suppresses apoptosis of erythroid progenitor cells? Several members of the bcl-2 family of apoptosis regulatory genes have been identified that function as inhibitors of apoptosis in hematopoietic cells.[71,72] Recently, our group has shown that *Bcl-x$_L$*, a *Bcl-2* family member that inhibits apoptotic cell death,[73] is expressed in human erythroleukemia cell lines and that it may be involved in the regulation of erythroid survival during differentiation.[74] Furthermore, we have demostrated that when the Epo-dependent erythroid progenitor cell line HCD-57, is cultured in the absence of Epo, the expression of both *Bcl-x$_L$* and *Bcl-2* is rapidly downregulated and this is accompanied by activation of an apoptotic process.[75] The constitutive (deregulated) expression of *Bcl-x$_L$* rescues erythroid progenitors from apoptosis induced by Epo deprivation, suggesting that Epo functions as a survival factor by repressing apoptosis through *Bcl-x$_L$* and *Bcl-2* in erythroid progenitor cells.[75]

How can we link the Epo-EpoR pathway with the expression of survival genes? Zhuang *et al.*, found that the Epo-dependent inhibition of apoptosis is blocked by the ectopic expression of kinase-deficient dominant-negative forms of Jak2, suggesting an essential role of this tyrosine kinase in the apoptotic pathway.[76] More recently, Fujio *et al.*, showed that leukemia inhibitory factor (LIF) induces *bcl-x* mRNA through Stat1 in cardiac myocytes,[77] which is consistent with the presence of a Stat binding ele-

ment, known as interferon-γ activation site (GAS), in the promoter region of the *bcl-x* gene.[78] Although all the Stats, with the exception of Stat2, are able to bind to the consensus GAS element,[79] Stat factors display clear specifities for distinct GAS-like elements, and therefore we do not know yet whether Stat5 can also bind to the *bcl-x* promoter. Taking all these data into consideration an attractive model would be that in which Epo triggers the activation of Jak2-Stat5 transduction pathway and Stat5 induces the expression of *bcl-x* that blocks the apoptotic machinary of the cell. Consistent with this model is the observation made above, or rather that the Jak/Stat signaling pathway seems to be necessary to prevent apoptosis of erythroid progenitors in response to Epo, but not to account for the proliferation of these cells.[58] Accordingly, any alteration of the Jak-Stat pathway could affect the survival of erythroid progenitors.

An interesting correlation has been established between protection from apoptosis and the IGF-I/IGF receptor system. It has been shown that IGF-I inhibits apoptosis induced by different stimuli in erythroid progenitors, embryo fibroblasts and myeloid cells.[80-82] Furthermore, the IGF-I receptor activation prevents apoptotsis and maintains the expression of Bcl-2 in myeloid progenitor cells[83] and neurons.[84] Recently, Parrizas *et al.*, suggested that IGF-I is capable of preventing apoptosis by activation of multiple signal transduction pathways including those of the kinases PI3K and MAPK.[85] In line with this, inhibition of PI3K accelerates apoptosis, and an activated form of the serine/threonine kinase Akt, a downstream effector of PI3K, blocks apoptosis.[86]

However, the correlation between these signaling pathways and the expression of survival genes is not clear yet (Figure 3).

Little is known about the mechanisms that maintain the survival machinary in PV erythroid progenitors; however it seems clear that an intracellular alteration makes that the Epo-dependent progenitors which would ordinarily die in the absence of growth factor, survive and mature normally. Our group, has recently studied this issue and found that the expression of *Bcl-$x_L$* is deregulated in PV erythroid progenitors. In normal erythropoiesis, only the immature erythroblasts (Epo-dependent stages) express *Bcl-$x_L$* whereas in PV erythroid cells *Bcl-$x_L$* is expressed in all stages along the erythroid differentiation pathway.[87] Since the expression of *Bcl-$x_L$* depends on the presence of Epo in normal erythroid cells[75] and the PV erythroid progenitors maintain high levels of this survival factor in the absence of Epo, we suggest that a deregulated expression of *Bcl-$x_L$* may be responsible, at least in part, for the abnormal erythropoiesis in PV.

## Conclusions

From the results obtained with mice carrying null mutations in the Epo or EpoR genes, we know that erythropoiesis is dependent on the presence of Epo and its receptor. However in polycythemia vera other growth factors and/or signaling molecules could play an important role in the generation of erythroid cells. Among the growth factors studied so far, IGF-I is an attractive candidate because of the following criteria: the IGF-I binding protein IGFBP-1, stimulates erythroid burst formation by markedly increasing progenitor cell sensitivity to IGF-I and interestingly, the levels of IGFBP-1 are more than four times higher in PV patients than in normal controls or patients with secondary erythrocytosis. Furthermore, activation of the IGF-I receptor inhibits apoptosis of erythroid progenitors and in neurons, it upregulates the levels of the anti-apoptotic protein *Bcl-$x_L$*. In addition, we have observed that the expression of *Bcl-$x_L$* is deregulated in PV erythroid cells. In consequence, it seems evident that a chronic increased level of IGFBP-1 may enhance the ability of IGF-I to induce the expression of negative regulators of apoptosis. Alternatively, IGF-I may regulate the activity of either inducers (i.e., caspases) or inhibitors (i.e., Bcl-2-family members) of apoptosis. Whether any other molecule involved in signal transduction (i.e., tyrosine kinases and phosphatases) or mRNA transcription (i.e., Stats) is responsible for or contributes to the PV phenotype is still unresolved.

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*JLF-L was responsible for the conception of the studies on polycythemia vera performed in the last years in the Laboratory of Immunology in Santander, and the conception and writing of this review article. The other authors contributed to the analysis of the literature and writing of the paper.*

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