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Erythropoietin receptor response circuits

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Abstract

Purpose of review—In 1985–1989 EPO, its receptor (EPOR) and JAK2 were cloned; established to be essential for definitive erythropoiesis; and initially intensely studied. Recently, new impetus, tools, and model systems have emerged to re-examine EPO/EPOR actions – and are addressed in this review. Impetus includes indications that EPO affects significantly more than standard erythroblast survival pathways; the development of novel erythropoiesis stimulating agents (ESA's); increasing evidence for EPO/EPOR cytoprotection of ischemically injured tissues; and potential EPO- mediated worsening of tumorigenesis.

Recent findings—are reviewed in four functional contexts: i) (Pro)erythroblast survival mechanisms; ii) New candidate EPO/EPOR effects on erythroid cell development, plus new EPOR responses; iii) EPOR down-modulation, and trafficking; iv) Novel ESA's.

Summary—As Current Opinion, this monograph seeks to summarize, and provoke, new EPO/EPOR action concepts. Specific problems addressed include: Beyond (and before) BCL-XL, what key survival factors are deployed in early stage proerythroblasts? Are distinct EPO/EPOR signals transduced in stage- selective fashions? Is erythroblast proliferation also modulated by EPO/EPOR signals? What functions are subserved by new non-canonical EPO/EPOR response factors (eg, PODXL, TRIB3, ROS, NFK-B)? What key regulators mediate EPOR inhibition, and trafficking? And for emerging ESA's, to what extent do activities parallel EPO's (or differ in advantageous, and/or potentially complicating ways)?

Keywords

erythropoietin (EPO); EPO receptor (EPOR); erythropoiesis; cytoprotection; ESA's

INTRODUCTION

In 1950 – 1955, EPO was first defined as a humoral erythropoietic factor in parabiotic experiments [1,2], and in anemic plasma preparations [3]. Nephrectomy experiments subsequently established the kidney as a prime site of EPO production [4] (EPO can also be expressed by early-stage erythroid progenitors, retinal, hepatic, and neural cells) [5–8]. In 1977, EPO was purified from urine of aplastic anemia patients [9]; was cloned in 1985 [10]; and was first used to treat the anemia of chronic renal disease in 1987 [11]. EPO's cell surface receptor (EPOR) was cloned in 1989 [12] and, despite a lack of kinase activity or consensus functional motifs, was subsequently demonstrated to function (in cell line models) as a single transmembrane protein to support EPO's survival and proliferative responses [13,14]. JAK2 was discovered in parallel [15], and was demonstrated to couple to EPOR dimers [16]. The EPOR, thus, formed a founder for a superfamily of JAK- coupled (hematopoietic) growth factor

receptors [17,18]. Pursuant EPOR (as well as EPO and JAK2) gene disruption experiments defined (pro)erythroblasts as essential EPO/EPOR targets [19,20]. One prime effect was on cell survival, and intense investigations followed to initially define intracellular networks of EPOR signaling.

The above era of EPO/EPOR studies expanded basic knowledge of HGF-R regulatory capacities; and defined select response pathways for EPOR/JAK2 complex activation, prime positive STFs, and major negative feedback mechanisms [for reviews, see [21–23]]. Furthermore, such work framed new concepts in transmembrane conformational signaling [16,24]; improved an understanding of roles of STAT action in normal and oncogenic contexts [25]; and provided initial insight into EPO's effects in the contexts of non-hematopoietic tissue cytoprotection [26–34] and the possible worsening of cancer [35–46]. Such studies have also stimulated the development of EPO orthologues and mimetics with promising new properties (plus design concepts that may advance other cytokine therapies) [47–63]. Reasons to intensify EPOR investigations therefore are perhaps as compelling as in 1955 [1–3], 1977 [9], and/or 1989 [12], and this review focuses on such emerging new EPOR actions. To avoid an inventorying of STFs and signaling pathways which the EPOR can modulate, an emphasis is placed on recent EPOR associated discoveries that promise to bring important new insight to bear on functional EPO/EPOR effects.

(Pro)erythroblast survival skills

Textbook models for EPO/EPOR erythropoietic actions appear straight-forward: Activated EPO/EPOR complexes provide basic survival signals to late CFUe- like (pro)erythroblasts, and this might predominately involve JAK2/STAT5 activation of *Bcl-xl* [64] and/or p85-alpha/PI3K effects on AKT (and downstream effectors) [65,66]. This simple EPO/EPOR action model, however, is complicated by a few additional considerations. In particular, studies of erythropoiesis in BCL-XL- null mice indicate survival roles selectively during late erythroblast development [71], and BCL-XL's effects on late-stage erythroblasts also have been suggested to be separable from EPO action effects [72]. In addition, recent studies of EPOR response genes in EPO- dependent primary bone marrow (BM) progenitors tend to discount *Bcl-xl* as a target in CFU-like (pro)erythroblasts (but point to several alternate new EPO/EPOR modulated regulators) [73–75]. For PI3K, a case for possible non-essential roles is based on hyper-morphic phenotypes exhibited by EPOR alleles which lack a well-defined distal EPOR PY479 docking site for p85-alpha [67–69] (p85-alpha knockout mice nonetheless display anemia during embryogenesis) [70].

The above observations raise basic questions concerning first, the timing of BCL-XL action in late-stage erythroblasts, and second the nature of alternate EPO/EPOR- activated survival factors. Recently, *Bcl-xl* has proven to comprise a moderate EPO/EPOR response factor in primary BM erythroblasts, but only in late stage Ter119^{pos} cells (Wojchowski and co-workers, manuscript in preparation). BCL-XL expression at late stages therefore may also be enforced by GATA1 plus GFI1B regulation [76]. In late- stage erythroblasts, Maeda et al [77] further have reported that transcriptional repression of *Bim* by LRF also constitutes an important route to survival. While broadly expressed, LRF in this context is further induced by GATA1. For early stage proerythroblasts, in contrast, EPOR's marked effects on survival remain incompletely understood. As mentioned above, and as discovered in primary BM erythroblasts, two strongly induced EPO/EPOR response factors (and novel candidate effectors) are an atypical intracellular serpin as Serpina-3G (S3G/Spi2a) [73]; and the pseudokinase adaptor protein TRIB3 [73]. Ectopic expression of each can promote erythroid progenitor cell survival [73], and for TRIB3 a new knockout model indicates non-redundant erythropoietic roles (especially when the erythron is challenged) [78]. S3G/Spi2a interestingly has been shown to play important roles in memory T-cell development [79] (potentially as a lysosomal cathepsin

inhibitor) [80]. For TRIB3, roles are emerging as a target- specifying adaptor for select ring-type E3 ubiquitin ligases [81]. Overall, one new biological concept therefore is that a hand-off may exist in erythroblast survival mechanisms which involves a transition from EPO/EPOR effects (and targets) in pro-erythroblasts, to subsequent GATA1- induced supporting factors at later stages of maturation.

Candidate EPO/EPOR effects on proliferation and development, plus additional new EPO/EPOR responses

In the early defining of EPO's actions, anti- apoptotic effects were highlighted, and possible proliferative roles were de-emphasized [82]. Possible effects on differentiation also have been debated, but for EPO and other HGFs were concluded to be nominal, and were relegated instead to the actions of lineage-restricted transcription factors [83–88]. No dramatic findings have emerged to date to suddenly reverse these concepts for EPO/EPOR action. However, certain recent studies argue to perhaps keep these cases open. With regards to effects on cell cycle progression, EPO has been shown to stimulate type-D cyclin expression [74] (and D1 and D2 cyclin also are known STAT5 targets) [89,90]. More recently, sharp EPO regulation of cyclin G2 has been discovered in primary bone marrow progenitors [74]. This specifically depends upon EPOR/JAK2/STAT5 signaling, but interestingly involves repression of this inhibitory cyclin. Repressive actions of STAT5 are atypical but have also been shown to be important, for example, for BCL6 regulation in B-lymphoma cells [91], and IL-4 mediated inhibition of IL2 in helper T cells [92]. In the EPOR system, EPOR-PY343/STAT5 signaling is particularly critical for accelerated erythropoiesis when the erythron is challenged [93]. Cyclin- G2 repression, therefore, may comprise one mechanism contributing to EPOR/JAK2/STAT5-supported erythroblast hyper-expansion. Apparent proliferative roles for EPO also have recently been reported in several non-hematopoietic contexts [94,95]. With regards to the question of whether HGF's can affect lineage decisions (and/or lineage progression) this also has recently been revisited, not yet within the EPO/EPOR system, but for related GCSF and MCSF cytokine receptors [96,97]. Rieger et al [96] first used a continuous bioimaging approach to monitor the fate (in liquid culture) of single primary murine BM granulocytic/monocytic progenitors (GMP's). Exposure to GCSF or MCSF not only proved to instruct 34% to 65% of bipotent GMP to granulocytic vs monocytic fates (respectively), but also accelerated differentiation. In related investigations, Sarrazin et al [97] advanced findings to a mechanistic level, and demonstrated that levels of the transcription factor MAFb enabled MCSF effects on myeloid commitment divisions. Specifically, decreases in MAFb levels allowed MCSF to signal more intensely, and to activate PU.1 (a dominant myelopoietic ETS- transcription factor) [98]. Thus, HGFR and transcription factor circuits can be integrated in ways that affect both rates and numbers of hematopoietic cells generated within a specific lineage.

Beyond effects on (pro)erythroblast proliferation and survival, increasing evidence indicates apparent EPO/EPOR effects on alternate cellular response pathways. One major new EPO/EPOR response factor is the cell surface adhesion and migration factor, Podocalyxin like-1 (PODXL). In transcriptome profiling of primary bone marrow erythroblasts, PODXL was discovered as a strong EPO/EPOR response factor, and this was confirmed at the cell surface level [75]. Follow-up studies tested the ability of PODXL-null fetal liver cells to reconstitute the erythron. These transplant experiments did not reveal obvious defects, and PODXL therefore may be non-essential for murine stress erythropoiesis [99]. For this sialomucin and CD34 orthologue [100,101], possible roles in adult bone marrow erythropoiesis (and hematopoiesis) nonetheless remain to be further tested. Interestingly, EPO cytoprotection of injured renal podocytes also has recently been reported [102,103]. Such observations raise the more general question as to possible specificity of EPO/EPOR responses not only in possible lineage and/or tissue specific contexts, but also in the context of erythroblast development per se. While working to extend EPO/EPOR studies to later stage erythroblasts, we, in fact, have

observed certain sharp differences in response pathways between CFUe- like cells, and their progeny. Examples include *Annexin a2*, *Podocalyxin* and *Bcl-xl* as early-, mid- and late- stage erythroblast predominant factors, respectively (unpublished data, and manuscript in preparation).

Interesting potential connections between EPO/EPOR signaling and ROS also have recently been described. First, in a 32D cell model, EPO has been observed to stimulate NAC- sensitive ROS formation, and ROS effects on G1- to S-phase progression have been outlined [104]. Second, gene disruption of one prime EPOR/P13K/AKT target, *Fox03a*, has been shown to dysregulate ROS, especially in late- stage erythroblasts [105]. This effect, however, may be uncoupled from EPO/EPOR action since *Fox03a* deletion sharply elevates ROS in late-stage cells (possibly due to compromised catalase, SOD and GPX1 expression) [105]. Third, in the context of endothelial progenitor cell mobilization, EPO's effects on this process interestingly have been shown to depend upon NOX2 [34]. This was observed in *Nox2*- null but not *Nox1*^{+/−} mice, and these studies also implicated SH2P as a candidate EPO/EPOR/ROS target. As an additional ROS- associated factor, NFK-B recently has been implicated in EPO/EPOR action and pro-survival effects during retinal regeneration [106], and previously in cerebrocortical neurons [107].

Within non-hematopoietic tissues, prime STFs and pathways which are at play also are of significant interest in at least two additional clinical contexts. Specifically, this relates to apparent abilities of EPO to cytoprotect select ischemically injured cardiac, renal, retinal, neural/glial tissues [26–34]; and to perhaps also promote tumorigenesis in select sets of chemotherapy patients (eg, head-neck and breast cancers) [35–46]. These latter effects are the topics of several recent reviews [and primary publications, [24–26] for example], and are therefore not addressed here in detail. One point which is raised, however, is that investigations in non-hematopoietic cells should be aided in basic and perhaps important ways via the advancing a more detailed understanding of EPO/EPOR actions (and signature responses) within primary (pro)erythroblasts (as a prime target). Finally, regarding possible additional non-erythroid effects, two recent studies also point to possible roles for EPO/EPOR signaling during megakaryopoiesis. Huang et al [108] have observed that mice expressing a knocked-in human EPOR allele are somewhat compromised in not only red cell, but also platelet production, while Klimchenko et al [109] have described an EPOR- positive bipotent erythro-megakaryocytic progenitor within differentiating hES cell cultures.

EPOR down-modulation, and trafficking

As essential feed-back mechanisms, the above EPO/EPOR growth and survival signals are balanced by likewise sophisticated networks of regulatory and/or inhibitory factors. One occurs at a level of EPOR – JAK2 complex assembly. Here, JAK2 may act as a chaperone in facilitating EPOR mobilization from intracellular pools [110]. Functional reciprocity also may exist in that EPOR interaction with JAK2's FERM domain recently has been shown to be important for potentiating JAK2's PTK activity [111]. Notably, this potentiating effect on HGF receptors also appears to apply for JAK2 V617F in clinical contexts of erythrocytosis [112]. Activated EPOR complexes also may be subject to JAK2 and/or STAT5 inhibition by the SH2 domain-encoding suppressors of cytokine signaling, SOCS 1,2 and/or 3 plus CIS. In particular, SOCS-3 and and/or CIS may inhibit JAK2 [113–115], and SOCS-3 and -2 further have been proposed to cross-regulate [116]. In ES cells, however, SOCS-3's actions recently have been shown to be sustained in the absence of SOCS-2 [117]. This is of potential clinical significance in that dysregulation via SOCS-3 hyper-phosphorylation may result from JAK2-V617F mutation, and this may limit SOCS-3's suppressing activity [112]. SOCS-2 knock-down in MDS cell lines, in contrast, leads to hyper-phosphorylation of STAT5, and hyper-methylation of *Socs-2* was observed in ~25% of MDS patients analyzed [118]. SOCS further have been

suggested to act as tumor suppressors [119] and SOCS-1 and -3 hypermethylation likewise has been reported among MDS patients [120]. A recent study of epigenetic modifications in patients with myeloid malignancies, however, failed to correlate reversal of SOCS-1 methylation with clinical response [121]. The complexity of SOCS effects extends further to their roles as components of cullin-type E3 ligase complexes [122,123]. Composite effects on EPOR/JAK2 assemblages therefore remain incompletely understood. Another molecular adaptor of recent increasing interest is the SH2-B family member, LNK. In erythroid progenitors, *Lnk* gene disruption limits erythropoiesis [124]. As proposed for SOCS-3, this has been indicated to involve inhibitory effects on JAK2. In a JAK2-V617F context, however, LNK (unlike SOCS-3) appears to retain its inhibitory effects [125]. Further attention to LNK is now being provided in stem cell and developmental contexts. Specific examples include discovered roles for LNK in hematopoietic stem cell expansion [126,127], and for *Drosophila* LNK in insulin/insulin-like growth factor and PKB signaling [128].

Recent progress also has been made towards understanding EPOR and HGF receptor trafficking as a sharply regulated down-modulating process. In the GCSFR system to date, roles have first been outlined for SOCS-3 in receptor turnover [129,130]. From an EPOR and trafficking vantage, three further candidate regulators recently have been initially described. The RING-domain ubiquitin ligase BTRC can bind at an EPOR DSG binding subdomain, and modulate functional levels of EPOR expression [131]. NOSIP/p33RUL is a second EPO-stimulated ubiquitin ligase which also appears to interact with the EPOR [132]. Finally, unexpected roles for p85- α recently have been described in which ligand-induced EPOR loss from the cell surface appears to depend upon p85, but not p110 PIK3 activity [133]. Such findings (as observed to date in cell line models) suggest that sophistication also exists in the regulation of EPOR trafficking. In future studies in primary erythroid cell models, this should be a productive area to advance.

Erythropoiesis stimulating agents (ESA's)

Several considerations have prompted efforts by several groups to develop ESA's beyond EPO per se. These include costs associated with recombinant EPO (due to mammalian cell expression, glycosylation concerns, purification) [47]; immune responses to EPO which are infrequent but nonetheless may involve conversion to red cell aplasia [134]; pharmacokinetic properties, and duration of erythropoietic effects [48]; and adjusted FDA guidelines for therapeutic goals of hemoglobin levels at 11–12 g/dL [36,47,48]. Properties of certain EPO orthologues including Aranesp/Darbepoietin and Mircera as supra-glycosylated and pegylated forms of α - and β - erythropoietin (respectively) have recently been reviewed [47]. Here, features of more recently developed ESAs are highlighted as CNTO-530/528 [59,60], Hematide [55–58], and HIF proline hydroxylase (PHD) inhibitors [61]. CNTO530 and Hematide build upon an EPOR peptide ligand concept, and overcome limitations of low peptide mimetic Kd [49–51], and limited half-life by incorporating dimeric peptide designs as well as stabilizing scaffolds. In CNTO530/528, a recombinant humanized IgG4 Fc region scaffold is used [59,60,135] while Hematide is a fully synthetic ESA built upon a PEG backbone [56]. In mouse and rat models, CNTO530 at single- dosing can promote erythropoiesis for up to one month, and is notably effective in lessening anemia due to paclitaxel plus carboplatin [60]. CNTO 528, a related orthologue, has advanced through phase-1 clinical trials [59]. Hematide has advanced to phase-3 trials for anemic patients with chronic kidney disease, and data to date indicate apparent achievement of sustained therapeutic effects at target levels [57,58]. By comparison, PHD inhibitors are designed to stabilize HIF's against turnover as accelerated by prolyl hydroxylation via EGLN 1–3 [136–139]. This enhances the capacity of HIF's to act as heterodimeric transcriptional activators of *Epo* expression. Increased levels of endogenous EPO production can then bolster red cell production. Interestingly, deletion of HIF2a and HIF1a genes in mouse models also indicates their direct (or indirect) linkage to iron transport

(eg, HIF2a regulation of DMT1) [140]. PHD inhibitors, therefore, may also have iron-mobilizing potential (as an advantage for heme, and hemoglobin synthesis). The extent to which PHD inhibition may affect other HIF pathways and/or other PHD targets [141] however, also merits careful attention. Finally, yeast display affinity maturation recently has been used to develop humanized anti-EPOR monoclonal antibodies with promising agonist activities in in vitro and in vivo pre-clinical studies [62,63]. Interestingly, anti-EPOR antibodies with increased off-rates exhibited heightened potencies [63].

CONCLUSIONS

To date, EPO and its receptor have served well as an important and highly informative paradigm for HGF action mechanisms, and effects. EPO has further found several important clinical niches. Currently, re-examinations of EPO/EPOR biology are being enabled via new transcriptome and phosphoproteome approaches, plus new genetic and primary cell models – and promise to continue to yield important new advances. As highlighted in this review, fertile areas for exploration include first a resolution of what series of apparently transitional survival factors support (pro)erythroblast survival. A GATA1/LFR/BIM axis is newly emerging for late- stage erythroblasts [77], but mechanistically how EPO cytoprotects proerythroblasts remains less clear. As unique pro-erythroblast survival routes are uncovered, certain may comprise new candidate targets for small molecule anti- anemia agents (which might circumvent issues of EPO in chemotherapy patients) [35–46]. Specific functional roles played by newly discovered EPO/EPOR response factors also now become important to critically determine (eg, via genetic GOF/LOF studies). These include PODXL, S3G, TRIB3, NFK-B and mediators of ROS [73,75,105]. For ROS, context plus balance may be important based on apparent positive cell cycle and cell migration effects on the one-hand [34,104], and potential untowards effects on the other [105]. Related basic questions also are raised concerning when the EPOR is expressed during erythroblast development, and whether EPOR ligation might engage distinct responses at discrete stages. New insight also continues to be gained into negative regulators of EPOR/JAK2 complexes. In erythrocytosis and JAK2-V617F contexts, how SOCS suppressors might be dysregulated [118,142], and how LNK [124,125,127] and possible additional molecular adaptors regulate EPOR/JAK2 signaling, also represent contemporary problems. Initial progress likewise has recently been made in understanding problems in EPOR trafficking [131–133]. More work is needed, however, to understand endocytotic and ubiquitinylation mechanisms and their possible specificity for EPOR and/or JAK- coupled receptors. How apparent intracellular EPOR pools [110,143] are regulated (and mobilized) also is of interest in erythroid cells, as well as ischemically injured tissues [26–34] (and potentially, certain cancers) [35–46]. Finally, it is likewise will be exciting to discover the utility, and potentially unique properties, of new erythropoiesis stimulating agents as they enter clinical and research avenues. These include new EPOR agonists [55–60] as well as inhibitors of HIF proline hydroxylases (PHD-I's) [61,139,141].

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