Erythropoietin, Thrombopoietin and Leptin Receptors: Signal transduction pathways

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8.ERYTHROPOIETIN,THROMBOPOIETIN AND LEPTIN RECEPTORS.

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INTRODUCTION

Erythropoietin (Epo), Thrombopoietin (Tpo) and leptin are hormones with distinct physiological properties. While the two formers regulate survival, growth and differentiation of erythroid and megakaryocytic progenitors respectively, leptin is crucial for mammalian body weight regulation[1-3]. The receptors for these different ligands have been isolated and well characterized and belong to the class I cytokine receptor family that contains most of the interleukin receptors involved in hematopoiesis as well as those of prolactin and growth hormone which play an important role in metabolism and reproduction[4]. This family of receptors shares structural similarities both in their extracellular and cytosolic parts and is characterized by the absence of a tyrosine kinase domain. Nevertheless, ligand binding to this type of receptor induces the tyrosine phosphorylation of many cellular substrates including the receptor itself leading to the activation of distinct signaling pathways. This review will focus first on the structure and biological properties of Epo, Tpo, leptin and their respective receptors. The second part will summarize the signal transduction pathways induced by

these different ligands and their respective role in cell proliferation, differentiation and survival.

STRUCTURE AND BIOLOGICAL PROPERTIES OF LIGANDS AND RECEPTORS.

Epo, Tpo and Leptin.

Epo has been purified at the end of the seventies and its cDNA derivative cloned several years after[5]. Epo cDNA was one of the first hematopoietic growth factor to be cloned and encoded a primary translation product of 166 amino-acids. This hormone is highly glycosylated. Its molecular weight is about 34 kDa while the respective part of the protein is of 18 kDa. Epo is highly conserved among different mammalian species with a 82% homology between human and mouse. The three dimensional structure of the protein has been determined and showed that Epo structure adopts a four helical conformation found in most of the cytokines which is essential for its activity[6]. During the adult life, Epo is expressed mainly in kidney and to a lesser extent in liver. In contrast, liver is the main site of Epo production during the embryonic life. In Brain, Epo expression has been detected in astrocytes[1]. Mice deficient in Epo die by embryonic day 13,5 due to a lack of a definitive erythropoieis in the fetal liver [7]. However, the presence of erythroid progenitors CFU-E and BFU-E is unaffected indicating that Epo is not necessary for the commitment to the erythroid lineage but affects the terminal differentiation of erythroid progenitors.

The c-Mpl receptor has been for a long time an orphan receptor. Inhibition of megakaryocytic colony formation *in* vitro by introduction of c-mpl anti-sense oligonucleotides in CD34+ cells have suggested that c-Mpl could encode a receptor for a megakaryocyte lineage specific growth factor[8]. Different strategies for cloning the putative c-Mpl ligand have been used ranging from its purification with different chromatography techniques and the determination of a partial amino-acid –sequence allowing cDNA cloning or by the use of cDNA libraries prepared from cells expressing c-Mpl and selected for their growth independency. The cDNAs isolated from different species encoded protein products with high degree of identity (69-77%)[2]. The predicted primary translation product of human Tpo cDNA contains 335 amino-acid with a peptide signal of 21 amino-acids at the N-terminus. The protein can be separated in two domains, one of 153 amino-acids which is highly conserved among different species sharing 23% identity with Epo and the second one with 181 amino-acids particularly rich in serine, threonine and proline residues and highly glycosylated. A truncated form of Tpo containing only the Epo like domain was shown to be active *in vitro* and a presumably spliced variant form of Tpo (Tpo2) with a four amino-acid

deletion within the Epo like domain is unable to confer growth of cells expressing the c-Mpl receptor indicating that the Epo like domain is crucial for Tpo activity[9]. Indeed this domain contains four cystein residues that form disulfide bridges required for activity. This amino-terminal domain would therefore adopt the helical conformation described for Epo and other cytokines. Tpo is expressed mainly in mouse and human liver and kidney. However, Tpo mRNA was also detected in skeletal muscle, brain, spleen and bone marrow. Hepatocytes and hepatoma cell lines express also Tpo mRNA. Mice lacking Tpo are severely thrombocytopenic with a 80 to 90% reduction in both platelets and megakaryocytes demonstrating the essential physiological role of Tpo in megakaryocytopoiesis and the production of platelets *in vivo*[10].

Among the loci identified in mice at which extreme obesity mutations occurs, *obese* (*ob*) and *diabete* (*db*) were the most intensively studied[11,12]. The parabiosis (cross-circulation) experiments of an *ob/ob* mice and a lean wild-type control resulted in *ob/ob* mouse weight normalization indicating that a circulating factor responsible for the weight regulation was absent in *ob/ob* mice. Purification of this factor was unsuccessful and a positional cloning strategy was used to isolate the *ob* gene and to identify its encoded 167 kDa amino-acid product, leptin (Ob)[13]. The mature polypeptide is secreted by adipose tissues and is found in the blood of various mammalian species including mice and humans. The predicted tertiary structure suggests that leptin adopts a helical cytokine like structure similar to Interleukin-2 (IL–2) and Growth Hormone (GH)[14]. Administration of recombinant leptin to *ob/ob* mice results in food intake reduction and weight loss. Leptin mRNA and protein expression are regulated in humans and rodents by changes in percentage of body fat but also by changes in food intake. Expression is up-regulated when the body fat increased and down-regulated when the body weight is reduced. Beside its metabolic and endocrine functions, leptin plays also a regulatory role in immunity, inflammation and hematopoiesis. Alterations in immune and inflammatory responses are present in leptin deficient mice[15].

Epo/Tpo/leptin receptors.

Epo, Tpo, and leptin receptors belong to the type 1 super-family of single transmembrane cytokine receptors (see Figure1). The Epo receptor (Epo-R) is the founder member of this family and is composed of a unique chain of 66 kDa [16,17]. The extracellular part of the receptor contains two Fibronectin III like sub-domains. Within the distal subdomain (called D1), two pairs of cystein residues form a disulfide bridge while the membrane proximal sub-domain (called D2) contains the conserved motif WSXWS known to be important for Epo receptor folding[18]. The cytoplasmic part ,

long of 236 amino acids, can be divided into two functional regions. The membrane proximal region contains two domains called box-1 and box-2 which are conserved in other cytokine receptors and are necessary for JAK kinase activation and Epo-induced mitogenesis [19-21]. The membrane distal region includes eight tyrosine residues which are phosphorylated upon Epo stimulation [22].

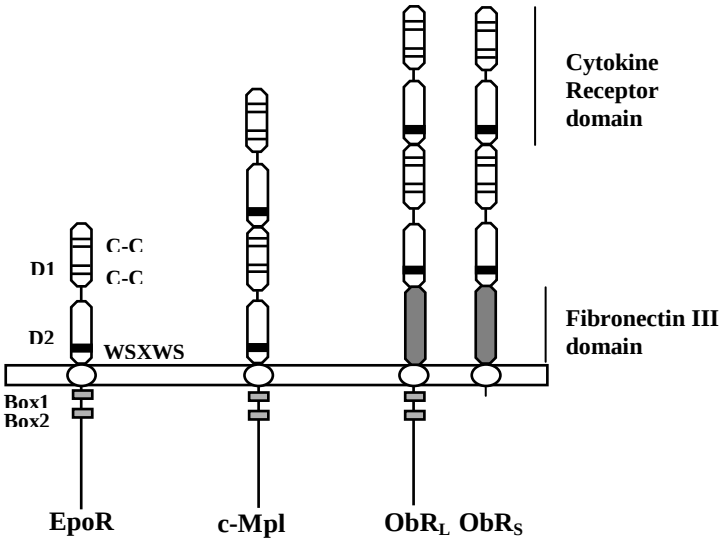

Figure1. Shematic representation of Epo-R, c-Mpl and Ob-R. The extracellular parts contain cytokine receptor domains with two characteristic features: two pairs of disulphide-linked cysteines and the WS motif. In the cytoplasmic domains, box1 and box2 are the only conserved motifs found in these receptors. The cytoplasmic region of Epo-R, c-Mpl and Ob-R contains also several tyrosine residues which are phosphorylated after ligand binding to the receptor.

The physiological importance of EpoR has been clearly demonstrated by gene knock-out studies in mice. Like *epo*-/- mice, *epo-R*-/- mice die at embryonic day 12,5 owing to severe anemia. EpoR is essential for definitive erythropoiesis in fetal liver but is not required for the development of primitive yolk-sac-derived erythrocytes. No defects were apparent in non-erythroid cell lineages in which Epo-R is expressed like megakaryocytes or endothelial cells[7,23].

The Tpo receptor was discovered as the product of the *c-mpl* gene, the cellular homologue of the oncogene v-*mpl*, the transforming principle of the murine myeloproliferative leukaemia virus (MPLV)[24]. Human and murine *c-mpl* cDNAs were isolated and sequenced and display approximately 81% identity at the amino-acid sequence level. Both human and murine sources show the presence of multiple isoforms of c-Mpl produced as a result of alternative splicing[25,26]. Three majors isoforms of c-Mpl have been described, Mpl-P encoding the full length Tpo receptor, Mpl-K which lacks most of the cytoplasmic region and is probably unable to transmit signal and

a soluble form of c-Mpl lacking the transmembrane and the cytoplasmic domains. The extracellular portion of c-Mpl (Mpl-P) is composed of a duplicated 200 amino-acid domain containing four spaced cysteine residues and a WSXWS motif adjacent to the transmembrane region, a feature shared with the common β-subunit of the IL-3, IL-5 and GM-CSF receptors[27]. Similarly to Epo receptor, the cytoplasmic part of c-Mpl can be divided into two domains: the membrane proximal domain containing the box-1 and box-2 crucial for Tpo-induced JAK kinase activation and proliferation and the membrane distal domain containing several tyrosine residues which are phosphorylated upon Tpo stimulation and are necessary for the recruitment of various signaling molecules[28,29]. Mice deficient in c-Mpl display a similar phenotype to *tpo*-/- mice and develop a thrombocytopenia. However, a residual platelet production (5-10%) is observed in these knock-out mice and it is sufficient to prevent spontaneous bleeding and allows a normal lifespan. This implies that Tpo-independent mechanisms of platelet production occurs *in vivo*. In addition to the deficit in megakaryocytes and platelet, progenitor cells of all hematopoietic lineages are produced in reduced numbers in *tpo*-/- and *c-mpl*-/- mice indicating an important role of Tpo signaling in hematopoietic stem cell regulation[10, 30]. Recently, point mutations or deletions in the coding region of c-Mpl have been found in a human congenital amegakaryocytic thrombocytopenia [31].

The leptin receptor (Ob-R) was first identified by expression cloning techniques. Sequencing of the original murine Ob-R cDNA isolated from the expression cloning screen revealed a single membrane-spanning receptor with homology to members of the class I cytokine receptor family, the closest relatives of Ob-R being the gp130-transducing chain of the interleukin 6 (IL-6-) receptor , the granulocyte colony stimulating factor (G-CSF) receptor and the leukaemia inhibitory factor (LIF) receptor α chain[32]. The predicted extracellular region of Ob-R was about 816 aminoacids while the intracytoplasmic part contained 34 amino-acids, suggesting that this protein might not have the ability to transmit signal (Ob-Ra). However, further screening and analysis of cDNA libraries allowed identification of several isoforms in both mice and humans, including a long form with an intracellular domain of 303 amino acids which is expressed preferentially in the hypothalamus (Ob-Rb). Differences in the receptor forms arising from alternate splicing results in Ob-R intracellular domains with differing length and composition. These Ob-R isoforms have been therefore divided in two classes: the long form ($Ob\text{-}R_L$: Ob-Rb) and the various short forms that have been isolated to date ($Ob\text{-}R_S$: Ob-Ra, -c, -d, -f)[33]. A soluble form of Ob-R (Ob-Re) has been also described and is believed to modulate circulating leptin levels [34]. The large extracellular region of Ob-R includes two cytokine binding domains, each containing a single copy of the characteristic WSXWS motif and a fibronectin type III domain adjacent to the transmembrane region. Deletion/substitution

experiments revealed that only the second cytokine binding domain is necessary for leptin binding and receptor activation. The intracytoplasmic region of the long form of Ob-R contains the classical box1 and box2 domains crucial for JAK kinase activation and several tyrosine residues which are phosphorylated upon leptin stimulation indicating that the long form is capable to transduce signal. In contrast the original short form OB-Ra retains only the box 1 and is deficient in signal transducing capacities[35-37].

The complete insensitivity of *db* mice to leptin administration and the identical phenotype of *ob* and *db* mice have suggested that the *db* locus encoded the leptin receptor. Indeed, the leptin receptor maps to chromosome 4 of the mouse in regions that contain the *db/db* mutation. The *db* mutation consists of a point mutation (GT transversion) within an exon containing the extreme C-terminus and the 3' untranslated region of the short form of leptin receptor resulting in the generation of a new splice donor site and inappropriate splicing of the long form of the Ob-R . As a consequence, the Ob-R_L has a truncated intracellular domain similar to the predominant isoform OB-R_S [38,39].

SIGNALING PATHWAYS INDUCED BY EPO, TPO AND LEPTIN.

Epo, Tpo and leptin receptor activation: early events in signal transduction.

The first observation that a mutant Epo-R (R129C) covalently dimerized by interchain disulfide bonds is constitutively active, provides strong evidence that receptor homo-dimerization is a crucial step in signal transduction [40]. Additional studies have demonstrated that the mutations E132C and E133C in the extracellular domain of Epo receptor also cause Epo receptor dimerisation via disulphide bond formation and render it constitutively active[41]. Recent data from crystallographic studies have shown that unliganded Epo receptors exist as a pre-formed dimer. This dimerisation is mediated by the D1-D2 intervening regions but the cytoplasmic portions of each paired monomers are so far apart that signal transduction cannot be initiated. In presence of Epo, these preformed dimers are brought into functional proximity and allow initiation of signal transduction[42]. Similarly it is believed that the Tpo receptor forms homodimers and mutants of c-Mpl that constitutively associate display also ligand–independent activity[43]. Given the relative structural similarities between Epo-R and c-Mpl, it is possible that preformed c-Mpl dimers exist in absence of ligand. Leptin receptors form also homodimers, both in the presence or absence of ligands. Each leptin receptor binds one molecule of leptin resulting in a tetrameric

complex. Activation of the receptor is thought to result from a ligand-induced conformational change[15].

One of the earliest responses detected within cells after ligand-induced receptor activation is a transient increase in tyrosine phosphorylation of cellular proteins including the receptor. Since Epo-R, c-Mpl and Ob-R like other hematopoietic cytokine receptors, lack intrinsic tyrosine kinase activity, these receptors must interact with and activate protein tyrosine kinases allowing signal transduction. Numerous studies over the last ten years demonstrated that the JAK tyrosine kinases are crucial for this obligatory step. The JAK kinases are usually associated with the membrane proximal domain of the cytoplasmic tail of the cytokine receptors. Presumably ligand-induced conformational change brings the associated JAK kinases into proximity and enables them to transphosphorylate and activate each other. Once activated, the JAK kinases then phosphorylate tyrosine residues within the cytoplasmic portion of the receptors which then provide docking sites for the recruitment of SH2-domain (Src Homology domain 2) containing molecules (Figure 2). These effectors are next eventually phosphorylated and activate their respective signaling pathways[44,45].

The JAK/STAT signaling pathway.

The JAK tyrosine kinases include four family members: JAK1, JAK2, JAK3 and TYK2. JAK2 is commonly activated after ligand binding to Epo-R, c-Mpl and Ob-R [46-50]. In addition activation of TYK2 during Tpo signaling has been also reported in cells expressing c-Mpl [51]. The absolute requirement of JAK2 in Epo or Tpo-induced signal transduction has been demonstrated in mice deficient in JAK2. *Jak2*-/- mice die as embryos between day 13 and 15 from severe anemia with a phenotype similar to *epo-R*-/- mice. However , both the total number of fetal liver cells and the number of erythroid progenitor cells are greatly reduced suggesting that JAK2 is required at an earlier stage of erythropoietic development than is the Epo-R. A possible explanation for this difference could be relied to the effect of Tpo signaling. Indeed, hematopoietic progenitors from the fetal livers of *jak2-null* mice fail to respond to Tpo. In addition JAK2 and not TYK2 is probably essential for most thrombopoietin-induced cellular functions because the genetic deletion of *jak2* in mice resulted in the complete absence of megakaryocyte development[52-54]. Since JAK2 deficient mice are not viable, it is difficult to predict its physiological role in leptin signaling events. The use of mice with conditional deletion of JAK2 in the hypothalamus will resolved this important question. The proximal region of the cytosolic domain in Epo-R, c-Mpl and Ob-R_L receptors as in many cytokines receptors contains two conserved segments identified as box1 and box2. Deletion or mutation of the box1 or box2 or the region between box1

and box2 in the Epo-R and c-Mpl impair activation of JAK2 and disrupt all measurable signal transduction and mitogenic stimuli although there are some exceptions to the rule[19,21,28,55].

Among the substrates activated by the JAK kinases are members of the Signal Transducer and Activator of Transcription (STAT) protein family. STAT proteins are latent cytoplasmic transcription factors which are activated by tyrosine phosphorylation following ligand binding to the receptors. Activated STATs form homodimers or heterodimers through a reciprocal interaction between the SH2 domain and the phosphorylated tyrosine residue present in the C-terminal region of these proteins. The complex then moves to the nucleus, where it binds to specific DNA elements and activates the transcription of target genes (figure 2).

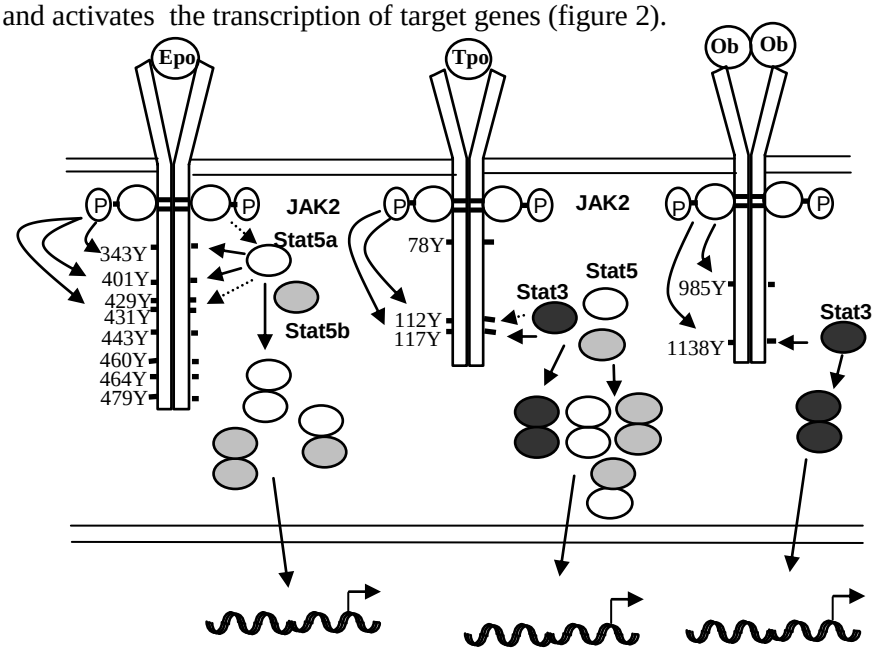

Figure2. The JAK/STAT pathway in Epo-R, Tpo-R and Ob-R signaling (See details in the text)

Seven mammalian members have been isolated (STAT1, 2, 3, 4, 5A, 5B and 6)[45]. Activation of STAT proteins by Epo, Tpo and leptin have been extensively described in the literature. It has been reported that STAT1, STAT3, STAT5 (5A and 5B) are activated following Epo or Tpo stimulation of different cell lines[56, 57-59]. In addition to STAT1, STAT3 and STAT5, STAT6 is also activated by leptin in cells expressing the long form of Ob-R[36,60,61]. However, the patterns of activated STAT observed for these ligands *in vitro* vary from one cell type to another and do not always follow the *in vivo* situation. STAT5A and STAT5B are the main STATs activated by Epo in normal primary erythroid cells while STAT3 and to a lesser extent STAT5 are activated in megakaryocytes or platelets in response to Tpo[56,62]. Leptin injection specifically induces activation of STAT3 in the

hypothalamus of wild type but not *db/db* mice[63]. Before their activation, STAT proteins are recruited to phosphorylated tyrosine residues of the activated receptors. Tyrosine residues which are necessary for STAT5 or STAT3 activation have been identified in the cytoplasmic tail of EpoR, c-Mpl and Ob-R. In the EpoR, mutation of the eight tyrosine residues revealed that phosphorylated tyrosine residues, Y343 or Y401, are independently sufficient for maximal activation of STAT5 while Y429 and Y431 can partially activate STAT5[64-66]. Interestingly , low level activation of STAT5 can occur in the absence of any tyrosine residues on the Epo-R suggesting that STAT5 may be activated independently of the receptor probably through a direct interaction with JAK2[67]. The use of cell lines expressing receptor mutants or dominant negative forms of STAT5 allowed to analyse the importance of STAT5 in Epo-stimulated cell proliferation and differentiation. Conflicting results emerged from these studies probably reflecting cell type specific differences. Some investigators have suggested a correlation between STAT5 activation and Epo-stimulated cell proliferation and/or survival whilst others have demonstrated a correlation with haemoglobin synthesis and differentiation [65,68-71]. The most surprising result came from the analysis of a combined *stat5a/5b* knock-out mouse. Hematopoiesis in these mice appears to be normal including normal number of red cells and normal bone marrow erythroid progenitors (CFU-E)[72]. However, a recent report demonstrated that STAT5 is essential for the high erythropoietic rate in the fetal liver of *stat5a/5b-/-* mice. *Stat5a/5b-/-* embryos are severely anemic and erythroid progenitors are present in low numbers, show higher levels of apoptosis and are less responsive to Epo[73]. These features may be explained by an important role of STAT5 in Epo-mediated inhibition of apoptosis. Indeed, STAT5 mediates the transcriptional induction of the anti-apoptotic *Bcl-x* gene in erythroid cells[73].

Tyrosine residues in the cytosolic portion of c-Mpl necessary for STAT activation have been also identified. Two phosphorylated tyrosine residues Y112 and Y117 are capable of STAT3 recruitment and phosphorylation, although the effect of Y117 on STAT3 activation is less pronounced. Similarly , Y112, plays a partial role in STAT5 phosphorylation. Like Epo-R, STAT5 activation occurs in absence of any cytoplasmic tyrosine residues. Mutations of Y112 and Y117 showed that both residues contribute but are not essential for Tpo dependent cell proliferation[29]. The role of STAT5 and STAT3 in megakaryocytic development and platelet formation remains unclear. In a human megakaryoblastic leukemia cell line, STAT5 is believed to mediate Tpo induction of the cyclin-dependent kinase (Cdk) inhibitor, p21 thereby contributing to Tpo-stimulated differentiation while in MO7e cells, constitutive activation of STAT5 has been correlated with ligand-independent growth[74,75]. However, *stat5a/5b-/-* mice show no obvious defect in the number of megacaryocytes , their precursors or their progeny. The role of STAT3 in Tpo signaling cannot be analysed because STAT3-/- mice are not viable, even at the earliest stages of embryonic development.

Leptin induces activation of STAT3 in cells expressing the long but not the short form of Ob-R and this requires the unique tyrosine residue Y1138 in the carboxyl-terminus of Ob-R_L[50] . For the same reason, the lethality of STAT3-/- mice does not allow to assess the physiological role of STAT3 in leptin signaling.

Other tyrosine kinases associated with the receptors .

Other tyrosine kinases have been shown to be activated during Epo signaling, including c-Fes/Fps, Syk, Tec and Lyn [76-79]. Lyn, a member of the Src tyrosine kinase, is involved in Epo-induced differentiation of the fetal liver cell line J2E [79]. Lyn interacts with the phosphorylated Y464 and Y479 of Epo receptor and to phosphorylated JAK2 through its SH2 domain. The kinase domain of Lyn binds also to the EPO-R membrane proximal 91 amino-acids. In addition Lyn was demonstrated to phosphorylate Epo-R and STAT5 and to interact with the hemopoietin-specific protein HS1 which is involved in Epo induced proliferation and differentiation of J2E cells[80,81]. Because mice deficient in Lyn do not show obvious hematopoietic defect, it is unclear whether Lyn is essential for Epo-R signaling. Tec is a member of the Tec/Btk (Bruton's tyrosine kinase) family of tyrosine kinase which are expressed primarily in hematopoietic cells. In FD-M6 cells stimulated with Epo, Tec has been shown to co-immunoprecipitate with the nucleotide exchange factor Vav and the small adaptor Grb2[78]. Phosphorylation of Tec in cells stimulated with Tpo has been also reported[82] .
Besides complex formation with cytosolic tyrosine kinases, functional and/or physical interaction with distinct receptor tyrosine kinases have been described. c-Kit which binds Stem Cell Factor (SCF) is co-expressed with Epo-R at BFU-E and CFU-E stages and SCF acts at each stage to enhance erythroid colony size and red cell production. Biochemical analysis in different erythroid cell lines have shown that SCF binding to c-Kit induces tyrosine phosphorylation of Epo-R and that c-Kit associates physically with the extended box2 of the cytosolic domain of Epo-R[83, 84].SCF stimulation of c-Kit also promotes Epo-R and STAT5 expression and enhances the STAT5-dependent expression of target genes [85,86].
A Functional interaction between Epo-R and IGF-1 (Insulin Growth Factor-1) receptor has been also reported [87].

Signaling through the phospholipid-modifying enzymes: PI 3-kinase, PLCγ and SHIP.

Phosphatidyl-inositol 3-kinase (PI 3-kinase) activation following cytokine stimulation of hematopoietic cells has been shown to play an important role in proliferation and survival. PI 3-kinase is composed of a p85 (regulatory) and a p110 (catalytic) subunit. Membrane localization and activation of PI 3-kinase is required to generate phosphatidylinositol 3,4-biphosphate and phosphatidylinositol 3,4,5-triphosphate, two lipid products that are needed to activate various isoforms of the serine/threonine kinases PKC and Akt. Activate Akt is known to induce phosphorylation of Bad, a Bcl2 family member and FKHRL1, a member of the forkhead transcription factor family, thereby generating distinct anti-apoptotic signals[88]. Activation of Akt and phosphorylation of its downstream targets Bad and FKHRL1 have been observed in erythroid progenitors and cell lines following Epo stimulation and LY294002, a specific PI 3-kinase inhibitor blocks the phosphorylation of Akt and induces apoptosis of human erythroid progenitors indicating that PI 3-kinase delivers an anti-apoptotic signal in these human primary cells[89,90] (Figure 3).

Epo-dependent association of Epo-R with PI 3-kinase has been reported and several mechanisms have been shown to activate the PI 3-kinase in Epo stimulated cells[91]. Phosphorylation of Y479 of the Epo-R is involved in binding the PI 3-kinase and studies in Epo-R-/- mice have demonstrated that a receptor containing only Y479 supports an almost normal number of fetal CFU-Es and BFU-Es [92]. However, mutant receptor lacking this tyrosine residue are capable to support normal erythroid differentiation. In addition, deletion or mutation of tyrosine 479 does not abrogate PI 3-kinase activation following Epo stimulation of different cell lines indicating that alternate pathways for PI 3-kinase activation without direct binding to Epo receptor exist. Indeed, several mechanisms have been described. One involves the nucleotide exchange factor Vav and the other the adaptor protein IRS-2 (Insulin-Receptor-Substrate 2). These two proteins are associated with the Epo receptor , are tyrosine phosphorylated in response to Epo stimulation and provide a binding site for PI 3-kinase[93,94]. More recently, the IRS-related proteins Gab-1 (Grb2 Associated Binder 1) and/or Gab-2 have been shown to be tyrosine phosphorylated in Epo-stimulated cell lines and primary erythroid progenitors and to recruit PI 3-kinase[95,96].

Tpo stimulation of c-Mpl receptor bearing cell lines, megakaryocytes and platelets leads also to activation of the PI 3-kinase and to the generation of an anti-apoptotic signal in human megakaryoblasts [97-99]. In contrast to Epo-R, no direct association of p85 with activated c-Mpl receptor has been detected so far and recent data have shown that IRS-2 and Gab2 in c-Mpl expressing Ba/F3 cells are tyrosine phosphorylated following Tpo stimulation, recruits p85 and enhance the enzymatic activity of PI 3-kinase. In addition, the tyrosine phosphatase SHP-2 which is thought to act as a positive effector of signaling, associates with Gab2 and PI 3-kinase in these cells. Disruption of SHP-2/Gab2 association by expression of a dominant negative form of SHP-2 decreases PI 3-kinase activity and the reduction of

Akt phosphorylation. Tyrosine phosphorylation of SHP-2 and its association with p85 have been also detected in primary murine megakaryocytes stimulated with Tpo, indicating the important role of SHP-2 as a regulator of PI 3-kinase activation in c-Mpl signaling. Neither Gab2 or IRS-2 are phosphorylated in murine megakaryocytes but instead a –100kDa tyrosine phosphorylated protein immunologically unrelated to Gab proteins co-immunoprecipitated with p85[100]. In contrast, in human megakaryocytes, Tpo induces the phosphorylation of Gab1[101]. Beside the role of PI 3-kinase activation in Tpo-induced cell proliferation and survival, it has been reported that Tpo enhances platelet activation *in vitro* and potentiates collagen receptor signaling through a PI 3-kinase dependent pathway [98].

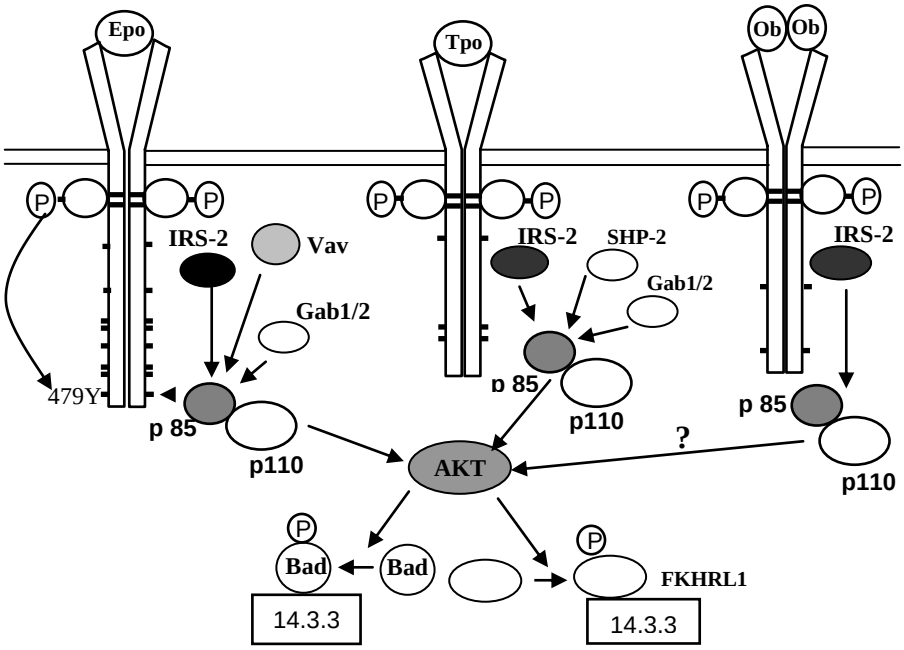

Figure3: EpoR, c-Mpl, Ob-R Signaling through PI 3-kinase. PI 3-kinase induces activation of AKT which can in turn phosphorylate Bad and FKHRL1. Phosphorylated Bad and FKHRL1 are sequestered in the cytoplasm by the protein 14.3.3 resulting in the inhibition of their pro-apoptotic function.

Leptin mimicks insulin effects on glucose transport and glycogen synthesis through a PI 3-kinase dependent pathway in C2C12 myotubes. This activation requires JAK2 and IRS-2. Leptin stimulates tyrosine phosphorylation of JAK2 and IRS-2 and IRS-2 associated PI 3-kinase activity has been found in C2C12 cells[102]. Leptin-induced K(ATP) channel activation in the rat CRI-G1 insulinoma cell line seems also to require PI 3-kinase activity[103].

Other phospholipid-modifying enzymes that have been found associated with Epo-R include phospholipase C-γ (PLC-γ) and phosphatidylinositol 3,4,5

triphosphate 5-phosphatase (Ship). In UT-7 cells, Epo rapidly induces the tyrosine phosphorylation of PLC-γ1 that is followed by diacylglycerol and 1,4,5 trisphosphate formation. These two second messengers activate PKC and increase intracellular calcium respectively indicating that PLC-γ1 activation could contribute to mitogenic signaling through the Epo-R[104]. In FDCP-1 cells engineered to express Epo-R, Epo induces the tyrosine phosphorylation of PLC-γ2, a process that contributes to glycosylphosphatidylinositol hydrolysis[105].
Ship is also tyrosine phosphorylated following Epo stimulation and associates with Epo-R[106,107]. Ship catalyzes the hydrolysis of inositol 3,4,5-trisphosphate and inositol 1,3,4,5-tetraphosphate to generate inositol 3,4-biphosphate and inositol 1,3,4,-trisphosphate respectively. Two distinct *ship* genes have been isolated. *Ship1* is expressed exclusively in hematopoietic tissues while *ship2* has a wider tissue distribution. In Ba/F3 cells expressing various tyrosine mutants of Epo-R, it has been recently reported that Ship1 is recruited to phosphorylated Y401, Y429 and Y431 through its SH2 domain, phosphorylated Y401 appearing to be the major site of Ship1 binding. In these studies, tyrosine phosphorylation of Ship1 induces its association with the SH2-containing adaptor Shc and the constitutive binding to the Grb2 adaptor protein, thereby linking Ship1 to activation of the Ras/MAP kinase pathway[108]. However, Ship1 recruitment to Epo-R does not directly correlate with activation of MAP kinase. Cells expressing Epo-R mutants containing only Y343 are also capable to activate Erks following Epo stimulation but Ship1 recruitment is abrogated in these conditions[109]. Association of Ship with PI 3-kinase, Gab-1 or IRS-2 following Epo stimulation has been also described[94,95]. *ship1* -/- mice exhibit a shortened life-span and a reduced number of mature but not primitive erythroid progenitors suggesting that Ship1 might be involved in a late stage of erythroid maturation[110].
In Ba/F3 cells engineered to express c-Mpl, Tpo induces the tyrosine phosphorylation of Ship. The residue Y112 of the cytoplasmic tail of c-Mpl is both a site of phosphorylation and a critical residue for the Tpo dependent phosphorylation of Ship. Ship could either interact directly with phopshorylated Y112 via its SH2 domain or it may be recruited indirectly though its association with the adaptor Shc[29].

Activation of MAP kinases through distinct signaling cascades.

Activation of the Ras/MAP kinase (Mitogen Activated Protein kinase) pathway is supposed to be initiated by the binding of the small linker Grb-2 to activated receptor complexes. The guanidine nucleotide exchange factor SOS (Son of Sevenless) preassociated to Grb-2 localises to the membrane and removes GDP from inactive Ras allowing GTP to bind and activate Ras.

Ras mediated membrane translocation of the serine-kinase Raf results in increased Raf kinase activity and the induction of a proteine kinase cascade involving the MAP kinase kinase (MEK1/2) and their downstream targets MAP kinases: Erk-1 and -2 (Extracellular signal Related Kinases). [111]. Grb-2 is recruited to the EpoR via the adaptor Shc[112,113]. The small adaptor proteins Shc are widely expressed as a 52-kDa and a 46 kDa form and contain a N-terminal phosphotyrosine-binding domain (PTB), a central effector domain and a C-terminal SH2 domain. Shc seems not to interact directly with the Epo-R but is recruited to Ship-1 or JAK2[108,113]. Following Epo stimulation, Shc is tyrosine phosphorylated, thereby providing docking sites for the SH2 domain of Grb-2. Grb-2 binds also to the scaffolding protein Gab-1 or the tyrosine phosphatase SHP-2 in Epo-stimulated cells[95] and it has been proposed that in CTLL-2 cells expressing Epo-R, Epo induces activation of Raf1/MEK/MAP kinase via a Shc independent pathway [114].

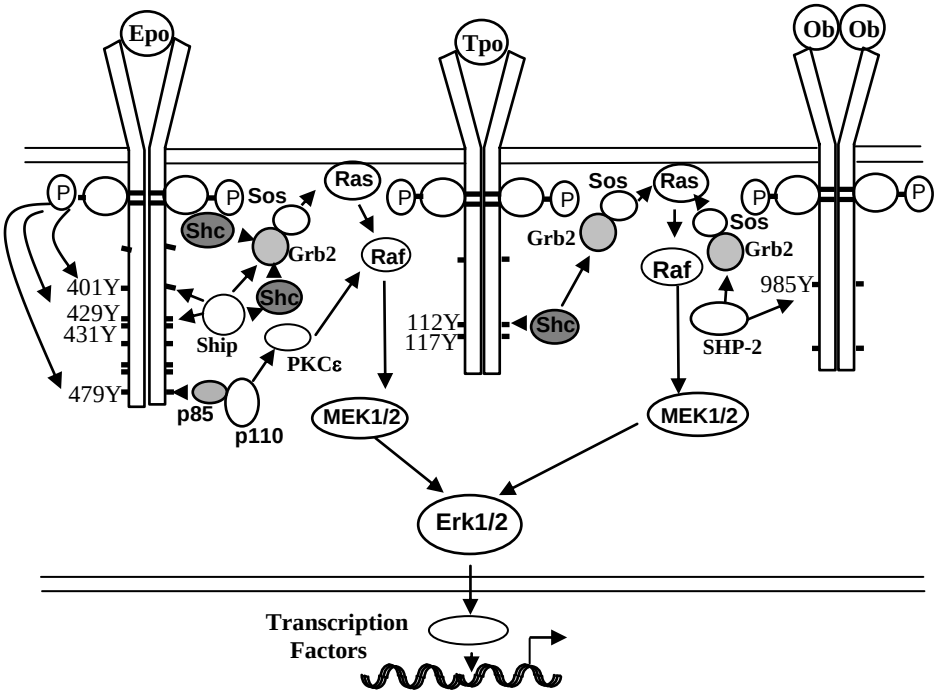

Figure 4. Activation of Erk1/2 through multiple signaling cascades.(See details in the text).

An alternate mechanism for Ras/MAP kinase activation involving the SH2/SH3 containing adaptor protein CrkL has been also reported. In 32D cells expressing the Epo-R, CrkL is tyrosine phosphorylated following Epo stimulation and associates with SHP-2, Ship-1 and Shc. In these cells, CrkL regulates the Epo dependent activation of the Ras/MAP kinase pathway through its interaction with the guanine nucleotide exchange factor C3G[115]. Ras independent activation of MAP kinase has been also

described. Binding of PI 3-kinase to phosphorylated Y479 of Epo-R results in MAP kinase activation via a PKCε dependent pathway[92]. The role of MAP kinase/Erk activation in Epo-R signaling remains unclear. In CTLL-2 cells expressing Epo-R or in HCD57 erythroid cells, Erk activity is required for the mitogenic effect of Epo[116,117].

The distal cytoplasmic domain of c-Mpl has been shown to be crucial for Ras/MAP kinase activation. Shc is recruited to phosphorylated Y112 present in this domain, is tyrosine phosphorylated following Tpo stimulation and associates with Grb-2[29]. Substitution of Y112 by phenylalanine within this c-Mpl domain is sufficient to abolish the normal differentiation response of WEHI3B-D+ and M1 cells expressing c-Mpl and the Tpo dependent phosphorylation of Shc , its association with Grb2 or the receptor and the induction of c-*fos* gene expression[118]. In 32D cells bearing c-Mpl receptors, introduction of a dominant negative form of Shc inhibits also Tpo mediated differentiation[119]. Interestingly, Tpo is capable to induce transformation of FRE rat fibroblasts engineered to express c-Mpl and Y112 and Shc phosphorylation have been shown to be crucial for the transforming capacity of c-Mpl [120]. In contrast to these different data, distinct C-terminal regions that do not include Y112 were also identified as necessary for Tpo dependent differentiation. In UT-7 cells expressing c-Mpl, a subdomain between cytoplasmic residue 71 and 94 has been shown to be responsible for megakaryocytic differentiation and prolonged activation of MAP kinase/Erk following Tpo stimulation[121,122]. In c-Mpl expressing F36P cells, prolonged activation of Ras plays also a central role in Tpo mediated differentiation[123]. In these different studies, the use of dominant negative or constitutively active forms of Ras or MAP kinase or the use of a chemical specific inhibitor of MEK1/2 further showed the requirement of Ras and MAP kinase in Tpo-induced differentiation. Although cell type specific differences exist that could explain the use of distinct cytoplasmic domains and signaling cascades, activation of the Ras/MAP kinase pathway makes an important contribution to differentiation signal triggered by Tpo. This was further evaluated in normal human and murine progenitors induced to differentiate into megakaryocytes by Tpo. In these primary cells, blockade of Erk1/2 activation reduces or delays the generation of megakaryocytes[124,125]. However, the role of the Ras/MAP kinase pathway in megakaryocytic differentiation *in vivo* remains uncertain. A recent report showed that mice expressing a mutant c-Mpl, without the cytoplasmic distal region have normal numbers of megakaryocytes and platelets compared to wild-type mice[126]. Thus, it is possible that activation of MAP kinase/Erk is not required or not essential for megakaryocytic differentiation *in vivo* or that other cytokines may compensate for thrombopoietin *in vivo* activation of Erks.

Leptin stimulation of cells expressing Ob-R induces activation of MAP kinase/Erk [50,127,128]. The tyrosine residue Y985 in the cytoplasmic

region of Ob-R plays a crucial role in this activation and mediates the recruitment of the tyrosine phosphatase SHP-2 which is tyrosine phosphorylated following leptin stimulation and associates with Grb2[50, 129]. The role of Erks activation in leptin receptor signaling remains largely undefined. In C2C12 muscle cells, activation of Erk2 appears to play a role in glucose uptake induced by leptin stimulation[130]. Leptin also stimulates the proliferation of Min6 cells and C3H10T1/2 cells through activation of MAP kinase[127,128].

The MAP kinase family is divided into four groups: Erk1 and -2 described above, the c-Jun amino terminal kinases (JNK), the p38 MAP kinase (p38) and Erk5/Bmk1. Epo and Tpo have been reported to activate JNKs in different cell line models and in certain instances p38 MAP kinase [131-133]. In SKT6 cells, Epo dependent activation of JNK and p38 have been shown to be important for Epo induced hemoglobinization while in HCD57 cells activation of p38 and JNKs is associated with Epo-induced proliferation[117,134]. The role of JNK and p38 in Epo-R remains to be established. Recent data showed that whereas some embryos from p38-/- mice die between day 11,5 and 12,5 , those that develop past this stage are anemic owing to failed definitive erythropoiesis. A role for p38 in the regulation of Epo gene expression and stress-induced erythropoiesis rather than a signaling effector of Epo-R has been proposed to explain the phenotype of these p38-/- mice[135].

Additional signaling adaptors and effectors.

Additional molecular adaptors that have been demonstrated to associate with Epo receptor complexes include Crk family members, c-Cbl, and APS. Crk proteins are the cellular homologues of v-crk which was originally described as an oncogene from the avian retroviruses CT10 and ASV-1. Three Crk protein variants are expressed in hematopoietic cells: CrkI, CrkII and CrkL. CrkII and CrkL proteins contain one SH2 and two SH3 domains while CrkI contains only one SH3 domain [136]. CrkL has been shown to be tyrosine phosphorylated in response to Epo stimulation of 32D cells and to associate with SHP-2 and Shc. Epo-induced tyrosine phosphorylation of CrkL is dependent on the membrane proximal Epo-R cytoplasmic region involved in the activation of JAK2 as well as the C-terminal 145 amino acid region which is required for tyrosine phosphorylation of SHP-2 and Shc [137]. In these cells, CrkL is constitutively associated through its SH3 domains with the guanine nucleotide exchange factor C3G and is believed to modulate activity of Ras and/or Rap1, a Ras family member involved in the regulation of beta1 integrin-mediated hematopoietic cell adhesion[115,138]. In Epo-stimulated human erythroid progenitors, CrkL associates also with DNA bound STAT5 and is thought to modulate its transcriptional

activity[139]. Similarly, CrkL become tyrosine phosphorylated in human platelets in response to Tpo stimulation and associates with STAT5 through an interaction between the SH2 domain of CrkL and tyrosine phosphorylated STAT5[140]. Finally CrkL and CrkII have been found to interact with the proto-oncogene c-Cbl following Epo stimulation of different cell lines[141]. c-Cbl was originally described as the transforming oncogene of the CAS NS-1 retrovirus. P120Cbl, the product of c-*cbl* is a scaffold protein containing a ring finger motif, a proline-rich region, conserved P(Y) sites and a PTB domain[142] . In Epo-stimulated UT-7 cells, c-Cbl is phosphorylated and associates with Grb2. This association is constitutive and involves probably an interaction between the prolin-rich region of c-Cbl and the SH3 domain of Grb2[143]. Similar results were obtained in the megakaryocytic cell line M-07E and in human platelets following Tpo stimulation[144-146]. An association between c-Cbl and PI 3-kinase has been also described in Tpo-stimulated cells [97]. Past studies have linked c-Cbl to negative regulation of growth factor signaling and to protein turn-over. Recent evidence showed that c-Cbl recruit components of the ubiquitin-conjugation pathway and act as an ubiquitin-protein ligase suggesting that c-Cbl may also negatively regulate Epo-R or Tpo-R signaling [142].

APS is a novel adaptor protein containing a Pleckstrin Homology (PH) domain plus one SH2 domain and upon Epo exposure is tyrosine phosphorylated at its C-terminus and associates with c-Cbl. Forced expression of APS in an Epo-dependent hematopoietic cell line results in reduced activation of STAT5 apparently by its binding to phopshorylated Y343 of Epo-R. Interestingly, co-expression of c-Cbl and APS is necessary to inhibit STAT5 activation in 293 cells[147].

Other candidate intracellular regulatory molecules are members of the protein kinase C (PKC) family of serine/threonine kinases. It has been proposed that PKC play a role in the lineage determination of erythroid and megakaryocytic differentiation[148]. Other studies in human and murine erythroleukemic cell lines have shown that the modulation of PKCδ and PKCε or both play an important role in erythroid maturation indicating that distinct isoforms of PKCs may be involved in Epo-R or Tpo-R signaling [149,150]. As mentioned above, PKCε is believed to link PI 3-kinase and MAP kinase and to regulate Epo dependent induction of c-myc and proliferation [92,151]. In contrast, down regulation of PKCα isoform expression inhibits Epo induced erythroid differentiation of human CD34+ progenitors cells[152]. More recently, it has been shown that PKCα controls erythropoietin receptor signaling and is important for Epo-dependent phosphorylation of Epo-R and of various effectors[153]. A role for PKCs in Tpo-mediated induction of c-myc and proliferation in UT-7 cells expressing c-Mpl and in the activation of Erks in human platelets has been also reported[154-156] . The precise role of each PKC isoform in Tpo-R signaling remains, however, to be investigated.

Positive and negative control of cytokine receptor signaling by the tyrosine phosphatases SHP-2 and SHP-1.

SHP-1 and SHP-2 are two structurally related tyrosine phosphatases containing two SH2 domains which bind directly at Epo receptor sites PY429/PY431 and PY401 respectively. SHP-1(also called HCP or PTP1C) is mutated in motheaten mice and is associated with hematopoietic cell hypertrophy, including an overexpansion of splenic CFU-E[157]. Cells expressing the Epo-R mutant Y429F do not activate SHP-1, exhibit prolonged Epo-induced activation of JAK2 in different hematopoietic cell lines and overexpression of SHP-1 inhibits Epo-induced differentiation and suppression of apoptosis in J2E erythroleukemic cells indicating that SHP-1 is an important negative regulator of Epo-R signaling and is responsible for JAK2 dephosphorylation and inactivation[158,159]. Familial and congenital polycythemia , an autosomal dominant benign erythrocytosis, has been reported to be associated with mutations of the Epo-R. Several of them causes the deletion of the last 59-83 C-terminal amino-acids which removes a negative regulatory domain including the binding sites of SHP-1, Y429 and Y431. Cell lines expressing these truncated receptors are hypersensitive to Epo and shows prolonged activation of JAK2[160]. In contrast to SHP-1, SHP-2 (also called Syp or PTP1D) is believed to positively regulate proliferation and mutation of Y401 of Epo-R abolish proliferative activity[161]. As mentioned above, SHP-2 is tyrosine phosphorylated and associates with Grb2, Ship, PI 3-kinase, Gab1, Shc, CrkL and Cbl in different cell lines following Epo stimulation[95,137,162]. SHP-2 is also involved in the activation of PI 3-kinase and MAP kinase in Tpo and Leptin-induced signaling events[50, 100,129,163]. It appears therefore that in addition to its phosphatase activity, SHP-2 plays a pivotal role as an adaptor molecule in the activation of distinct signaling pathways elicited by Epo, Tpo and leptin.

Negative regulation of Epo-R, c-Mpl and Ob-R signaling by the CIS/SOCS family members.

Members of the supressor of cytokine signaling (SOCS) family were discovered as negative regulators of cytokine signaling by inhibition of the JAK/STAT pathway. Eight structurally related family members have been isolated: Cis-1 and SOCS-1 to-7, each of which contains a conserved C-terminal domain of 40 to 50 amino acids called the SOCS box and a central SH2 domain[164]. The N-terminal region is the most divergent part of Cis-1/SOCS proteins and is believed to be important for the ability of SOCS proteins to inhibit tyrosine kinases and/or other signaling effectors. The gene

encoding Cis-1, the founder member of this family, has been isolated as a STAT5 target[165]. Epo, Tpo and other cytokines like IL-2, IL-3, GM-CSF rapidly induces *cis* gene expression and Epo-induced transcription is mediated in part by STAT5 binding sites present in the human and murine *cis* gene promoters[166]. In the Epo receptor system, Cis-1 binds to phosphorylated Y401, a major STAT5 binding site and has been proposed to blunt proliferative signaling by inhibiting STAT5 activation[167,168]. Cis-1 is also ubiquinated and is involved in the proteasome degradation of the Epo-R[169]. In FDCP-2 cells bearing c-Mpl, Tpo induces Cis-1 expression and its binding to tyrosine phosphorylated c-Mpl, a process involving probably the SH2 domain of Cis-1. Like Epo-R, Cis-1 could be important for c-Mpl degradation in this cell line[170]. The physiological role of Cis-1 in Epo-R and Tpo-R remains unclear. As in *stat5a/5b* -/- mice, transgenic mice expressing constitutively Cis-1 from a β-actin gene promoter, show defects in Prl-dependent mammary gland formation, T cell proliferation and growth hormone signaling, but adult erythropoiesis is apparently normal [168].

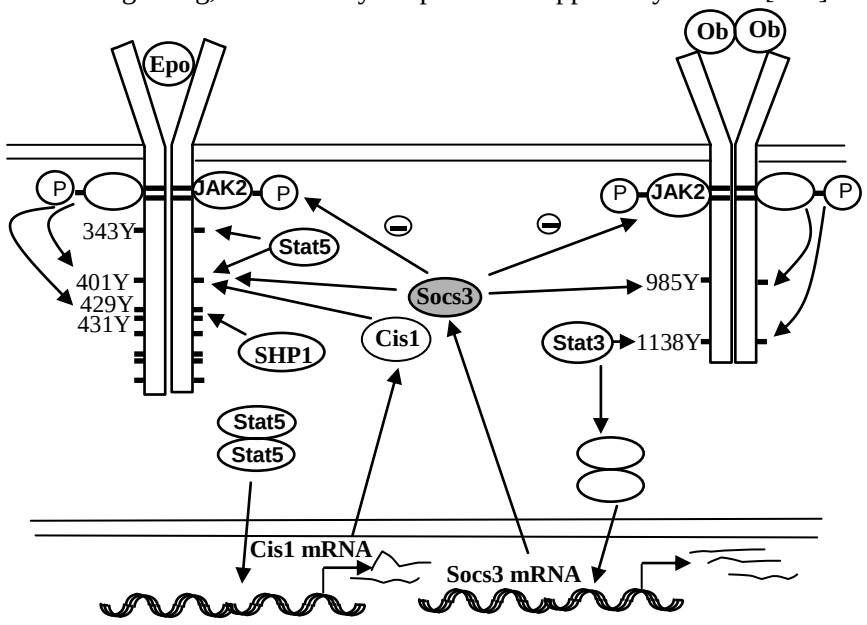

Figure 5. Negative regulation of Epo-R and Ob-R signaling.(See details in the text)

Epo-induced SOCS-1, SOCS-2 and SOCS-3 expression has been also reported. SOCS-1 also called JAB (JAK binding protein) binds to Y1007 of JAK2 and inhibits JAK2 activity and tyrosine phosphorylation of Epo-R in 293 cells [171]. In erythroid progenitors and in Ba/F3 cells expressing Epo-R, SOCS-3 binds to phosphorylated Y401 of Epo-R and to JAK2 and inhibits Epo-dependent proliferation and STAT5 activation. Binding of SOCS3 to Epo-R and JAK2 requires the N and C-terminal regions adjacent to the SH2 domain. Deletion of the Y401-containing region of Epo-R reduces the

inhibitory effect of SOCS-3[172]. Mice deficient in SOCS-3 die as embryos at days 12-16 and a marked erythrocytosis is associated with this embryonic lethality. Moreover, the *in vitro* proliferative capacity of progenitors is greatly increased. Transgene-mediated expression of SOCS-3 results also in embryonic lethality and blocks fetal erythropoiesis[173]. *Socs-1*-/- mice were also generated but showed a completely different phenotype and develop a complex fatal neonatal disease due to an hyperresponsiveness to IFNγ[174]. Thus, SOCS-3 but not SOCS-1 plays a crucial role in negatively regulating fetal liver erythropoiesis indicating that the specific inhibitory role of SOCS-3 in erythropoiesis may be explain by its ability to bind to both Epo-R and JAK2.

In Tpo-dependent hematopoietic cell lines, IFNα supresses Tpo-induced phosphorylation of c-Mpl, STAT5 and Tpo-induced megakaryocytopoiesis by induction of SOCS-1[175]. However, the identity of SOCS proteins involved in the negative regulation of Tpo signaling *in vivo* remains to be determined.

Peripheral leptin administration rapidly induces SOCS-3 mRNA in the hypothalamus of *ob/ob* but not *db/db* mice but has no effect on Cis-1, SOCS-1 and SOCS-2 expression. In addition, in Ay/a mice, a model of leptin-resistant murine obesity, an increase in SOCS-3 mRNA expression was observed in the arcuate and dorsomedial hypothalamic nuclei[176]. In cell lines expressing Ob-R, induction of SOCS-3 expression by leptin is mediated by STAT3 and expression of SOCS-3, but not Cis-1 or SOCS-2 blocks leptin-induced signal transduction[50, 177]. The use of cells expressing Ob-R tyrosine mutants demonstrated that SOCS-3 binds to phosphorylated Y985 which was previously shown to be a docking site for SHP-2. Mutation of Y985 or overexpression of SHP-2 blunts SOCS-3-mediated inhibition of Ob-R signaling indicating that SOCS-3 inhibits OB-R signaling by binding to phosphorylated Y985[178]. In conclusion, SOCS-3 is a leptin inducible inhibitor of leptin signaling and a potential mediator of central leptin resistance.

CONCLUSIONS

Since the discovery of Epo, Tpo, leptin and their receptors, a remarkable progress has been made toward understanding, at the molecular level, the signaling mechanisms that underlie the physiological properties of these different cytokines. Epo, Tpo and leptin signal transduction pathways appear to be similar and most of the signaling molecules so far identified to be activated by Epo, Tpo and leptin are also found in the repertoire of signaling pathways used by other cytokine receptors. Questions that concern the molecular mechanisms of cytokine specificity and the permissive versus instructive role of cytokines in cell differentiation are still under debate. The

permissive model proposes that cytokine receptors provide only signals for survival and/or proliferation. Several data from the literature seem to favor the permissive role of Epo and Tpo on erythroid and megakaryocytic differentiation. The c-Mpl cytoplasmic domain can be replaced by that of granulocyte colony-stimulating factor receptor *in vivo* without a measurable effect on megakaryocyte differentiation and expression of prolactin receptor in Epo R-/- progenitors fully supports erythroid differentiation indicating that signals emanating from these receptors are qualitatively sufficient to mimic those required for the biological properties of Epo and Tpo[179, 180]. In addition, it became evident that one of the primary action of Tpo or Epo is to prevent induction of apoptosis and that some of the signaling pathways induced by these ligands, e.g PI 3-kinase, deliver an anti-apoptotic signal in erythroid progenitors and megakaryocytes. One other persistent question concerns the identification of the key components downstream the receptor and JAK2 which are necessary for Epo or Tpo-induced proliferation, survival and differentiation *in vivo*. Past studies with Epo receptor mutants in which all the tyrosine residues of the cytoplasmic part of the receptor were mutated have indicated that the tyrosine residues are required for erythroid colony formation from murine CFU-E[181,182]. Contrasting results were however obtained with similar mutants in the erythroid differentiation of human CD34+ progenitors[183]. Epo-R mutants containing any one of the five tyrosine residues Y343, Y429,Y431,Y443 are capable to support the formation of significant numbers of murine CFU-E although to a reduced level while Epo-R mutant retaining only Y479 support the formation of an almost normal number of CFU-E. These results indicate that proliferation, differentiation or survival of erythroid progenitors are at least in part mediated by redundant signaling pathways[18]. In addition, the conclusions that can be drawn from signaling studies in cell lines show that the signaling pathways described in this review are activated through different mechanisms, can interact and probably regulate each other through different signaling adaptors or effectors and are able to act together to fully support proliferation, differentiation or survival. Activation of PI 3-kinase through its binding to phosphorylated Y479, for instance, results in MAP kinase activation. This may explain the relatively high activity of this receptor mutant retaining only Y479 on erythroid differentiation [66]. Activation of both JAK2/STAT5 and Ras pathways is also required for full erythroid differentiation[183]. In a similar vein, although mice expressing a c-Mpl mutant without the cytoplasmic membrane distal region have normal numbers of megakaryocytes and platelets, they exhibit a smaller increase in platelet numbers than wild-type mice in response to exogenous Tpo treatment indicating that signals emanating from the distal part determine the quantitative but not the qualitative response to Tpo and that co-operation between the proximal and the distal domain of the c-Mpl cytoplasmic region is necessary for an appropriate acute response to Tpo[126]. Co-operation between different signaling pathways may therefore explain the final

outcome that optimise the biological properties of Epo, Tpo and leptin. The use of mice with deletion or conditional deletion of one or more genes encoding various signaling effectors or adaptors will be helpful in the future to determine the downstream component(s) that typify the biological action of Epo, Tpo and leptin.

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