

The effect of erythropoietin on normal and neoplastic cells

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Abstract: Erythropoietin (Epo) is an essential hormone that binds and activates the Epo receptor (EpoR) resident on the surface of erythroid progenitor cells, thereby promoting erythropoiesis. Recombinant human erythropoietin has been used successfully for over 20 years to treat anemia in millions of patients. In addition to erythropoiesis, Epo has also been reported to have other effects, such as tissue protection and promotion of tumor cell growth or survival. This became of significant concern in 2003, when some clinical trials in cancer patients reported increased tumor progression and worse survival outcomes in patients treated with erythropoiesis-stimulating agents (ESAs). One of the potential mechanisms proffered to explain the observed safety issues was that functional EpoR was expressed in tumors and/or endothelial cells, and that ESAs directly stimulated tumor growth and/or antagonized tumor ablative therapies. Since then, numerous groups have performed further research evaluating this potential mechanism with conflicting data and conclusions. Here, we review the biology of endogenous Epo and EpoR expression and function in erythropoiesis, and evaluate the evidence pertaining to the expression of EpoR on normal nonhematopoietic and tumor cells.

Keywords: erythropoietin, erythropoietin receptor, tumor, anemia, angiogenesis

Introduction

Erythropoietin (Epo) is a hormone, so named because of early studies demonstrating that Epo had a singular effect on stimulation of erythropoiesis, the formation of red blood cells.¹ Epo functions by binding to and activating the Epo receptor (EpoR) expressed on the surface of committed erythroid progenitor cells. This in turn induces erythroid progenitor cell survival, proliferation, and differentiation into circulating enucleated hemoglobin-containing red blood cells (RBCs), which are critical for oxygen transport.

The cloning of the *EPO* gene in the early 1980s allowed for the development of recombinant erythropoietins and analogs (erythropoiesis-stimulating agents [ESAs]), offering an alternative to transfusion as a method of raising hemoglobin levels in patients with anemia. However, in some clinical trials, the treatment of cancer patients with recombinant human Epo (rHuEpo) or other ESAs has been associated with decreased locoregional control of tumor growth and/or decreased survival.^{2,3} Some investigators have reported that ESAs may have nonhematopoietic effects via direct activation of EpoR on nonhematopoietic cells, including tumor cells. This hypothesis was used as one possible explanation for the decreased locoregional control of tumor and decreased survival reported in some ESA clinical trials in anemic cancer patients.

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In this review, we examine the mechanisms by which ESAs stimulate the formation of normal erythroid cells, and explore the hypothesis that ESAs can stimulate growth or survival of other nonhematopoietic cell types, including tumor cells.

Erythropoiesis

Maturing erythroid progenitor cells expand in number and decrease in size as they progress through a series of differentiation stages (Figure 1). The first committed erythroid cell type forms characteristic “burst” colonies in semi-solid medium, and was therefore called a burst-forming unit-erythroid cell (BFU-E). BFU-E cells are present at 40–120 cells per 10^5 bone marrow cells,⁴ and further differentiate into colony-forming unit-erythroid (CFU-E) cells. CFU-E cells, present at concentrations of 200–600 cells per 10^5 bone marrow cells,⁴ begin synthesis of hemoglobin and differentiate into erythroblasts. Erythroblasts enucleate forming reticulocytes, so named because of the “reticulin” associated with the residual ribosomal RNA detectable with

dyes such as methylene blue. After several days, mitochondria are degraded, reticulin declines, and the cells become mature RBCs. RBCs lack DNA, and therefore can neither divide nor alter gene expression in response to stimuli.⁵

Erythropoiesis occurs in specialized niches in the bone marrow, encompassing a macrophage surrounded by maturing erythroid cells.⁶ In healthy humans, 2×10^{11} RBCs are generated per day and constitute 99% of circulating cells and approximately 40%–45% of the blood volume. To sustain this level of RBC production, a substantial fraction (25%) of the cells in a normal bone marrow smear are erythroid precursors.⁷ However, erythroid precursors in the “liquid” portion of bone marrow represent a smaller proportion (0.01%–1%).^{8–11} RBCs have a lifespan of 3–4 months under normal conditions in humans,¹² but can be decreased in such disease states as renal failure.¹³

Erythropoietin

Erythropoiesis is stimulated when Epo, a glycoprotein hormone expressed primarily in the kidney, binds and activates

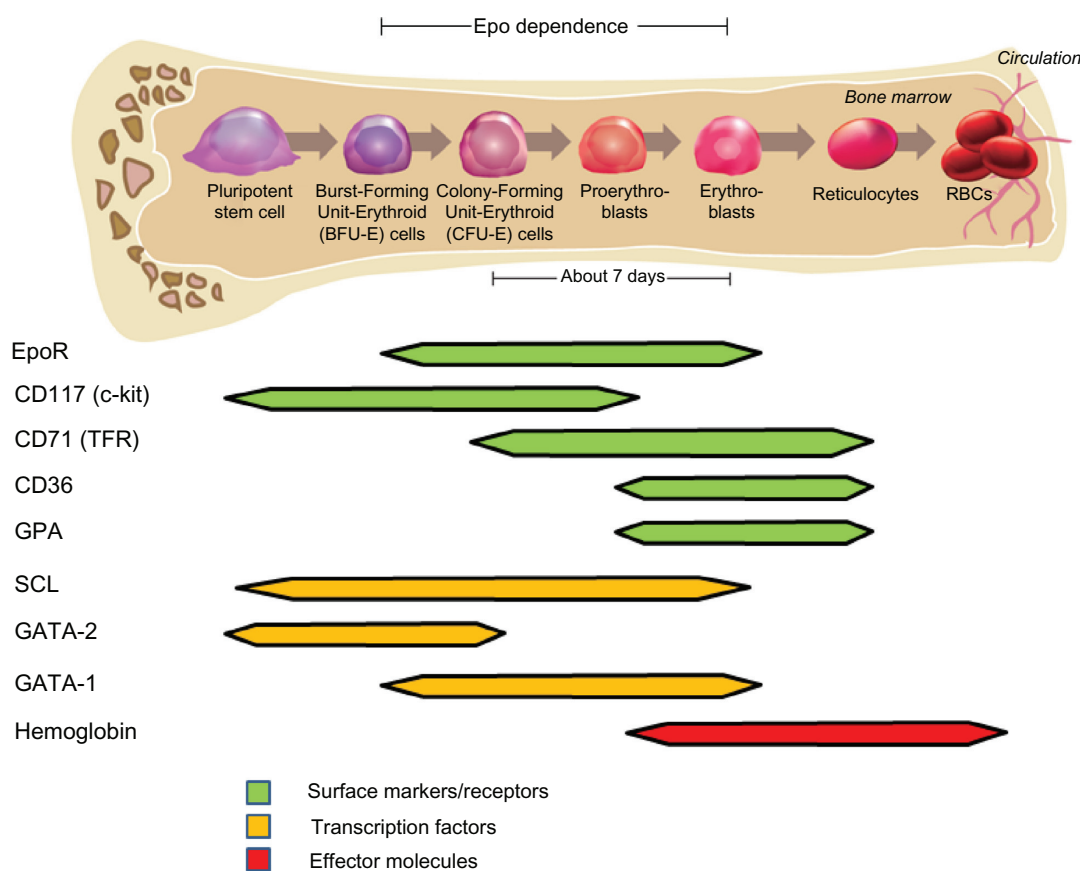


Figure 1 Erythropoiesis and the expression of stage-specific markers.

Notes: Shown is a schematic diagram of the various stages of human erythropoiesis that results in the formation of mature red blood cells (RBCs). Time-dependent expression of various proteins is shown, including erythropoietin receptor (EpoR) and other cell surface markers (green), transcription factors (orange), and effector molecules such as hemoglobin (red).

the EpoR expressed on the surface of erythroid progenitor cells. HuEpo is encoded by a single gene on chromosome 7¹⁴ (mouse chromosome 5) that is transcribed into a 1.6–2.0 kb mRNA¹⁵ and translated into a 193 amino acid (aa) precursor protein. During transit through the secretory apparatus, the 27 aa signal peptide and C-terminal arginine are removed, carbohydrate chains are added (3 *N*-linked and 1 *O*-linked) and the ~30-kDa glycoprotein is released into the surrounding fluids. This process occurs rapidly, and Epo does not typically accumulate intracellularly.¹⁶

The normal level of circulating Epo in humans is approximately 5 pM (~20 mU/mL; 100 pg/mL), substantially below the K_d of the Epo–EpoR interaction (~100 pM), indicating that only a fraction of the EpoR is Epo bound under normal conditions. However, this level of binding is sufficient to sustain erythropoiesis at a rate that will maintain normal RBC levels. Increased Epo concentrations result in an increased rate of erythropoiesis,^{17–19} thereby resulting in an increase in circulating RBCs with a maximal rate of erythropoiesis achieved at Epo concentrations of approximately 0.5–1 U/mL.^{18,20} Low Epo concentrations, on the other hand, result in apoptosis of precursor cells.²¹ Epo concentrations below the normal circulating concentration therefore result in a decline in RBC numbers in peripheral blood because the rate of loss (~0.8%–1% per day) exceeds the rate of production.

Epo expression increases with decreasing oxygen tension (hypoxia), and this mechanism appears to be the primary driver of erythropoiesis. Hypoxia by itself has little effect on erythropoiesis in vitro.²² Hypoxia inducible factor (HIF), a heterodimer comprised of α - and β -subunits, is one of several transcription factors that regulate *EPO* gene expression,^{23,24} though HIF-2 α has been shown to be the primary regulator of *EPO* transcription.^{25–28} HIF α (subunits HIF-1 α or HIF-2 α) protein levels are controlled by enzymes (HIF-prolyl hydroxylases [HIF-PH]) that hydroxylate the α -subunit of HIF, targeting it for ubiquitination by the Von Hippel–Lindau (VHL) protein and subsequent degradation by the proteasome.^{29–34} HIF-PH activity increases with increased levels of oxygen, iron, and 2-oxoglutarate, and thus HIF-PH can act as a “sensor” of oxygen tension, iron levels, and metabolic activity. As HIF protein levels increase due to decreased HIF-PH activity, the rate of Epo production in the kidney and liver as well as mobilization of iron to support increased erythropoiesis also increases. The renal Epo-producing cells appear to be either “on” or “off” (Figure 2), and thus increased Epo production is due to recruitment of increased numbers of producing cells and

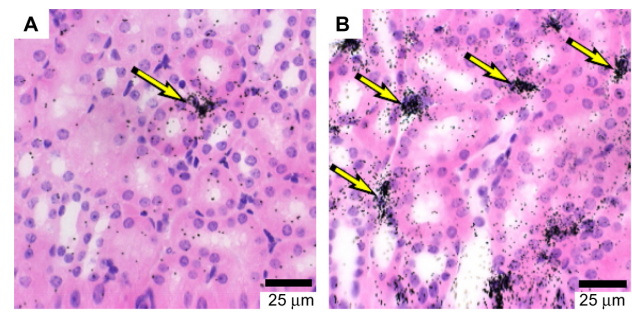


Figure 2 (A and B) Erythropoietin (*Epo*) mRNA is expressed in kidney interstitial cells. Mice were made anemic by withdrawing 0.5 mL blood and replacing with 0.5 mL saline 8, 16, and 24 hours prior to sacrifice. Standard in situ hybridization (ISH) on kidney sections was performed with an antisense ³³P-labeled *Epo* probe. **(A)** ISH for mouse *Epo* mRNA in a control mouse; **(B)** ISH for mouse *Epo* mRNA in an anemic mouse.

Notes: Increased number of cells expressing *Epo* transcripts in kidney from anemic mice vs normal mice, but with a similar number of grains over renal *Epo*-producing cells from both normal and anemic kidneys. Data and figure kindly provided by Sheilah Scully, Amgen.

not due to an increase in rate per cell.^{35,36} Under conditions of severe anemia and therefore low O₂ concentration, Epo levels can increase up to 1000-fold.³⁷

The administration of Epo increases erythropoiesis, but has limited effects on other aspects of hematopoiesis. This conclusion is supported by a number of studies. Epo and EpoR knockout mice had an absence of post-CFU-E erythroid cells but numbers of earlier progenitor cell types – CFU-E, BFU-E, CFU-granulocyte macrophage, and CFU-megakaryocyte – in fetal liver were normal.³⁸ These observations indicated that Epo was not essential for the generation of these progenitor cells. Though administration of Epo to animals and humans resulted in a rapid stimulation of erythropoiesis, the total bone marrow (BM) cellularity and numbers of myeloid, lymphoid, and megakaryocytes remained unchanged.^{17,39–43} Epo was also unable to stimulate early murine multipotential hematopoietic progenitor cells (Lin[–], Sca⁺).⁴⁴ Finally, in humans, constitutive overexpression of Epo affected erythropoiesis but not other hematopoietic lineages,⁴⁵ and subjects with polycythemia due to a hypersensitive EpoR had normal white blood cell and platelet counts.⁴⁶

Epo is expressed primarily in the kidney and liver,^{47,48} with minimal levels of transcripts detected in most other tissues, including brain, heart, and lung.^{36,49–57} In a normal adult animal, the kidney produces 70%–90% of the total Epo, with much of the remainder produced in the liver.^{57–60} The Epo-producing liver cell is a hepatocyte,³⁶ while in the kidney, it is a neuronal fibroblast cell type found in the interstitial region near the proximal tubular cells (Figure 2).^{36,51,55,61,62} Consistent with the detection of Epo transcripts primarily in kidney and liver, transgenic mice expressing LacZ or green fluorescent protein (GFP) under control of an *Epo* promoter

showed B-gal activity/GFP in liver and kidney but not other tissues, including brain and lung.^{36,63} Although there are some reports that Epo expression may extend to other tissues and cell types (including cells in the brain), these data were based on Western immunoblot and immunohistochemical (IHC) methodologies that used nonspecific or insensitive antibodies or reverse transcription-polymerase chain reaction (RT-PCR).^{64–71} Therefore, the results of antibody studies are inconclusive. Furthermore, the significance of mRNA detection by nonquantitative RT-PCR is unclear, because there was no evidence provided that the transcripts were translated into significant amounts of Epo protein.

Erythropoietin receptors

The *EPOR*^{72–74} is encoded by a single gene found on human chromosome 19p and mouse chromosome 9.^{72,75} It expresses a 2.0–2.2-kb mRNA that is translated into 508 aa (human) and 507 aa (mouse) proteins.^{20,74} After the removal of the 24 aa signal peptide, 484 aa (human) and 483 aa (mouse) proteins with a calculated molecular weight of approximately 53 kDa are generated.⁷⁶ Addition of an *N*-linked carbohydrate chain results in a protein with an estimated size of 56–57 kDa, which is comparable to the size of mature human and murine EpoR as determined by Western immunoblot analysis (~59–61 kDa).^{76–78} The mature form is then transported to the cell surface, making it accessible for binding to Epo.

However, transport of EpoR to the cell surface is inefficient, and the majority of EpoR is detected in the endoplasmic reticulum, Golgi, and endosome-like structures.⁷⁹ Less than 10% of the total EpoR protein synthesized appears on the cell surface.^{80–83} The remainder is degraded, but EpoR “fragments” can be detected by Western blotting with specific anti-EpoR antibody A82.⁷⁸

Cloning of the mouse and human *EPOR* genes^{73,74} allowed for the further identification of potential EpoR-expressing and Epo-responding cells. According to in situ hybridization studies using *EPOR* probes, *EPOR* transcripts were detected in erythroid progenitor cells, with no EpoR transcripts detected in other hematopoietic cell types or in nonhematopoietic tissues, including adult liver, heart, skeletal muscle, and kidney.^{20,74,84–86} High-level *EPOR* mRNA expression was detected by Northern blot analysis in megakaryocyte/erythroid cell lines, but levels were low to undetectable in other types, including pluripotent embryonic stem/carcinoma cells, multipotent hematopoietic cells, myeloid progenitors, and committed lymphoid and macrophage precursors.⁸⁷ With the advent of more sensitive PCR and microarray methodologies, *EPOR* transcripts were detected in multiple nonerythroid cell types from the BM compartment as well as in various normal and tumorous tissues.^{56,64,84,85,88–94} However, compared to erythroid progenitor cells and tissues containing them, levels are relatively low, as shown in Figure 3.

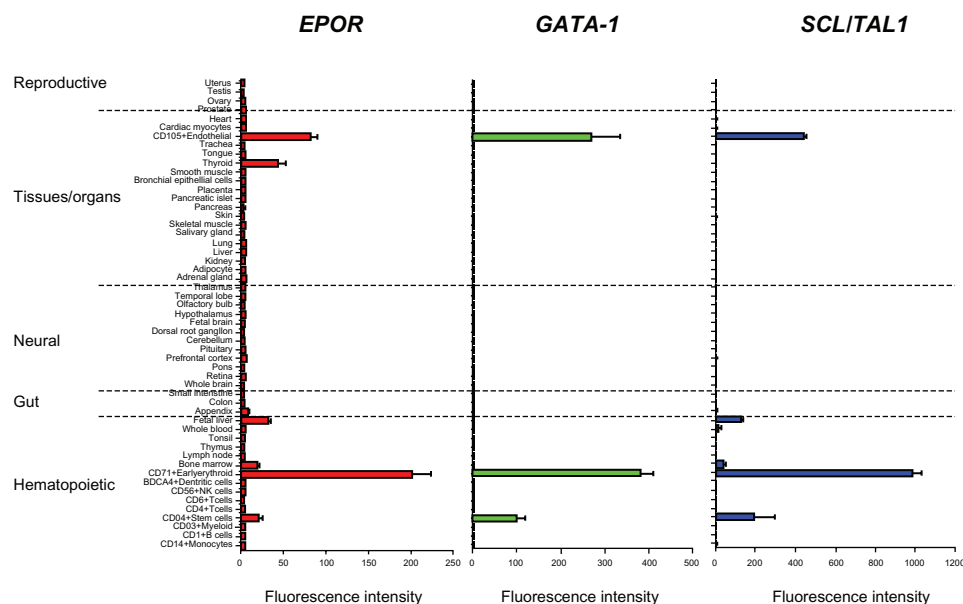


Figure 3 Erythropoietin receptor (*EPOR*), *GATA-1*, and *SCL/TAL1* have similar transcript profiles in normal human tissue.

Notes: Illustrated are levels of transcript (average of $n = 2$) and standard error obtained through microarray analysis of normal human tissue for *EPOR*, *GATA-1*, and *SCL/TAL1*. Levels of expression are in mean fluorescence units. Data were obtained from the publicly available database <http://biogps.org>. Probes shown are: *EPOR*, 209962_AT; *GATA-1*, 210446_AT; *SCL*, 206283-s_AT. Similar intensities were observed with other probes. Note that high-level expression of *EPOR* mRNA is found primarily in tissue/cell types containing erythroid cells. CD105 (endoglin) is expressed in endothelial cells, but it is also coexpressed with CD71 in erythroid cells.^{343,344,461} Thus the *EPOR* detected in CD105+ cells is likely due to erythroid cell-specific expression.

The observation that *EPOR* transcripts could be detected at low levels outside the erythroid compartment suggested that EpoR protein could be generated and that therefore Epo could potentially have effects in nonerythroid tissues. Indeed, initial Western immunoblot and IHC experiments with anti-EpoR antibodies suggested that EpoR protein was widely expressed in nonerythroid cells at relatively high levels.⁹⁵ However, these results were confounded, as nonspecific antibodies with poor sensitivity and specificity were used.^{76,91,96–98} Concerns regarding anti-EpoR antibody specificity and sensitivity first became apparent when the reported size of putative EpoR proteins detected by Western blot differed from the calculated molecular size of EpoR in positive controls.⁷⁶ Furthermore, putative EpoR proteins were also detected in EpoR negative control cells with these anti-EpoR antibodies.⁷⁶ The use of nonvalidated anti-EpoR antibodies has caused significant confusion and conflicting data in the literature.^{99,100} This issue is not unique to EpoR, as nonspecificity of antibodies has caused issues in the reliable detection of many proteins.^{101,102} This has resulted in misdirected research and unnecessary or inappropriate clinical decisions.

Another reason why the detection of EpoR protein has been problematic is that in nonerythroid cells, the levels of EpoR expression are generally very low, and therefore sensitive and specific detection methods are needed. For example, according to radiolabeled [¹²⁵I]rHuEpo-binding assays, which are very sensitive, in erythroid progenitors EpoR was found to be expressed at $<2 \times 10^3$ surface receptors/cell.^{103,104} This contrasts with other receptors such as EGFR, which is expressed in epithelial cells at 1×10^5 to 1×10^6 receptors/cell.^{105,106} Using live freshly derived cells, Epo binding was detected on the surface of erythroid progenitor cells,^{107,108} but not on unfractionated bone marrow, macrophage, thymocytes, monocyte, granulocyte, or late myeloid precursor cells;^{104,108–113} or on cells from normal tissues, including heart, kidney, brain, and lung.⁸ Recently, a sensitive and more-specific anti-EpoR monoclonal antibody (A82) suitable to detect low levels of EpoR by Western immunoblot was described.⁷⁸ Results with A82 indicated that only erythroid cells had high levels of EpoR protein, with low to undetectable levels in other nonhematopoietic tissues and hematopoietic cell types (Figure 4).^{80,94}

Regulation of EpoR

During normal erythroid differentiation, EpoR mRNA and surface protein increase as cells progress through the BFU-E to CFU-E stage,^{11,20} with a decline thereafter and an absence of detectable expression on reticulocytes and

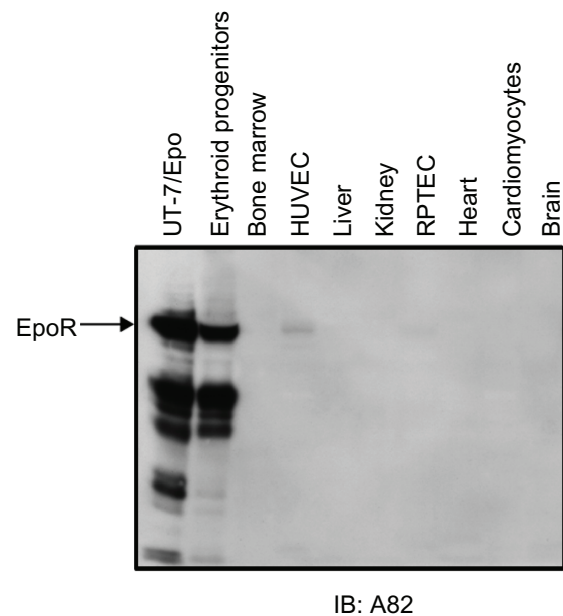


Figure 4 High-level erythropoietin receptor (EpoR) protein expression is found in erythroid cells but not in other tissues. EpoR expression was analyzed by Western immunoblot analysis with anti-EpoR antibody A82 that was shown to specifically detect human EpoR in erythroid cells.⁷⁸ The arrow shows the location of full-length EpoR. Smaller proteins have been shown elsewhere to be EpoR fragments.⁷⁸ UT-7/Epo cells (EpoR positive control) are derived from a megakaryoblastic leukemia and are Epo-dependent.¹⁶²

Notes: This research was originally published in *Blood*. Sinclair AM, Coxon A, McCaffery I, et al. Functional erythropoietin receptor is undetectable in endothelial, cardiac, neuronal, and renal cells. *Blood*. 2010;115(21):4264–4272. © American Society of Hematology.⁹⁴

Abbreviations: HUVEC, human umbilical vein endothelial cells; RPTEC, renal proximal tubule epithelial cells.

RBCs^{104,110,111,114,115} (Figure 1, Figure 5). In knockout mice, neither *Epo* nor *EpoR* were required for the formation of BFU-E cells or the transition to the CFU-E stage.¹¹⁶ EpoR is required for the Epo-dependent expansion and survival of erythroid progenitors as they differentiate from CFU-E into mature hemoglobinized RBCs, and Epo responsiveness correlates with EpoR expression level.^{20,104,113,115,117} The observation that BFU-E grew with GM-CSF or interleukin (IL)-3 plus Epo but not with Epo alone, but did grow with Epo alone if EpoR expression was increased by forced overexpression using retrovirus-mediated gene transfer,¹⁰⁸ suggests that increased *EPOR* mRNA and protein expression is an important step preceding Epo responsiveness. However, increased *EPOR* mRNA is necessary but not sufficient for surface EpoR expression,¹¹⁸ and other factors are required, such as JAK2, which acts as a key signaling intermediate as well as a chaperone.¹¹⁹

EPOR mRNA has a relatively long half-life, approximately 90 minutes in human cells and 75 minutes in murine cells,^{20,120} and the half-life is not affected by Epo or by cellular differentiation. The *EPOR* promoter was found to be

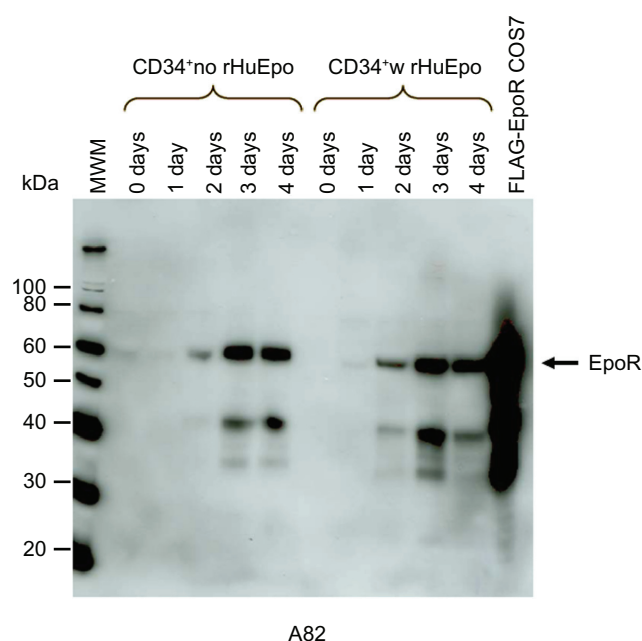


Figure 5 Erythropoietin receptor (EpoR) expression in differentiating CD34+ hematopoietic progenitor cells grown with or without Epo.

Notes: CD34+ cells were cultured in medium containing 50 ng/mL stem cell factor, 10 ng/mL interleukin (IL)-3, 10 ng/mL IL-6 with (w/) and without (no) 5 U/mL recombinant human erythropoietin (rHuEpo) for the indicated number of days. The arrow denotes the position of full-length EpoR (59 kDa). FLAG-EpoR COS7 is an EpoR positive control cell lysate from COS-7 cells expressing a FLAG-tagged version of EpoR.⁷⁸ Data and figure kindly provided by Leigh Busse, Amgen.

Abbreviation: MWM, molecular weight marker in kilo Daltons (kDa).

active in erythroleukemia cell lines MEL and HEL, but not in nonerythroid cell types, including NIH3T3, HeLa, EL4, S194, WEHI-3, or COS.^{121–125} These findings suggested that *EPOR* gene transcription is controlled by essential erythroid-specific transcription factors that are limiting or absent in some cell types. In one study, the sequence of the *EPOR* in Epo-responsive and -unresponsive mouse erythroleukemia cells was the same,¹²⁶ suggesting that lack of response was not due to defects in EpoR itself.

Reporter experiments have been performed in transgenic mice to track the *in vivo* expression of endogenous EpoR in different cell populations. Using the Cre-Lox system, EpoR Cre mice were crossed to Lox Rosa26 enhanced yellow fluorescent protein (eYFP) reporter mice, and expression of eYFP was found to correlate with activity of the *EpoR* promoter.¹²⁷ In hematopoietic cells, eYFP was detected in erythroid cells up to the erythroblast stage. However, no eYFP was detected in megakaryocytes, platelets, macrophages, granulocytes, monocytes, or leukocytes. Further, eYFP was not detected in highly purified hematopoietic stem cells, mesenchymal, or osteoblastic enriched populations from the bone microenvironment. In a similar experiment, GFP-Cre was introduced into the *EPOR* locus by homologous recombination.⁸⁸ With

this construct, *EpoR*-driven Cre activity was observed in Ter119⁺ erythroid cells but not in other hematopoietic lineages, including granulocytes, macrophages, monocytes, leukocytes, lymphoid cells, megakaryocytes, or platelets, nor in early Sca-1⁺ hematopoietic “stem cells.” Cre activity was observed in fetal liver and bone marrow, but not in any other tissue, including brain, heart, lung, and kidney. These observations are consistent with *in situ* *EpoR* hybridization experiments with tissues and purified hematopoietic cell types (see above) where high-level *EpoR* mRNA expression was detected only in erythroid cells or tissues containing erythroid cells.

EpoR expression does not appear to be controlled by Epo. In support of this, EpoR protein is increased in the absence of Epo in differentiating erythroid cells (Figure 4), and in nonhematopoietic tissues, *EpoR* mRNA levels were not altered in Epo-deficient skeletal muscles,¹²⁸ nor were EpoR levels changed when endothelial cells were cultured with Epo.¹²⁹

EpoR also does not appear to be regulated by hypoxia. Neither *EPOR* transcripts^{22,80,91,130–135} nor protein levels⁸⁰ were increased under hypoxic conditions. The lack of elevated *EPOR* transcription with hypoxia is consistent with the absence of a consensus hypoxia response element in the *EPOR* transcriptional regulatory regions. However, some reports have suggested EpoR expression is regulated by hypoxia.^{132,134,136–140} These latter data are confounded, because the studies were not appropriately controlled and conclusions were based on the use of nonspecific anti-EpoR antibodies to detect EpoR by IHC.

Several different transcription factors have been reported to play a role in regulating *EPOR* transcription, including GATA-1.^{43,123} GATA-1 knockout mice do not develop erythroid cells, but are able to develop other hematopoietic cell types.^{141–143} GATA-1 expression is primarily restricted to the erythroid lineage and is essential for high-level *EPOR* promoter activity.¹²³ Indeed, this relationship can be seen when *EPOR* and *GATA-1* mRNA levels in various tissues are compared (Figure 3). *EPOR* transcript levels correlate with *GATA-1* transcript levels across tissue and cell types, levels of both change concomitantly during cell division,¹⁴⁴ both are expressed in the same cell types during erythropoiesis,¹⁴⁵ and GATA-1 levels correlate with Epo responsiveness in cell lines.^{146,147} However, GATA-1 alone is insufficient to drive *EPOR* expression, and other factors appear to be essential, including Friend of GATA (Fog1),¹⁴⁸ a factor that forms a complex with GATA-1,¹⁴⁹ the erythroid specific factor *SCL/TALI*,^{150–153} which demonstrates a similar expression profile

as *EPOR* and *GATA-1* (Figure 3); and *ETV6/RUNX1*, which when overexpressed can also increase *EPOR* gene transcription.¹⁵⁴ Consistent with a similar tissue expression profile, *SCL/TAL1* is coexpressed with *GATA-1* in the same hematopoietic cells.¹⁵⁵ Another possible regulator is SP1, a transcription factor found in lysates from erythroid but not in nonerythroid cell lysates.¹²⁴

The *EPOR* promoter appears to be leaky because transcript levels are detected in numerous cell types, albeit at lower levels compared to erythroid cells. This is consistent with the finding that the *EPOR* gene promoter has characteristics of a ubiquitously expressed gene (ie, lacks a TATA box) and thus should have low basal transcription in nonerythroid cells.^{156,157}

Activation of EpoR

Activation of EpoR is initiated by the direct binding of a single Epo molecule with two membrane-spanning EpoR proteins^{158–160} that form a homodimer (Figure 6). The binding of Epo induces a conformational change in EpoR that brings the transmembrane and intracellular regions

of the receptor in close proximity. Following binding, the Epo–EpoR complex is activated, internalized, and some is degraded in lysosomes, with the remainder recycled to the cell surface.^{8,161} However, EpoR can also be internalized and degraded in lysosomes without Epo binding and activation.¹⁶²

EpoR does not contain intrinsic tyrosine kinase activity but instead requires an accessory tyrosine kinase (JAK2) to induce the signaling cascade.¹¹⁹ JAK2 interacts with EpoR at the juxtamembrane region,¹¹⁹ and the conformational change induced by Epo binding to EpoR^{163,164} brings the JAK2 molecules into close proximity, resulting in their transphosphorylation.¹⁶⁵ The activation of JAK2 results in the phosphorylation of tyrosine residues in EpoR, which serve as docking sites for mediators of the STAT5, MAP kinase, and PI3 kinase/Akt signaling pathways¹⁶⁶ (Figure 6). Following activation, negative regulators of EpoR, including Src homology region 2 domain-containing phosphatase 1 and suppressor of cytokine signaling proteins SOCS-1 and SOCS-2, down-modulate signaling responses.^{167,168} Further control of Epo-induced signaling in cells is mediated through

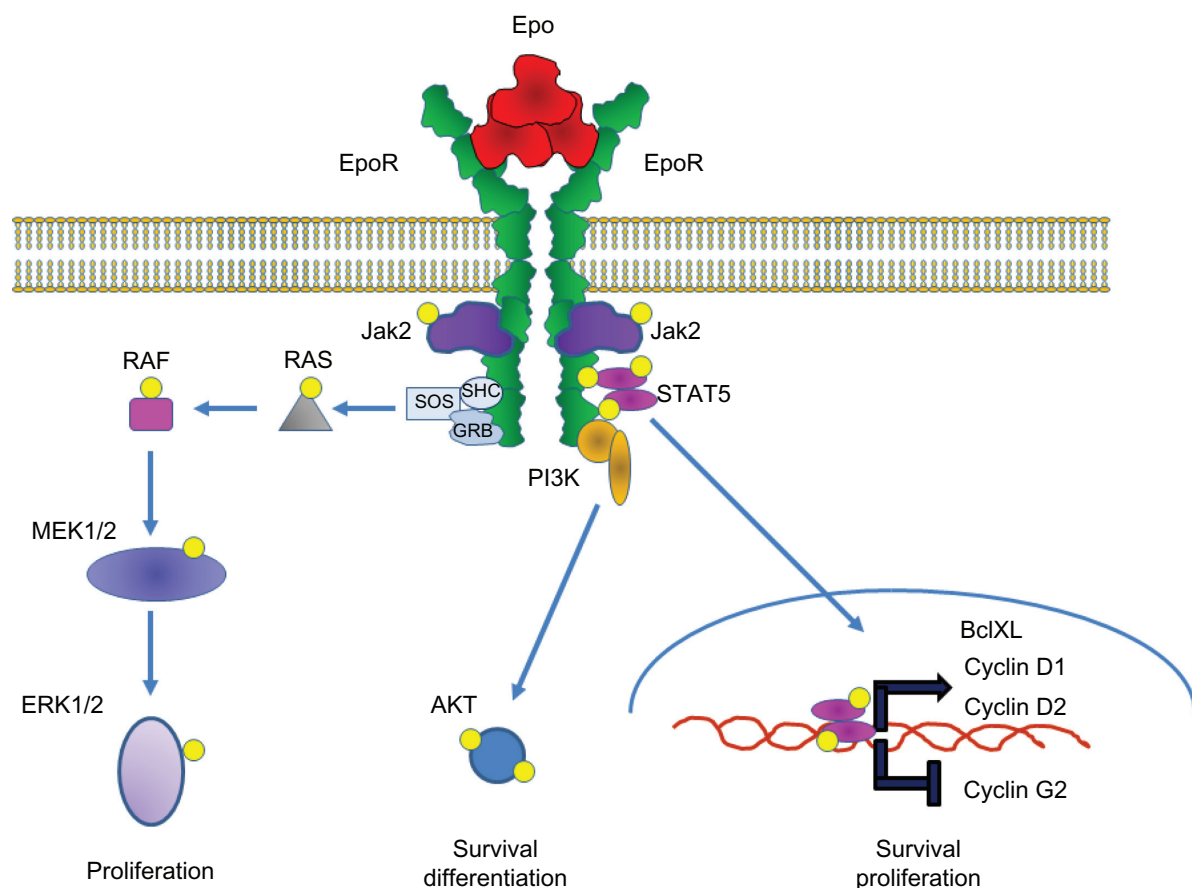


Figure 6 Erythropoietin receptor (EpoR) activation and signaling with Epo in erythroid progenitor cells.

Note: Schematic diagram of the signaling cascades and effector responses observed in erythroid progenitor cells when EpoR is activated with erythropoiesis-stimulating agents.

inhibition of EpoR cell surface expression through ubiquitination and subsequent proteosomal degradation.¹⁶⁹

The rate of assembly of a functional EpoR homodimer is EpoR concentration-dependent.^{158,170} In HEL cells, the magnitude of increase in phosphorylated JAK2 after Epo treatment, minimal in the parental cells, is increased with overexpression of EpoR.¹⁷¹ However, levels of surface EpoR are not always correlated with *EPOR* mRNA level.¹⁷² Thus, low-level protein production and/or inefficient EpoR processing and surface translocation may be limiting factors for Epo–EpoR responses. In support of this possibility, increasing levels of EpoR in growth factor-dependent cell lines caused them to become demonstrably Epo-responsive.^{20,104,108,147,171,173,174} EpoR levels also appear to affect magnitude of response to Epo in vivo. For example, mice that were haplo-insufficient (*EpoR*^{+/-} mice) had reduced hematocrit and reduced responsiveness of CFU-E to Epo compared to normal mice.¹⁷⁵ While these studies indicate that a minimal level of EpoR expression is required for a functional response, the absolute level of EpoR required is unclear. SH-SY5Y cells (a neuroblastoma cell line) were reported to respond to rHuEpo despite very low levels of surface EpoR, less than 50 surface EpoR/cell.^{176,177} However, others could not detect responses in SH-SY5Y cells.^{91,94,178}

Another possible explanation for the lack of functional EpoR in some cells even though the receptor protein is expressed is that other accessory factors for functional responses are missing. Consistent with this proposal, the leukemia cell lines K562 and OCIM-1 do not respond to Epo (signaling or proliferation/survival) despite detectable EpoR expression on the cell surface using Epo-binding assays.^{103,112,115} In addition, EpoR was detected at ~1000 receptors/cell in other cell lines derived from patients with acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), and erythroleukemias, but only some were responsive to Epo.^{73,103,179–182} This may be at least partly explained by constitutive activation of pathways making them nonresponsive to cytokine stimulation.¹⁸³ For example, K562 cells have the Bcr/Abl fusion,¹⁸⁴ while OCIM-1 cells have constitutive phosphorylation of STAT5, though the pathways contributing to this constitute activation are unknown.¹⁸⁵ However, other processes could also be defective in those cells, explaining the lack of Epo–EpoR response.

EpoR overexpression can confer Epo dependence in some cell types but not others, indicating EpoR expression is necessary but not sufficient for a response. For example, forced overexpression of EpoR resulted in Epo dependence

for growth in factor-dependent murine progenitor cell lines (FDCP-1, 32D, BaF3) but not in others, such as mouse IL-2-dependent T-cell lines HT-2 and CTLL2^{186–195} or in NIH-3T3 cells,¹²¹ which are dependent on platelet-derived or fibroblast growth factor for growth.¹⁹⁶ Infection of BM cells with virus expressing EpoR or a constitutive-active EpoR variant (R129C) resulted in an increase in erythroid, macrophage, and megakaryocyte cells but not other lineages, including lymphocytes, granulocytes, mast cells, and eosinophils.^{108,197–199} This suggests that macrophage and megakaryocyte progenitors cells are programmed for a response but lack sufficient EpoR expression, while other cell types lack programming. For example, HT-2 cells expressing EpoR failed to grow with Epo despite Epo-induced phosphorylation of EpoR and JAK2. However, these cells had a deficit in Epo-induced STAT5 phosphorylation,¹⁸⁶ suggesting a deficiency in downstream signaling pathways. A somatic fusion of EpoR-expressing HT-2 cells with BaF3 cells resulted in Epo dependent growth and signaling, suggesting addition of an essential factor by BaF3 cells. Taken together, these observations suggest that in addition to the accumulation of a certain level of EpoR, the cells must contain the required intracellular signaling networks for a “programmed” response.

Is functional EpoR expressed in tumor cells?

The potential for ESAs to stimulate tumor growth has been of significant controversy since 2003, when it was reported that patients with head and neck cancer receiving rHuEpo had reduced locoregional control of their tumors compared to control subjects.^{2,200} This was followed by an analysis of patient samples for expression of EpoR,²⁰¹ in which an association between staining with the anti-EpoR antibody C-20 and negative clinical outcomes was reported. This raised the hypothesis that EpoR was expressed on tumors and that ESAs directly stimulated tumor growth. This hypothesis appeared to be supported by preclinical data that suggested that most tumors and cell lines expressed high levels of EpoR, and further that ESAs directly promoted tumor cell growth and survival.^{100,202,203} However, these data contrasted with data from other groups that reported EpoR was not present on tumor cells and that ESAs did not have a direct tumor-stimulating effect.^{99,204–206} Further, with clinical data from other trials and meta-analyses, there was not a significant association between ESAs and tumor progression end points.² These conflicting data have caused considerable confusion and have led to calls for additional research. Here, we provide

a critical evaluation of the research that pertains to the expression and function of EpoR in tumor cells.

Tumor growth is commonly driven by oncogenes, which are marked by shared characteristics, including overexpression due to genomic amplification, mutations that induce constitutive activation, and increased transcriptional/translational activity. Although *EPOR* genomic amplification and gene rearrangements have been described in some erythroleukemia and megakaryoblastic leukemias and derived cell lines (eg, UT-7 F36E and TF-1),^{172,207–209} *EPOR* amplification is thought to be a rare event. Several studies failed to show amplification of *EPOR* or alterations to chromosome 19, the location of the *EPOR* gene,^{209,210} even in erythroleukemia, the disease above all others in which involvement of Epo/EpoR might have been predicted. Furthermore, in contrast to oncogenic receptors such as HER2 and EGFR, in a screen of >1000 different solid tumors, EpoR gene amplification was rarely found, and when observed was similar to the frequency and magnitude of amplification of other nononcogenes.⁹²

Constitutive activation of EpoR could theoretically also provide a growth advantage to tumors. This has been observed with Friend virus infection, which results in constitutive activation of EpoR through the binding of Env protein gp55 to EpoR, and has been shown to induce erythroleukemia in mice.^{211,212} An activating mutation in murine EpoR was identified (R129C) in a mutagenesis screening study that induced constitutive activation and conferred growth factor independence in IL-3-dependent BaF3 cells.²¹³ However, activating EpoR mutations do not appear to play a role in tumorigenesis, and naturally occurring activating EpoR mutations have not been found in human erythroleukemias.^{209,210} For example, EpoR sequence analysis was performed on six tumor cell lines (UT-7/Epo, MCF-7, 769-P, CAKI-2, SH-SY5Y, and HeLa), and no activating EpoR mutations were found (Amgen data on file). Moreover, while EpoR hyperactivating mutations^{214,215} have been reported in patients with congenital erythrocytosis, these subjects had normal platelet and white blood cell counts and no increased incidence of tumors or leukemic transformation,^{192,209,211,216–218} and were otherwise normal.

A prerequisite for a direct effect of ESAs on tumor cells is that they must express EpoR. *EPOR* mRNA was detected in multiple tumor cells and cell lines using RT-PCR.^{20,90,96,134,219–228} However, *EPOR* transcript levels were 10–1000-fold lower in tumor tissues and cell lines compared to Epo-responsive positive control cells.^{64,80,91,229–234} These results were consistent with Northern analysis of solid tumor and leukemic cell lines, in which *EPOR* mRNA

was expressed at low to undetectable levels.^{87,235} One group reported a direct correlation between *EPOR* transcript levels and poor clinical outcome in a subset of patients treated with ESAs, but definitive prognostic conclusions could not be made.²³⁰ Moreover, levels of *EPOR* mRNA in tumors were similar to that of their normal counterpart.^{92,134} These data demonstrate that though the *EPOR* gene is expressed in normal tissues and tumor cells, *EPOR* mRNA transcripts are not overexpressed in tumors, with levels detected representing the low basal transcription seen in normal tissues.

As *EPOR* mRNA was detected in tumors, it seemed likely that EpoR protein was also present on tumor cells. Indeed, Henke et al reported that high levels of EpoR protein was expressed in tumors from head and neck cancer patients who had poor outcomes when treated with ESAs using IHC studies.²⁰¹ EpoR expression was also reported by multiple groups in various tumors and tumor cell lines by Western immunoblot and IHC using the same antibody (C-20).^{236–242} Over 30 different studies have been published with putative detection of EpoR in tumors and tumor cell lines that all used the C-20, M-20 and H194 antibodies (produced by the same manufacturer – Santa Cruz Biotechnology). These studies were thought to indicate that ESAs may stimulate EpoR expressed in tumors and thereby promote tumor growth and survival. However, analysis of the Henke et al clinical samples indicated that the level of EpoR protein expression suggested by the C-20 staining did not correlate with the level of *EPOR* mRNA.²³⁰ In addition, not all groups reported correlations between C-20 antibody staining of other clinical tumor specimens and adverse clinical events.^{243–246} Further, in cells deemed to be EpoR-positive through staining with C-20 antibody, no cellular responses, such as changes in proliferation or viability, were observed.²⁴⁷ These discordant results were highlighted in a study in which tumor cells from patients with B-CLL were reported to express EpoR using a nonspecific anti-EpoR antibody, but no EpoR protein was detected on the cell surface using a more specific digoxigenin-labeled rHuEpo binding method.⁹⁶

Several issues have recently come to light in the analysis of anti-EpoR antibodies, including C-20: the putative EpoR proteins detected with the antibodies varied in size by Western immunoblot analysis, were detected in negative control cell lines, differed in size from the EpoR detected in positive control samples, and in control studies many were shown to be nonspecific.^{76,91,97,98,230,248,249} Therefore, it is likely that the putative EpoR detected with these antibodies were non-EpoR cross-reacting proteins, thereby giving false-positive results. One of the proteins detected by C-20 was 66 kDa in size

and thought to be EpoR, but was subsequently shown to be heat shock protein (HSP)70.⁷⁶ Since HSP70 is ubiquitously expressed and expression is increased when cells and tumors undergo stress responses, the IHC results reported with C-20 may have reflected HSP70 biology and not EpoR. The use of nonspecific antibodies in general,¹⁰¹ and anti-EpoR antibodies in particular,⁷⁶ is a well-recognized problem in research that has resulted in recommended guidelines for antibody validation.^{250–254}

Recently, a specific and sensitive anti-EpoR antibody (A82) suitable for detecting EpoR by Western immunoblot analysis was described.⁷⁸ Using A82 in Western analyses of total protein lysates (intracellular and cell surface protein), EpoR was undetectable in normal nonhematopoietic human and mouse tissues^{94,185} and in tumor specimens from breast, lung, ovary, colon, and skin.²⁵⁵ In another analysis of 66 tumor cell lines with A82, 80% of the lines had over 100-fold lower or undetectable levels of EpoR compared to a positive control hematopoietic cell line.⁸⁰ The remaining cell lines had relatively low levels (5–100-fold lower) compared to that observed with a positive-control hematopoietic cell line. Only one tumor cell line (the NSCLC line NCI-H661), which had the highest level of total EpoR, had detectable EpoR on the cell surface according to [¹²⁵I]rHuEpo-binding experiments. However, neither NCI-H661 nor any of the other solid tumor lines examined responded to ESAs in signaling studies.⁸⁰ Mouse monoclonal antibody MAB307 has also been used to detect cell surface EpoR by flow cytometry. While EpoR was detected on positive controls, including primary erythroid progenitors with MAB307, no EpoR was detected on the surface of viable tumor cells from over 180 different biopsies from patients with tumors including breast, colon, ovary, lung, head and neck, and kidney.²⁵⁶ These findings are consistent with Western immunoblot data generated with A82.

Another method used to examine surface EpoR in tumor cells and cell lines is competitive binding experiments with labeled rHuEpo. Specific rHuEpo binding to some hematopoietic cells and certain myeloid and erythroleukemia cells and cell lines was reported.^{103,107,112,257} However, surface EpoR was not detected in primary hematopoietic leukemias, such as B-CLL or multiple myeloma,²⁵⁸ or in most hematopoietic cell lines and nonhematopoietic cancer cell lines.^{78,80,92,103,113,115,180,259,260} In a controlled flow cytometry study using biotinylated rHuEpo, 81/136 samples from AML patients were reported to bind rHuEpo, of which only 13 of 81 had an increase in growth with rHuEpo treatment.²⁵⁷ However, there was no correlation between the amount of

EpoR and the *in vitro* proliferative response to rHuEpo. In the same study, 4/14 acute lymphoblastic leukemia patient samples were reported to bind rHuEpo, but none proliferated with rHuEpo. In other studies, one group reported that rHuEpo increased colony number and plating efficiency with cells from CML patients.²⁶¹ In contrast, in other studies, no proliferative effect of ESAs in AML and B-cell leukemic cell types were found,^{258,262} and rHuEpo did not have an effect on STAT5 phosphorylation on those cells.²⁶³

A few studies have evaluated [¹²⁵I]rHuEpo binding in epithelial tumor cell lines. While some studies have reported specific binding to solid tumor cell lines,^{235,264,265} other studies reported none.^{80,99} In Epo-responsive hematopoietic cell lines and primary erythroid cells, rHuEpo has a high binding affinity (K_d ~50–400 pM).^{103,104,109,172,266,267} In contrast, in the studies with solid tumor cells that reported binding, the rHuEpo binding affinity was unusually low (K_d ~1400–16,000 pM). The low affinities reported in these studies may be due to nonspecific interactions of rHuEpo²⁶⁸ related to the hydrophobic nature of rHuEpo.

To independently determine if functional EpoR was present on the cell surface, investigators have also examined EpoR downstream signaling events after treatment of cells with ESAs *in vitro*. Signaling through EpoR is dependent on JAK2, which transduces downstream signaling through the STAT5, PI3K, and MAPK pathways²⁶⁹ (Figure 5). Thus, positive results showing phosphorylation of JAK2 or STAT5 with ESAs in tumor cells would be important evidence for activation of EpoR with Epo. However, there are a number of reports indicating no increased phosphorylation of JAK2 or STAT5 with rHuEpo in tumor cell lines,^{80,193,270–272} with only rare positive reports: SH-SY5Y (neuroblastoma), H838 (lung cancer), and several head and neck cell lines.^{132,224,273,274} However the results in the SH-SY5Y and H838 cell lines were not reproducible by others.^{91,94,255}

In other attempts to demonstrate specificity of potential responses to EpoR, a putative JAK2 inhibitor (AG490) has been used and effects on rHuEpo signaling and other functional effects in cell lines reported.^{132,246,275–278} However, AG490 shows minimal JAK2 inhibitory activity *in vitro*.²⁷⁹ Further, AG490 has been reported to also inhibit JAK3, EGFR, HER2, guanylyl cyclase C, and BCR-ABL.^{279–283} These data raise significant questions as to the validity of results from studies that have used AG490 to ascribe effects mediated through EpoR and JAK2.

In the studies reporting positive signaling effects of ESAs on tumors or tumor cell lines, increases in phosphorylation of ERK or AKT were reported.^{205,229,272,275,276,28}

⁴ However, those results are in conflict with results from other groups who reported no effect on the same pathways using the same or similar cell types.^{80,91,223,232,233,259,285} Interestingly, there are several reports where rHuEpo had no effects on phosphorylation of JAK2 or STAT5, but did have effects on ERK phosphorylation.^{271,272,276,284,286–288} In those experiments, cells were serum-starved to increase the signal-to-noise ratio, making them sensitive to minor manipulation/stimulatory effects. Because the MAPK, PI3K/AKT, and JAK2-STAT5 pathways are stimulated by multiple receptor ligand complexes beyond Epo,^{289–291} contaminating factors could produce similar effects. Indeed, signaling that had been suggested to be mediated through EpoR was mimicked in cell lines using a media change alone.²⁹² ESA-induced signaling can also be mimicked with endotoxin, which can accumulate in contaminated preparations and can enhance AKT and ERK phosphorylation.^{293,294} Bovine serum albumin (frequently used to stabilize ESA preparations), can also support cell growth as well as stimulate ERK phosphorylation of cell lines, particularly when serum-starved cells are used,^{292,295} due to contaminants such as IGF1²⁹⁶ and insulin.²⁹⁷

ESAs have also been evaluated for potential chemotaxis activity. In some studies, ESAs were reported to increase movement of cells in Matrigel *in vitro*.^{271,276,278,288} These data supported the hypothesis that ESAs could promote metastases of tumor cells. However, others reported no effect of ESAs on migration with the same or similar cell types.^{232,233,298–300} In some of the cell lines reported to migrate in Matrigel with ESAs (eg, MCF-7, HeLa), EpoR protein was undetectable,^{78,80} raising questions about the significance of data generated with those cell lines. Furthermore, the effects reported to be mediated by ESAs were generally small compared to molecules known to induce migration, such as EGF or FGF,^{298,300} and could be a result of endotoxin, a contaminant that can similarly stimulate migration.^{301–304}

Effects of ESAs on tumor cell line proliferation have also been evaluated. However, in most studies, ESAs had no effect.^{99,205} For example, in a controlled study, though estradiol increased the proliferation of 29 tumor cell lines derived from multiple tissue sources, rHuEpo treatment did not.³⁰⁵ These findings were supported by studies in other groups that evaluated multiple different cell lines.^{80,91,300} However, in one study, rHuEpo was reported to enhance proliferation in a head and neck cell line LU-HNSCC-7 in serum-free medium (<1.4-fold increase). Notably, the authors commented that the effects observed could have been due to the medium change, although no control for that was presented.²³³

In primary tumors from renal and colorectal tumors, rHuEpo was also unable to stimulate proliferation.³⁰⁶ More recently, in a study with biopsies from a large cohort of patient samples with epithelial tumors (>180) from breast, colorectal, lung, ovary, head and neck, and kidney, rHuEpo was unable to increase the phosphorylation of AKT, ERK, or STAT5 *ex vivo*.²⁵⁶ The lack of response may be explained by the lack of EpoR expression on those cells²⁵⁶ or the high incidence of constitutive activation of pathways rendering them insensitive to growth factor stimulation.²⁶³

In vivo xenograft studies have been used to examine the effect of exogenously administered ESAs on cell growth or the ability to prevent cell ablation with chemotherapeutic agents or radiotherapy in rodents. In 31 different studies, there was no tumor growth or survival-promoting effects observed, even when high doses of ESAs were used^{99,205} (Table 1). This may be explained, in part, because most of the cell lines examined expressed little to no EpoR, and therefore would not be expected to directly respond to ESAs. However, the lack of a tumor-promoting effect was not solely explained by insufficient EpoR, because even with cells (eg, in ovarian carcinoma line A2780) having tenfold-higher levels of EpoR due to forced overexpression, no growth-promoting effects with rHuEpo were observed.²³² Further, one group performed studies using mice that produced spontaneous tumors, but again no increase in tumor incidence or growth with rHuEpo treatment was observed.³⁰⁷

In contrast to xenograft studies with ESAs, *in vivo* Epo antagonism studies have been described where the blockade of Epo–EpoR inhibited tumor growth.^{64,227,272,308} However, these reports are inconsistent with *in vitro* experiments demonstrating that the cell lines used expressed little/no EpoR and had no detectable response when treated with ESAs. Antagonism studies can be impacted by other inhibitors and factors, such as endotoxin in the preparations, that can inhibit tumor cells.³⁰⁹ The possibility that the tumor growth inhibition reported was due to the experimental design also cannot be excluded, as negative controls were not included in those studies. Taken together, these data suggest that functional EpoR is not expressed on tumor cells.

Epo–EpoR autocrine/paracrine loops

Paracrine stimulation of EpoR in cells has been reported to support growth of Epo-responsive cell lines.^{310,311} Accordingly, some groups have also suggested that both Epo and EpoR are coexpressed in tumor cells and this may be a mechanism that drives autocrine tumor growth.^{312–314}

Table 1 Effect of erythropoiesis-stimulating agents in xenograft or syngenic tumor models

Tumor type and origin	EPO/DA dose	Reported tumor and survival outcomes	Study
Tumor regression alone			
Murine myelomas MOPC-315, 5T33 MM	30 U Epo QD	Tumor regression and prolonged survival	Mittelman et al ⁴⁴⁰
Murine BCL-I leukemia/lymphoma	30 U Epo QD	Tumor regression and prolonged survival	Mittelman ⁴⁴¹
Enhanced tumor ablation			
Neurogenic sarcoma ENE2	750 U/kg Epo TIW	Improved RT therapy in anemic mice	Stuben et al ⁴⁴²
Lewis lung carcinoma	60 U/kg Epo (two doses)	No effect alone; enhanced CT	Sigounas et al ⁴⁴³
Rat DS-sarcoma	1000 U/kg Epo	No effect alone; improved ablative RT	Thews et al ⁴⁴⁴
Ovary adenocarcinoma	20 U Epo TIW	No effect alone; improved CT	Silver and Piver ⁴⁴⁵
Glioblastoma HTZ II	1000 U/kg Epo TIW	No effect alone; improved RT in anemic mice	Stuben et al ⁴⁴⁶
Rat DS-sarcoma	1000 U/kg Epo TIW	No effect alone; improved CT in anemic rats	Thews et al ⁴⁴⁷
Colon adenocarcinoma	1000 U/kg Epo QD	Restored PT in anemic mice	Golab et al ⁴⁴⁸
Human glioblastomas GBM-Nan I and U87	300 U/kg Epo QD	No effect on tumor alone; enhanced RT in both lines	Pinel et al ⁴⁴⁹
Murine SCC VII squamous cell carcinoma and RIF-I fibrosarcoma	30 µg/kg DA QW or Q2W	No effect alone; improved RT in anemic mice in both lines	Ning et al ²³¹
Lewis lung carcinoma	10 µg/kg DA QW	No effect alone; improved CT	Shannon et al ²⁷⁰
Human squamous cell A431, colorectal carcinoma HT25	150 U/kg Epo TIW	No effect alone; enhanced CT in both models	Tovari et al ⁴⁴⁹
Rat breast cancer line LCM 2388 into rat	60 IU Epo QW ± tamoxifen	No effect Epo alone; increased regression Epo + tamoxifen	Sairah et al ³⁵⁶
Human squamous cell carcinoma (A431)	150 U/kg Epo TIW	No effect alone; improved RT in anemic mice	Lovey et al ⁴⁵⁰
No enhanced tumor ablation			
Murine MmB16 melanoma	20 U Epo BID	No effect alone; no enhanced IL-12 therapy	Golab et al ⁴⁵¹
Rat R3230 mammary carcinoma	2,000 U/kg Epo TIW	No effect alone	Blackwell et al ⁴⁵²
Rat I3762 mammary adenocarcinoma	50 µg/kg Epo TIW	No effect alone	Bianchi et al ⁴⁵³
Rat DS-sarcoma	1000 U/kg Epo TIW	No effect	Kelleher et al ⁴⁵⁴
Rat R3230 mammary carcinoma	3 µg/kg DA TIW	No effect to enhance RT	Kirkpatrick et al ⁴⁵⁵
Murine C26-B colon adenocarcinoma	25 U Epo QD to 25 U TIW	No effect on tumor; decreased body weight loss	van Halteren et al ⁴⁵⁶
Rat R3230 mammary carcinoma, murine CT26 colon carcinoma, human HCT-116 colon carcinoma, human FaDu head and neck carcinoma	2000 U/kg Epo TIW	No effect alone	Hardee et al ³⁵⁵
Rat R3230 mammary carcinoma	2000 U/kg Epo TIW	No effect alone; no enhanced CT	Hardee et al ⁴⁵⁷
Human breast carcinomas MDA-MB-231 and MCF-7	2.5 mg/kg epoetin-α, 7.5 mg/kg DA, and 2.5 mg/kg epoetin-β	No effect alone; no enhanced CT in either model	LaMontagne et al ³⁰⁰
Head and neck squamous cell carcinoma LU-HNXSCX-7	400 U/kg epoetin-β Q3D	No effect alone; slight increased tumor growth with surgical transection	Kjellen et al ⁴⁵⁸
Human breast MCF-7, renal 786-0, gastric SCH, lung A549, ovary SK-OV-3 tumor cell lines into mice	1000, 3000, or 10,000 IU/kg epoetin-β QW	No effect alone; no enhanced effect on bevacizumab on A549 and MCF-7 (avastin)	Kataoka et al ²²³
Murine B16F10 melanoma	30 mg/kg DA QW	No effect alone	Miller et al ³⁰⁷
Human glioblastoma U87	5000 U/kg Epo TIW	No effect alone	Hassouna et al ²⁹⁹
Human breast MDA453β and MCF7-HER18 (engineered)	100 U rHuEpo daily (weekdays)	No effect alone; antagonized trastuzumab effect on tumor regression	Liang et al ²⁷⁵
Promoted tumor growth			
Murine MCA-induced fibrosarcoma	100 IU/kg Epo QW	Epo promoted tumor growth	Okazaki et al ⁴⁵⁹
Murine colorectal cancer cells in 50% hepatectomized mice	10 mg/kg DA once	Increased tumor growth after hepatectomy	Rupertus et al ⁴⁶⁰

Abbreviations: TIW, three times per week; BID, twice per day; QD, once daily; QW, once per week; Q2W, every two weeks; Q3D, every three days; RT, radiotherapy; CT, chemotherapy; PT, photodynamic therapy; DA, darbepoetin alfa; Epo, erythropoietin.

Consistent with this possibility, some erythroleukemia cells were reported to express Epo^{315,316} and Epo was reported to support their growth.³¹⁷ Erythrocytosis is observed in some patients with renal carcinomas, liver carcinomas, in Wilms'

tumors and cerebellar hemangioblastomas.^{47,48,318–320} In VHL syndrome patients that contain pVHL mutations, paraneoplastic Epo production and erythrocytosis is associated with renal carcinoma, cysts, cerebellar hemangioblastoma, and

pheochromocytoma.³²¹ However, in many of these cases, it is likely that Epo production is secondary to activation of the HIF pathway, or alternatively, secondary to tumor formation in cell types that normally produce Epo (eg, hepatocytes). Alternatively, tumors may produce other substances that can synergise with Epo and promote erythropoiesis, such as thyroid hormone, glucocorticoids, SCF, IL-3, or GM-CSF.

The possibility that tumors express both Epo and EpoR and that this is a driver of their growth is not supported by other data. Indeed, anemia and not erythrocytosis is a general characteristic of patients with solid tumors, suggesting that most tumor cells do not express significant amounts of Epo. Several groups reported that an Epo–EpoR cytokine loop is not a general property of tumors.^{80,322} Forced expression of Epo in mouse erythroid cells, using a human *EPO* gene under the control of a human β -globin locus control regulatory element, resulted in autocrine stimulation of erythropoiesis and erythrocytosis in transgenic mice. However, those mice did not develop erythroleukemia.⁴⁵ Similarly, constitutive Epo expression in the bone marrow of mice using retroviral vectors with *EPOR* expression cassettes resulted in erythrocytosis but not erythroleukemia,⁴⁵ and *Epo* gene therapy in mice did not result in tumors when Epo was overproduced.^{323,324}

The suggestion that tumor cells may express Epo at levels sufficient to activate resident EpoR is based almost exclusively on IHC experiments on tumor sections or Western immunoblot analysis on tumor cells using nonvalidated anti-Epo polyclonal antibodies. In the kidney, where Epo is expressed at relatively high levels, Epo is secreted efficiently, resulting in very low intracellular stores. Consequently, attempts to identify the Epo-producing cell type by IHC with anti-Epo antibodies would be difficult and have been unsuccessful.^{16,51} This indicates that it would be even more difficult to detect Epo in tissue sections that have even lower Epo expression levels than in the kidney.⁵⁰ In addition, similar to anti-EpoR antibodies, many available anti-Epo antibodies used by investigators are also nonspecific (Amgen, unpublished data) raising further questions about the significance of positive IHC or Western data with anti-Epo antibodies.

Epo and angiogenesis

Blood vessel development consists of two distinct phases – vasculogenesis and angiogenesis. Vasculogenesis is the assembly of vessels de novo and angiogenesis arises through the proliferation, movement, and incorporation of endothelial cells into existing vessels.³²⁵ Given the important role that Epo and EpoR play in regulating oxygen delivery, hypothetically Epo may also play a role in regulating blood flow through effects

on the endothelium or through stimulation of blood vessel formation. Supporting this possibility, in *EpoR* and *Epo* knock-out mouse embryos, though de novo vasculogenesis remained intact,^{326,327} a defect in angiogenesis was reported. Positive effects of Epo on vasculogenesis or angiogenesis using bone marrow-derived endothelial progenitor cells (EPCs) in vitro and in vivo have also been reported by some groups,^{328–332} but positive effects were not observed by others.^{333–336} ESAs have been reported to increase circulating levels of EPCs,^{337–341} and in the case of a subject with erythrocytosis caused by a mutation in *EpoR* resulting in hypersensitivity to Epo, there were increased levels of circulating EPCs.³⁴² However, interpretation of some of this positive data can be confusing, because a surface marker found on endothelial cells (endoglin: CD105)³⁴³ is also expressed on erythroid cells,^{343,344} resulting in possible false-positive identification of EPCs with that marker.

In contrast to the data described above, there are other reports that ESAs did not affect the vasculature. For example, rHuEpo did not affect endothelial progenitor levels^{345,346} or endothelial markers in patients receiving hemodialysis in clinical studies,³⁴⁷ and Epo did not recruit BM-derived endothelial progenitor cells in BM-transplanted mice to neointima in arteries with wire-induced injury despite accelerating reendothelialization.³⁴⁸ Further confounding the data are other studies suggesting BM-derived endothelial progenitor cells do not contribute to the vasculature.^{349,350} These included a study where *EpoR*^{−/−} mice had normal vascular endothelium,³⁸ as did *EpoR*^{−/−} mice crossed with transgenic mice where EpoR expression was restricted to the erythroid compartment.³⁵¹ Therefore, if EPCs do not even contribute to the vasculature, the role of Epo itself in possibly mobilizing the EPC becomes irrelevant. These conflicting studies raise questions about the significance of reports that ESAs affect endothelial progenitors.

In several independent studies, endothelial cells were reported neither to express significant levels of EpoR nor to respond to ESAs. In one study using a specific anti-EpoR antibody, A82, endothelial cell preparations expressed very low levels of total EpoR protein, with no detectable protein on the cell surface and no response to ESAs in vitro.⁹⁴ In other studies, rHuEpo had no effect on endothelial cell preparations in controlled in vitro and in vivo experiments.^{94,352–354} In tumor xenograft studies, no effect on angiogenesis was observed when animals were administered ESAs.^{355–357}

While several groups have reported that EpoR was present in endothelial cell preparations, the studies were based on the detection of EpoR using anti-EpoR antibodies that suffered from the same antibody nonspecificity issues described above. In ESA response studies, effects were only observed at

supraphysiologic and suprapharmacologic levels of rHuEpo (>10 U/mL), a concentration which may be more prone to provide false-positive results. Some groups reported that [125 I]rHuEpo bound to endothelial cell preparations,^{129,358,359} but the binding properties included unusually high EpoR density and low affinity, characteristics more consistent with nonspecific or off-target binding.²⁶⁸ Further, the high EpoR density reported did not correlate with the relatively low EpoR transcript levels or EpoR protein levels detected by Western analysis with a specific anti-EpoR antibody.⁹⁴ Increased thymidine incorporation into brain capillary endothelial cells following addition of rHuEpo was reported in one study, but only if the addition was accompanied by a change in growth medium,³⁵⁸ raising concerns about potential artifacts. Artifact was most likely the reason that rHuEpo reportedly induced increased vascularization in chicken eggs (chick chorioallantoic membrane assay),^{337,360,361} because there is no evidence of cross-species activity between human Epo and chicken EpoR.^{362–364}

Cytoprotective effect of Epo on normal nonhematopoietic cells and tissues

In addition to erythropoietic defects in *Epo* or *EpoR* knockout mice, nonhematopoietic developmental defects in the heart and vasculature were also reported, suggesting a functional role for Epo–EpoR in those organs.^{326,365} This possibility was further evaluated in transgenic mice with *EpoR* expression limited to the hematopoietic compartment using a GATA-1 promoter linked to the *EpoR* gene.³⁵¹ Though the *GATA-1-EpoR* transgenic mice had no detectable *EpoR* mRNA expression outside the erythroid compartment using RT-PCR analysis, the mice developed normally and had normal organ function and vasculature. These data suggested that EpoR was not required for normal nonhematopoietic organ development, and that reported nonhematopoietic effects may have been mediated through indirect mechanisms, such as insufficient oxygen delivery due to the defect in erythropoiesis.

Cytoprotection studies in animals have been performed to evaluate the possibility that ESAs have nonhematopoietic effects. Overall, in a number of different animal studies (rodents, pigs, rabbits), ESAs were reported to enhance angiogenesis after injury in models of hypoxia-induced hypertension³⁶⁶ and peripheral hind limb ischemia,³⁶⁷ and reduce tissue injury in heart,^{368–374} brain,^{375–377} kidney,^{378,379} and other organs^{204,367,380–383} using different injury model systems. Though these data suggest that ESAs have direct effects on nonhematopoietic tissues, the positive findings

from these studies may be related to RBC increases, such as enhanced oxygen delivery or changes in ferrokinetics.³⁸⁴ In the particular case of neuroprotection by ESAs, cerebrospinal fluid (CSF) Epo levels did not correlate with plasma Epo levels,³⁸⁵ ESAs were not transported into the brain at significant levels,³⁸⁶ and even though there was some increase in CSF levels of Epo where there was blood–brain barrier dysfunction, Epo concentrations were still very low^{385,387} (1–3 mU/mL vs 10–30 in serum), raising questions about possible direct effects of ESA addition on brain function in animal or human studies.

In a conditional *EpoR* knockout study in mice with brain-specific inactivation of the *EpoR* gene, endogenous Epo–EpoR was found nonessential for protecting neurons from ischemic injury, though a role was suggested in poststroke neurogenesis.³⁸⁸ In this study, mice with no *EpoR* expression in the brain had a slight reduction in proliferation and migration of neuroblasts to the peri-infarct cortex. A similar role of endogenous Epo–EpoR was suggested using another conditional *EpoR* knockout system.³⁸⁹ In the absence of neural *EpoR*, a twofold increase in neural cell apoptosis and a two- to threefold decrease in neural progenitor cell proliferation compared to wild type was reported. However, the functional neurological impact of the findings in these two studies was not reported.

Although ESAs were reported to have cytoprotective activities by directly interacting with EpoR present on cells, the data supporting this hypothesis are confounded by a number of issues similar to those associated with the hypothesis that ESAs directly stimulate tumor cells. Some investigators reported *EpoR* mRNA was expressed in nonerythroid tissues and suggested functional EpoR protein may also be present.^{176,286,358,365,370,389–392} However, *EpoR* mRNA levels in nonhematopoietic tissues were 5–1000 times lower than in bone marrow (see also Figure 3), and detection of *EpoR* mRNA in cell lines and endothelial cells did not predict surface expression.⁹⁴ Many of the investigators that reported EpoR protein expression in normal nonhematopoietic tissues^{390,391,393} used antibodies known to be nonspecific, most likely resulting in false-positive results.^{76,91,97,98,248,249,394} Alternative approaches to determine surface protein, such as radiolabeled [125 I]rHuEpo binding studies, found EpoR characteristics (high receptor number, low affinity) that are substantially different from EpoR characteristics on erythroid progenitor cells (low receptor number, high affinity).^{11,129,235,358,359,391} Recently, results using a specific anti-EpoR antibody (A82) indicated that EpoR was undetectable in most nonhematopoietic tissues from humans and mice (see Figure 4), raising further questions about the

potential for ESAs to have a direct effect on nonhematopoietic tissues.^{94,255}

ESAs were reported to activate downstream antiapoptotic signaling pathways in nonhematopoietic tissues, a mechanism that could inhibit cell death associated with tissue insult (eg, ischemia, reperfusion injury, and exposure to cytotoxins) *in vitro*.^{369,372,375,376,389} For example, rHuEpo was reported to activate AKT and ERK signaling in cardiac myocytes *in vitro*, reducing apoptosis by ~30% upon exposure to hydrogen peroxide.³⁹⁵ In studies evaluating the effects of ESAs on nonhematopoietic cell proliferation, signaling, or inhibition of apoptosis, modest effects (two- to threefold increases that are within the experimental noise of the system) were reported.^{368,375,378,395,396} Many of these studies used cells starved of serum and did not describe the use of an appropriate vehicle control, both of which raise the possibility of nonspecific effects.^{286,375,395,397,398} Furthermore, rHuEpo doses used for the *in vitro* studies were approximately tenfold higher (>10 U/mL) than levels achievable in patients with modest responses reported, raising the possibility of artifacts as well as questions about the physiological and clinical relevance of these findings.^{286,368,370,378,396,399}

While the possibility that ESAs may be cytoprotective is supported by some studies, many of the *in vivo* studies with ESAs are conflicting. For example, though in two studies rHuEpo reduced ischemia reperfusion-induced renal injury and preserved renal function,^{400,401} in another study rHuEpo did not preserve renal function.⁴⁰² In studies using the same transgenic mouse model of amyotrophic lateral sclerosis, mixed findings have been reported. In one, rHuEpo delayed symptom onset and prolonged survival times.⁴⁰³ In a second, rHuEpo delayed disease onset in females but not males,⁴⁰⁴ and in the third, rHuEpo had minimal improvement in motor neuron function, with no effect on motor neuron loss or overall survival.⁴⁰⁵ In another central nervous system (CNS) model, though high doses of rHuEpo (500–5000 U/kg daily) were reported to inhibit CNS inflammatory effects rats with experimental autoimmune encephalomyelitis,⁴⁰⁶ no protective effect was found in animals with adjuvant arthritis, even when the same high-dosing regimen was used.⁴⁰⁶

In other *in vivo* animal studies, ESAs did not provide nonhematopoietic protective effects. Pretreatment of rats with darbepoetin alfa did not alter endotoxin-evoked myocardial depression or the expression of proapoptotic or antiapoptotic genes in the heart.⁴⁰⁷ rHuEpo was unable to provide neuroprotective effects in a rabbit bacterial meningitis model, even though the systemically administered rHuEpo was reported to penetrate the CNS in infected rabbits.⁴⁰⁸ rHuEpo

was also unable to prevent endotoxemia-induced liver and kidney damage in rats.⁴⁰⁸ Human clinical studies with tissue-protective end points have also been performed. To date, the cytoprotective effects reported in animal models have generally not translated into a clinical benefit in humans (reviewed in Sølling⁴⁰⁹) who had injury to brain,^{410–412} heart,^{413–419} or kidney.^{420–426} Further, in a recent study, rHuEpo had no effect on intracellular signalling with human skeletal muscle.⁴²⁷ Taken together, these data suggest that ESAs may not have the broad, reproducible, robust, nonhematopoietic protective abilities described by some investigators.

Alternative receptor complexes for Epo and Epo derivatives

An alternative receptor complex that can bind ESAs and mediate cytoprotective activity has been proposed based on the unusual binding affinities of ESA reported on nonhematopoietic cells. The proposed alternative receptor was reported to consist of a heteromeric complex of EpoR and the GM-CSF/IL-3/IL-5 β -common chain (βc).³⁹³ It was further proposed that a chemically modified Epo molecule (carbamoylated Epo [cEpo]) bound the alternative receptor complex and provided tissue-protective effects in the absence of stimulation of erythropoiesis.⁴²⁸ Similar to rHuEpo, a number of model systems with various cytotoxic insults have been used to describe this cytoprotective activity of cEpo, such as inhibition of cardiac-myocyte apoptosis,^{393,429} improvement in cardiac function after permanent ischemia,⁴²⁹ inhibition of renal tubule apoptosis, improvement in renal function after ischemia-reperfusion or obstructive injury,^{430–432} and reduction in neural lesions and apoptosis in the CNS with various rodent model systems.^{433–435} Data used to support the hypothetical cytoprotective role of the βc -EpoR heteromer were generated using mice in which the GM-CSF βc had been knocked out. Based on these data, cEpo and ESAs were reported to bind to the heteromer, activate signaling pathways, and prevent apoptosis in several normal nonhematopoietic tissues.^{393,397,428} However, this hypothesis is controversial, as other investigators have found βc does not play a role in preventing apoptosis with ESAs.¹⁷⁶ It is particularly noteworthy that the investigators who initially generated the GM-CSF βc knockout mice⁴³⁶ examined the receptor status and responsiveness of those animals thoroughly and concluded that there was no evidence of an interaction between the GM-CSF βc and EpoR.^{437–439}

Summary and conclusions

Epo is an essential cytokine that binds and activates EpoR resident on the surface of erythroid progenitor cells, thereby

promoting erythropoiesis. To this end, ESAs are currently indicated for treatment of anemia in patients with chronic kidney disease and chemotherapy-induced anemia. Epo has also been reported to have effects beyond erythropoiesis, such as tissue-protective effects and promotion of tumor cell growth or survival. This Epo–EpoR tumor stimulation hypothesis has been used to explain the safety signals seen in some clinical trials in anemic cancer patients treated with ESAs. However, putative positive results for this hypothesis are generally confounded by the absence of controls to detect false-positive effects and the use of nonspecific reagents in many studies. EpoR levels outside the erythroid compartment are very low, and the data that such low-level EpoR can bind significant amounts of Epo and promote a functional response are unconvincing. Further, in controlled clinical trials, the cytoprotective benefits observed in animal studies have not as yet translated into benefit in the clinic. The totality of evidence suggests that ESAs do not directly stimulate tumor cells and that similarly the cytoprotective and other nonhematopoietic effects of ESA treatment reported are not a direct effect of ESAs acting through EpoR on nonerythroid cells.

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Disclosure

Both authors are employees and/or hold stock in Amgen, Inc, a manufacturer of ESAs.

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