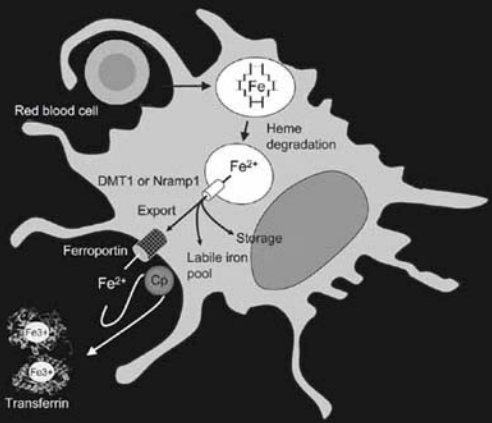
Milestones in Drug Therapy
Michael J. Parnham
Jacques Bruinvels
Series Editors

Erythropoietins, Erythropoietic Factors, and Erythropoiesis

Molecular, Cellular, Preclinical, and Clinical Biology

2nd Revised and Extended Edition

Steven G. Elliott
MaryAnn Foote
Graham Molineux
Editors



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Milestones in Drug Therapy
MDT

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Contents

Erythropoietin receptor

Clinical utility

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Preface to the first edition

Research on, and interest in, red blood cell formation spans several centuries and was thought to have peaked in the 1980s with the cloning of the erythropoietin (EPO) gene. In the years subsequent to the cloning of EPO and its expression as a recombinant protein, much was written about EPO. Although much has been learned and published, new, exciting data are becoming available on almost a daily basis. *Erythropoietins and Erythropoiesis: Molecular, Cellular, Preclinical, and Clinical Biology* compiles both pertinent historical and very recent research on this molecule and its clinical utility.

The book is divided into two sections: Background and Basic Science and Clinical Uses of Recombinant Erythropoietins. To begin, Israels and Israels describe the biology of red cells, the hierarchy of erythropoietic progenitor cells, their development to mature cells, and the effects of endogenous EPO on their development. Foote summarizes the historical interest in, and search for, an erythropoietic factor. Once EPO was identified, cloned, and expressed, the path was set for the study of other aspects of EPO biology both within erythropoiesis and other cellular systems.

The structures of recombinant human erythropoietin (rHuEPO) and its receptor (EPOR) have been studied and modeled using X-ray crystallography and other techniques, and the chapter by Osslund provides three-dimensional structural information. Activation of EPOR by EPO is essential for the survival, proliferation, and differentiation of red blood cells. EPOR is also expressed in many organs, including the brain, heart, endothelium, and ovaries, and may have physiological roles in these organs. Although studies are underway to establish the role of EPOR signaling in various organs, it is becoming increasingly apparent that red cells are not the only targets of EPO. Dame provides further data on the effects of both endogenous EPO and rHuEPO on hematologic and non-hematologic tissues. EPO has long been known to have a direct effect on the formation of red blood cells, and more recent work suggests that it may have a myriad of diverse effects that may allow the use of rHuEPO in clinical settings of neurological, cardiac, neonatal care, and as well as in other settings. Heatherington presents detailed information on the pharmacokinetics of EPO and rHuEPO in various patient populations. The pharmacokinetic properties of rHuEPO are some of the major factors that determine dosing regimens and mode of administration, and this literature review offers extensive information. Molineux reviews the basic biology of EPO and rHuEPO. The effects of treatment with rHuEPO are wide-ranging, especially in patients with degenerating kidney performance, suggesting effects beyond mere replacement of the missing endogenous EPO.

This section ends with a description by Chuck et al. of the production techniques for rHuEPO, from establishment of a cell bank to purification of the final clinical product.

Several clinical settings are discussed in detail, including treatment of the anemias of nephrology (Macdougall), oncology (Glaspy), and chronic diseases (Means) and also use in surgery (Cushner). Glaspy discusses the design of clinical trials using rHuEPO and offers insight into some published clinical data with rHuEPO and darbepoetin alfa, an erythropoiesis-stimulating protein that persists in the circulation three times longer than rHuEPO. The anemias of chronic diseases are among the most common syndromes in clinical medicine, but they often are under-recognized and under-treated. Clinical studies have demonstrated the efficacy of rHuEPO in the management of anemias of chronic diseases and have established a role for iron therapy as an adjunct to rHuEPO in this syndrome. Blood loss is inherent to the surgical setting. Using the orthopedic surgery model, Cushner focuses on the use of rHuEPO not only to decrease allogeneic transfusions but also to maximize blood parameters, such as hematocrit and hemoglobin concentration, during the peri-operative period.

Because rHuEPO is so effective in stimulating production of red blood cells, it has the potential for abuse by athletes, particularly Olympic athletes in endurance sports, such as running, cycling, and cross-country skiing. Catlin et al. describe how laboratories test for the illegal use of rHuEPO and how athletes are monitored and charged.

In 2001 and 2002, numerous cases of pure red cell aplasia, a rare but potentially life-threatening condition, were suddenly reported to health authorities worldwide. These cases of pure red cell aplasia were first noted in patients with kidney disease who were receiving rHuEPO. Mayeux and Casadevall describe this adverse event and how they test patients for the presence of anti-EPO antibodies, and they provide a possible explanation for the occurrence of pure red cell aplasia.

Finally, Elliott discusses current approaches in construction of EPO analogs to stabilize or increase activity, chemical modification of rHuEPO, gene delivery, and development of slow-release formulations. One successful strategy discussed is glycoengineering of rHuEPO, which involves construction of glycosylation analogs with increased content of sialic acid-containing carbohydrate. One such glycoengineered molecule, darbepoetin alfa, has been approved for marketing in the United States, the European Union, Australia, and Canada. Clearly, more than 20 years after the initial identification, isolation, cloning, and expression of the gene for EPO, research continues.

This book contains much information on erythropoiesis and red blood cell production, its regulation, and areas of continued or possible research, and is a resource for new and veteran researchers. Since different perspectives allow readers to arrive at their own conclusions and serve to stimulate scientific thought, we have not removed areas of controversy or overlap among chapters. We hope that this book proves useful and we invite your comments.

We have tried to acquire the necessary permissions and authorizations before publication, and great care has been taken in the preparation of the chapters. Nevertheless, errors cannot always be avoided. The editors and publishers, therefore, cannot accept responsibility for any errors or omissions that have inadvertently occurred. The views and opinions expressed in the book are those of the participating individuals and do not necessarily reflect the views of the editors, the publisher, Amgen Inc., or any other manufacturer of pharmaceutical products named herein. The package insert should be consulted before administration of any pharmaceutical product.

Graham Molineux, PhD
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Thousand Oaks, California
February 2003

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Preface to the second edition

Cloning of the erythropoietin (EPO) gene was accomplished in the 1980s, allowing a breakthrough in the treatment of anemia. Now more than 2 decades later, important new understandings on erythropoiesis and EPO are revealed as a consequence of continued research in this area. The first edition of this book provided a review of the field in 2003. This new edition continues the examination of this branch of learning with new information, updates, and changes.

To begin, Torbett and Friedman provide an overview of both normal and abnormal erythropoiesis as a basis for understanding various disease states and the role and action of recombinant erythropoiesis-stimulating agents (ESA). Mole and Ratcliffe discuss the production of endogenous EPO by defining *cis*-acting regulatory sequences that control *EPO* gene expression. The biology of EPO is covered in the chapter by Molineux and Sinclair. Prchal and Gregg have provided a new chapter for this edition concerning genetic abnormalities in erythropoiesis, and Constantinescu has written a new chapter on the mechanism of EPO receptor activation. Another new chapter was provided by Doshi, Perez-Ruixo, Jang, and Chow that contains updated information on the pharmacokinetics and clearance of ESA.

In the clinical section of the book, the use and abuse of ESA is described. Catlin and Hatton provide an update on the abuse of ESA by athletes. Goodkin has written a new chapter on the use of ESA in the setting of renal disease. Arvedson and Sasu have contributed a new chapter that discusses the roles iron and regulation of iron metabolism in both normal erythropoiesis and disease states. Another new chapter is by Jelkmann, Depping, and Metzen on the non-hematopoietic effects of ESA.

As with the first edition, we have allowed different perspectives and some overlap of material to allow readers to arrive at their own conclusions and to stimulate scientific thought and discussion. The views and opinions expressed in the book are those of the participating individuals and do not necessarily reflect the views of the editors, the publisher, Amgen Inc., or any other manufacturer of pharmaceutical products named herein. We have tried to acquire the necessary permissions and authorizations before publication, and care has been exercised in the preparation of the chapters. Nevertheless, errors cannot always be avoided. The editors and publishers, therefore, cannot accept responsibility for any errors or omissions that have inadvertently occurred.

The book is a review of ESA which are widely used in the treatment of anemia. The views and information contained herein are not meant to be a guide in the use of these agents. Instead, the package insert should be consulted before administration of ESA or any other pharmaceutical products.

Steven G. Elliott, PhD
MaryAnn Foote, PhD
Graham Molineux, PhD

Thousand Oaks, California
November 2008

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Abbreviations

ADM	adrenalmedulin
AGM	aorta-gonad-mesonephros
AIDS	acquired immunodeficiency syndrome
Akt	protein kinase B
AMPK	AMP kinase
ANP	atrial naturetic peptide
ARNT	aryl hydrocarbon nuclear receptor translocator
ASGR	asialoglycoprotein receptor
ATM	ataxia telangiectasia mutant
2,3-BPG	2, 3 biphosphoglycerate
B-CLL	B-cell chronic lymphocytic leukemia
bHLH	basic-helix-loop-helix
BFU–E	burst-forming unit–erythroid
BPA	burst-promoting activities
BTG-1	B-cell translocation gene 1
CAD	*C*-terminal activation domain
CAIX	carbonic anhydrase-9
CAS	Court of Arbitration for Sport
CEPO	carbamoylated EPO
CFU–E	colony-forming unit–erythroid
CFU–GEMM	colony-forming unit–granulocyte, erythroid, macrophage, megakaryocte
CHMP	Committee for Medicinal Products for Human Use
CHO	Chinese hamster ovary
CHr	mean corpuscular hemoglobin
CKD	chronic kidney disease
CP	Chuvash polycythemia
CR1	complement receptor 1
CSF	cerebrospinal fluid
DFO	desferrioximine
DHFR	dihydrofolate reductase
DOPPS	Dialysis Outcomes and Practice Pattern Study
DMT1	divalent metal transporter 1
DcytB	duodenal cytochrome b
eEPO	endogenous EPO
EMEA	European Medicines Evaluation Agency
EMP	EPO mimetic peptide
eNOS	endothelial nitric-oxide synthase

EPAR	European Public Assessment Report
EPO	erythropoietin
EPOR	erythropoietin receptor
ERK	extracellular signal-regulated kinase
EryP-CFC	primitive erythroid colony-forming cell
ESA	erythropoiesis-stimulating agent
ET	essential thrombocythemia
EU	European Union
Fab	fragment of antibody
FGF	fibroblast growth factor
FIH-1	factor-inhibiting HIF-1
FIS	International Ski Federation
FOB	follow-on biologics
FRET	fluorescence resonance energy transfer
Gas6 protein	growth arrest-specific 6 protein
GC-MS	gas chromatography-mass spectrometry
G-CSF	granulocyte colony-stimulating factor
GFR	glomerular filtration rate
GLUT	glucose transporter
GMP	Good Manufacturing Processes
HCP	hematopoietic cell phosphatase
HCP-1	heme carrier protein-1
HIF	hypoxia inducible factor
HNF	hepatic nuclear factor
HO-1	hemoxygenase-1
HRE	hypoxia-responsive element
HUVEC	human umbilical vein endothelial cell
IEF	isoelectric focusing
IFN	interferon
IL	interleukin
INN	International Nonproperity Name
IOC	International Olympic Committee
iPAS	inhibitory PAS
IRS	insulin receptor substrate
IU	international unit
JAK2	Janus-type tyrosine kinase
JAK-STAT	Janus kinase/signal transducers and activators of transcription
JH	JAK homology
kDa	kilodalton
KDOQI	Kidney Disease Outcomes Quality Initiative
LC-MS-MS	liquid chromatography coupled to tandem mass spectrometry
LDHA	lactate dehydrogenase A
LPS	lipopolysacharride
MALDI	matrix-assisted laser desorption/ionisation
MAPK	Ras/mitogen-activated kinase

MCH	mean corpuscular hemoglobin
MCVr	reticulocyte mean corpuscular volume
MCV	mean corpuscular volume
MDS	myelodysplastic syndromes
MEC	minimum effective concentration
miRNA	microRNA
mTORC	mammalian target of rapamycin complex
NADO	National Anti-Doping Organization
NDRG	N-myc downstream regulated
NHCT	Normal Hematocrit Cardiac Trial
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NO	nitrous oxide
ODDD	oxygen-dependent degradation domains
PAI	plasminogen activator inhibitor
PDGF	platelet-derived growth factor
PDK	pyruvate dehydrogenase kinase
PFCP	primary familial and congenital polycythemia
PH	pleckstrin homology
PHD1, 2, or 3	prolyl hydroxylase domain proteins 1, 2, or 3
PI3K	phosphoinositide-3 kinase
PIAS	protein inhibitor of activated stat
PKC	protein kinase C
PRCA	pure red cell aplasia
PTEC	proximal tubular epithelial cells
PV	polycythemia vera
pVHL	von Hippel-Lindau protein
rHuEPO	recombinant human erythropoietin
rHuGH	recombinant human growth hormone
RIA	radioimmune precipitation assay
ROS	reactive oxygen species
RXR	retinoic acid receptor
SAPK	c-Jun *N*-terminal kinase
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulfate-polyacrilamide gel electrophoresis
SH2	SRC homology 2
SHIP	phosphatidylinositol 3,4,5 triphosphate 5-phosphatase
siRNA	small interfering RNA
SNP	single nucleotide polymorphisms
SOCS	suppressors of cytokine signaling
STAT	signal transducer and activator of transcription
TAD	transactivating domains
sTfR	soluble transferring receptor
TGF	transforming growth factor
TIMP	tissue inhibitors of matrix metalloproteinase

TMDD	target-mediated drug disposition
TNF	tumor necrosis factor
TOF	time-of-flight
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRALI	transfusion-related acute lung injury
TRIM	transfusion-related immune modulation
Tsat	transferrin saturation
UCI	International Cycling Union
USADA	United States Anti-Doping Agency
VEGF	vascular epithelial growth factor
VO_2 peak	peak oxygen

Background

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
Edited by S.G. Elliott, M.A. Foote, and G. Molineux

Erythropoiesis: an overview

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Normal erythropoiesis

Red blood cell production in vertebrates is dynamic and tightly regulated with losses due to senescence or bleeding in normal individuals being balanced by generation of new red cells. The main function of the red cells is to transport oxygen from the lungs to tissues in all parts of the body and to transport carbon dioxide back for exchange. Healthy adult humans maintain a total of $2–3 \times 10^{13}$ erythrocytes at any given time, with men having 5–6 million and woman having 4–5 million erythrocytes/microliter blood [1]. The normal life span of an erythrocyte is approximately 120 days in the blood requiring a daily replacement of about 1% of the circulating erythrocytes [1]. To put the numbers of red cells needed daily into perspective, in an adult human maintenance of steady-state red cell numbers requires the generation of 2×10^6 erythrocytes every second. Moreover, basal oxygen consumption is 4 mL/kg/min and body stores are approximately five times greater, emphasizing the need to maintain a stable red cell mass while having the capacity to increase red cells during times of tissue hypoxia [1, 2]. Changes in the production of erythrocytes from a steady-level will occur in response to blood loss, increased oxygen demand, altered erythrocyte lifespan, or diseases.

Erythropoiesis is a regulated process whereby hematopoietic progenitor cells give rise to committed erythroid progenitors, proliferate, and differentiate to mature red cells to maintain steady state red cell mass (Fig. 1). Erythropoiesis occurs in three defined stages: generation of erythroid committed blast cells from multipotent hematopoietic progenitors; division and differentiation of these morphologically identifiable erythroid progenitor cells; and terminal cellular morphologic changes, including enucleation, to produce reticulocytes and ultimately mature red cells [3, 4]. In adult humans, erythroblasts are produced from committed progenitors arising from hematopoietic stem cells present in the bone marrow. As the progeny of a stem cell progress through development, there is a loss of multipotency and increasing lineage restriction [3–5]. The various cellular stages in erythrocyte development are identified by their ability to form colonies in semisolid medium supplemented with specific cytokines and by cell surface markers (Fig. 1). The response to

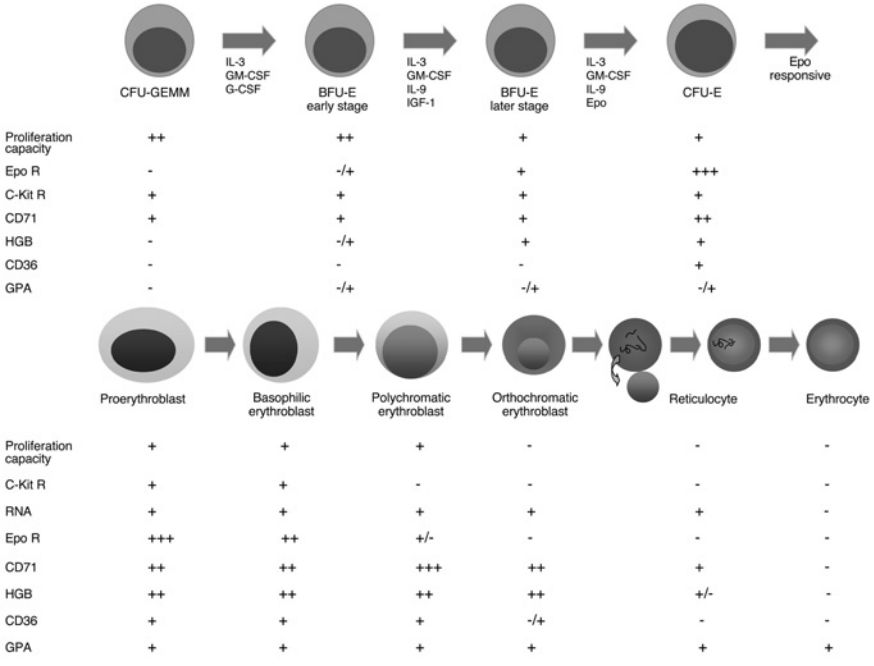

Figure 1. Lineage specific stages of erythropoiesis. Stages of erythrocyte differentiation are shown with defining cellular characteristics based on relative proliferation, cytokine responsiveness, cell surface markers, and/or presence of RNA. CFU-GEMM = colony-forming unit–granulocyte, erythroid, macrophage, megakaryocte; BFU–E = burst-forming unit–erythroid; EPOR = erythropoietin receptor; CFU–E = colony-forming unit–erythroid; C-Kit R = stem cell factor receptor, CD = CD117; CD71 = transferrin receptor; HGB = hemoglobin synthesis; CD36 = gpIIIb, thrombospondin receptor; GPA = glycophorin A present on human cells only.

selected cytokine presumably correlates to the normal environmental conditions controlling growth and differentiation of selected lineages: at early stages of development erythroid progenitors respond to multilineage cytokines; as differentiation progresses, cytokine responses become more limited and specific to the erythroid progenitors [4, 6, 7].

Hematopoietic stem cells give rise to a common myeloid progenitor that in turn, gives rise to bipotential progenitors restricted to either the granulocyte/macrophage or the erythroid/megakaryocte pathways [8–11]. A comparable stage of development defined *in vitro* using cytokine containing semisolid medium is the colony-forming unit – granulocyte, erythroid, macrophage, megakaryocte (CFU-GEMM) precursor that gives rise to bipotential progenitors restricted to either granulocyte/macrophage or erythroid/megakaryocte pathways [8–11]. Progenitors committed to the erythroid/megakaryocte pathway express erythropoietin receptor (EPOR) and are responsive to erythropoietin (EPO), whereas cells committed to the myeloid pathway do not express EPOR. The most immature erythroid-restricted progenitor is the burst-forming

unit–erythroid (BFU–E) which gives rise to colony-forming unit–erythroid (CFU–E) [12, 13]. Early BFU–E are blast-like cells, highly proliferative, and give rise to clustered burst colonies of up to 20,000 cells (Fig. 1) [12, 13]. BFU–E express low amounts of EPOR but daughter cells derived from them express high amounts of EPOR, become EPO responsive, transferrin receptor positive, and begin to express hemoglobin [1, 12]. CFU–E are highly responsive to EPO, generate smaller colonies, and express many of the gene products required for definitive erythroid development (Fig. 1). EPO has been found to stimulate division and prevent apoptosis of late-stage erythroid cells generated from CFU–E [6, 7, 12]. Moreover, in tissue culture systems at this stage of development cells begin hemoglobin synthesis and acquire cytoskeletal proteins and cellular adhesion molecules, steps that help define them as nucleated erythroblasts [4, 6, 7].

The next steps of erythroid differentiation involve morphologically identifiable nucleated precursors that progress from the proerythroblast to basophilic, polychromatophilic, and orthochromatic cell forms leading to the reticulocyte (Fig. 1) [1, 13]. During these distinct stages of erythroid differentiation, four major cellular processes ensue: the accumulation of hemoglobin that participates in driving the basophilic to acidophilic cytoplasmic changes seen during maturation, limited erythroblast expansion, a continued decrease in cell size, and nuclear condensation and finally enucleation [1, 14]. Erythroblasts mature in a microenvironmental niche in bone marrow termed ‘erythroblast islands’ where they are closely associated with macrophages that serve as stromal or nurse cells [15–17].

In the final stages of erythroid development, reticulocytes exit the marrow and enter the circulation where they mature into erythrocytes. This stage of development is characterized by disassembly of ribosomes, golgi bodies, and other cellular machinery, removal of organelles, enucleation, changes in the cytoskeleton leading to the classic biconcave discoid shape, and then release into the circulation [1]. The last steps of differentiation again take place in the erythroblast islands, where macrophages participate in terminal maturation of erythrocytes [15–17]. The mature erythrocyte persists in the circulation for up to 120 days until ‘senescent’, when changes in surface antigen expression or physical characteristics of the cell trigger removal from the blood by macrophages of the reticuloendothelial system [18].

During mammalian development from embryo to adult, the body compartment responsible for hematopoiesis changes, thus providing precise regulation in specialized niches depending on developmental age [3, 4, 19–22]. In the mouse, primitive red cells arise in the yolk sac blood islands very soon after gastrulation [23, 24]. Erythroblasts originate from unique, developmental stage-specific progenitors, termed primitive erythroid colony-forming cells (EryP-CFC), that are distinguishable by their growth, maturation properties, and globin gene expression patterns [25, 26]. This cell population is short lived during development and provides red cells until hematopoiesis is established in the fetal liver by hematopoietic stem cells that give rise to definitive erythropoiesis

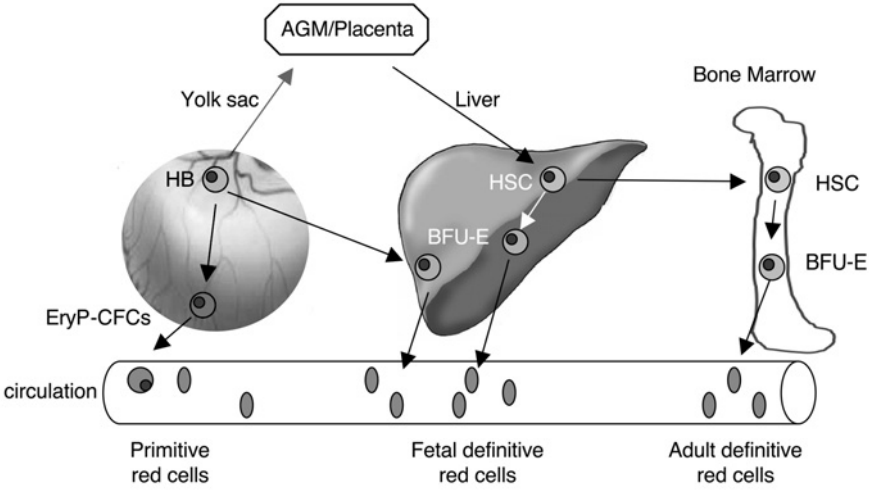

Figure 2. Model of primitive and definitive erythroid development in the mammalian embryo. The current findings in the field support a model whereby three distinct phases of erythroid progenitors emerge in the mammalian embryo. The first phase consists of primitive, nucleated erythrocytes, derived from erythrocyte precursor colony-forming cells (EryP-CFC), generated from yolk sac hemangioblasts (HB). These cells mature in the circulation to become enucleated red blood cells. The second phase is the seeding of the fetal liver by yolk sac-derived, definitive BFU–E that gives rise to red cells through the developmental pathway presented in Figure 1. In the last phase aorta-gonad-mesonephros (AGM) and placental derived HSC seed the fetal liver, which in turn, give rise to the HSC-erythroid developmental program as described in Figure 1. It should be noted that HSC that seed the fetal liver may originate in the yolk sac, migrate to the AGM where they mature into HSC, then seed the fetal liver. Determining the origin of HSC that give rise to the definitive hematopoietic system is an area of active investigation. At the end of gestation, long-term repopulating HSC from the fetal liver seed the bone marrow that then becomes the site of definitive erythropoiesis for fetal and adult red cell production.

(Fig. 2) [27–29]. Since the first primitive erythroid cells arise with endothelial cells during primitive erythropoiesis in the embryonic yolk sac, a common progenitor, the hemangioblast, has long been proposed to be the precursor. Only recently has this concept been verified experimentally in the mouse [30].

Adult hematopoiesis is characterized by the continuous generation of mature blood cells from the long-term repopulating hematopoietic stem cells. In the mouse, the developmental origin of long-term repopulating hematopoietic stem cells is associated with the appearance of clusters of cells arising from the dorsal aorta in the aorta-gonad-mesonephros (AGM) region [31, 32]. It appears that the placenta also functions as a site of hematopoietic stem cell expansion and perhaps origin [21, 33]. Within a short time after appearance of hematopoietic stem cells in the AGM/placenta, they are found in the fetal liver [32, 34, 35]. Two reports using mice bring into question the origin of the stem cells that seed the fetal liver establishing definitive hematopoiesis, and ultimately erythropoiesis [36, 37]. Both studies reply on different gene disrupted mice that severely limit dissemination of hematopoietic cells from the yolk sac

to AGM/placenta and other hematopoietic sites, thus providing a developmental window to observe tissue seeding of hematopoietic stem cells before the embryo dies [36, 37]. Given the caveats of the experimental design as to whether hematopoietic cells can develop in or seed a dying embryo, the studies might suggest that the stem cells originate in the yolk sac and then must migrate to the AGM to undergo maturation before seeding the fetal liver and other tissue spaces. Lastly, these studies do not formally exclude the development of hematopoietic stem cells in the AGM and placenta.

Interestingly, definitive erythroid potential has been reported to be present in the embryo before long-term engrafting stem cells are present in the fetal liver [25, 26]. It has been reported that in mice, BFU–E arise before circulation, and after circulation formation are found in increasing numbers in the liver [25, 26]. These findings might suggest that the yolk sac produces an additional wave of immature erythroid progenitors that enter the circulation and take up residence in fetal liver [25, 26]. A similar developmental pattern has been described for the developing human embryo [20]. The findings in the mouse and human embryo would support the premise that yolk sac-derived BFU–E migrate to the fetal liver where they complete development to produce the first definitive erythroid cells in the embryo (Fig. 2) [25, 26]. During the end of gestation, hematopoiesis and definitive erythropoiesis begin to take up residence in the bone marrow [4, 5]. The current findings in the field support the model of erythroid ontogeny as depicted in Figure 2. These exciting findings underscore that there is still much to understand about hematopoietic development, which remains an area of active investigation. The reader is referred to a number of excellent classical and current reviews of the field to provide more information on the ontogeny of hematopoiesis and erythropoiesis than can be provided herein [4, 5, 21, 22, 38].

Erythropoietin

EPO, an approximately 30–34 kDa glycoprotein hormone, is the primary hematopoietic growth factor governing the rate of red cell production. The action of EPO depends upon the presence of an adequate number of responsive progenitor cells, availability of sufficient iron and cofactors (folate, B12) to support heme and globin synthesis, and an appropriate microenvironment to support erythroid development. The rate of red cell production is dynamically responsive to demand in order to replace cells lost through bleeding, destruction, and senescence. EPO concentrations are inversely related to hemoglobin concentration, ranging from approximately 10 mU/mL in nonanemic conditions up to 10,000 mU/mL in severe anemia [39, 40]. In response to EPO stimulation after blood loss, marrow production of red cells can increase 3- to 5-fold in the presence of an adequate iron supply [41]; in the case of a more severe hemolytic insult, production may increase even further (7- to 8-fold) due to more efficient recycling of iron from lysed red cells [42].

Interestingly, while EPO values are closely tied to the rate of red cell production, studies in knockout mice lacking EPO show that it is not required for development of BFU–E, the earliest committed red cell progenitors (that express only a small number of EPOR on their cell surface) [43] (Fig. 1). As such, EPO does not recruit cells to the erythroid lineage nor does it specify cell fate. Rather, EPO exerts its dynamic control over red cell formation by supporting the growth, survival, and differentiation of the progeny of erythroid-committed progenitor cells [6, 44], with the highest amounts of EPOR being found on late progenitor cells such as CFU–E [45].

Production

In adult mammals, the major physiologic site of EPO production is the kidney, although *EPO* gene transcription can be detected in liver [46], and at lower amounts in many other tissues, including brain. Within the kidney, *in situ* hybridization for EPO mRNA localizes production to peritubular interstitial fibroblasts [47, 48]. While expression in the adult liver echoes the role of this organ as the major source of EPO during embryogenesis, expression in the brain and other tissues suggest nonhematopoietic functions for this hormone/growth factor, including possible autocrine or paracrine effects in cells and tissues that express both EPO and EPOR.

The mechanism of regulation of EPO expression at the molecular level involves an ingenious coupling between oxygen sensors (prolyl hydroxylase domain proteins 1, 2, or 3 [PHD1, 2, or 3] [49] and/or the asparagine hydroxylase, factor-inhibiting HIF [FIH-1] [50]) and the transcription factor primarily responsible for *EPO* gene activity, hypoxia inducible factor [HIF]. HIF are heterodimeric transcription factors composed of a constitutively expressed β subunit and a labile α subunit [51, 52]. The O_2-dependent enzymatic activity of PDH2 is directed toward proline residues 402 and 564 of HIF-1α. Under normoxic conditions, PDH2 constitutively hydroxylates HIF-1α promoting the binding of the von Hippel-Lindau protein (pVHL), leading to the ubiquitination and degradation of HIF-1α [53] (Fig. 3). In a parallel regulatory pathway, the O_2-dependent enzymatic activity of FIH is directed toward asparagine residue 803 of HIF-1α, blocking interaction with the transcriptional coactivators p300 and CBP [54]. Thus, under normoxic conditions there are two tonic inhibitory influences on HIF-1 activity – one affecting the transcriptional activity of HIF-1α by FIH mediated hydroxylation of asparagine 803, and one affecting protein stability of HIF-1α through PDH2-mediated hydroxylation of prolines 402 and/or 564. A specific mutation in pVHL (R200W) is causal for familial erythrocytosis type 2 (also known as Chuvash polycythemia) [55, 56], and a number of additional mutations in *VHL* resulting in erythrocytosis have been described [57].

When tissue oxygenation decreases, these tonic inhibitory influences are relieved – with stabilization of transcription competent HIF-1α protein, dimer-

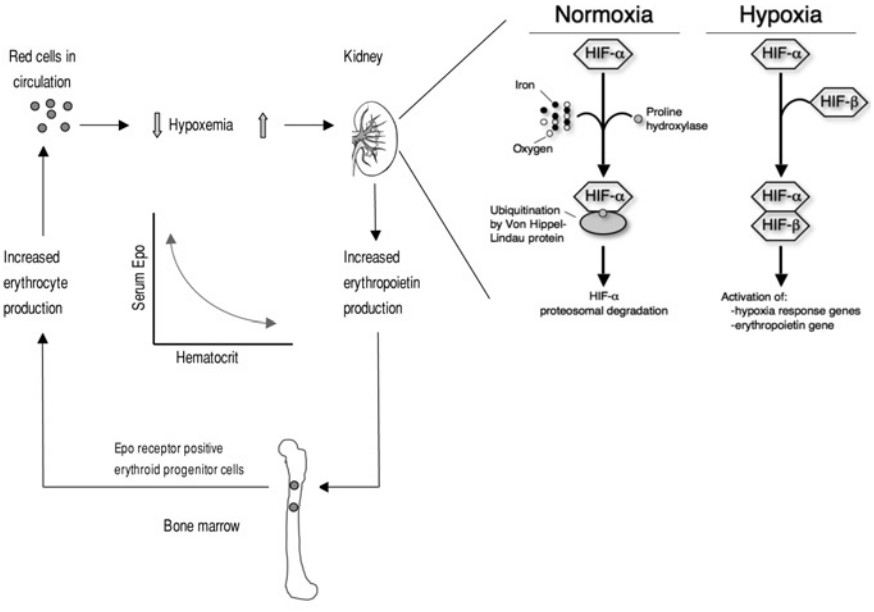

Figure 3. Regulation of erythropoietin production during normoxia and hypoxia. In adults, hypoxia resulting from decreased oxygen transport by red cells is primarily sensed in the kidneys, and to a low level in the liver, leading to an increase in EPO production. Renal produced EPO stimulates proliferation of erythroid committed precursor cells in the bone marrow resulting in the production of increased numbers of mature red cells capable of carrying oxygen and correcting tissue hypoxia. The inset graph depicts the relationship between serum EPO concentrations and the hematocrit as an indicator of hypoxia. The inset diagram to the right of the kidney depicts the roles of HIF in regulating activation of EPO and hypoxia response genes. In hypoxic situations the α-subunit is stabilized, and forms a transcriptionally competent heterodimer with the constitutively expressed HIF-1α subunit. HIF then interacts with additional nuclear factors to activate expression of erythropoietin and hypoxia response genes. The α-subunit is rate limiting in the transcription complex, and its destruction by proteosomal degradation is controlled by the amount of cellular oxygen. During normoxia, hydroxylation of two specific proline residues in HIF-1α occurs by three cellular prolyl hydoxylases in an iron, oxygen, and 2-oxglutarate-dependent fashion. This posttranslational modification, in combination with acetylation of a specific lysine residue, permits binding of the pVHL to HIF-1α promoting ubiquitination and degradation. In addition, oxygen-dependent hydroxylation of an arginine in HIF-1α blocks binding of transcriptional co-factors required for gene transcription. While both HIF-1α and HIF-2α can regulate erythropoietin gene expression, recent evidence suggests that HIF-2α is the physiologic regulator in adult mammals.

ization with HIF-1β, and transcriptional activation of responsive promoters. Two hypotheses have been put forward to explain the loss of PDH2 enzymatic activity with hypoxia: one suggests substrate limitation (O_2 and/or α-ketoglutarate) [49], the other suggests oxidation of Fe (II) in the catalytic center of PDH2 by mitochondrial-derived reactive oxygen species [58–60].

HIF affects transcription of hundreds of genes, coordinating adaptive tissue responses to hypoxia, directing cellular metabolic shifts to favor glycolysis, stimulating growth of new vessels (through induction of growth factors such as vascular endothelial growth factor [VEGF]), and regulating mitochondrial autophagy [61] in addition to increasing oxygen-carrying capacity through

induction of erythropoietin production in the kidney. Two additional HIF-1α homologues exist in mammals – HIF-2α and HIF-3α, as well as two additional prolyl-hydroxylase domain proteins, PDH1 and PDH3 [49, 62]. While there is evidence of overlapping function and crosstalk between these proteins, PDH2 is the primary regulator of HIF-1α stability [63]. The importance of PDH2 as a regulator of HIF and subsequent erythropoiesis in a clinical setting has been demonstrated by the discovery of mutations (Pro317Arg and Arg371His) in PDH2 in families with hereditary erythrocytosis (now designated familial erythrocytosis type 3) [64, 65]. The functional activity and regulation of HIF-2α and HIF-3α are being investigated. Two studies demonstrate that HIF-2α is an important regulator of EPO production by the liver [66], and that postnatal ablation of HIF-2α results in anemia, suggesting that HIF-2α is the physiologically important regulator of EPO production in adult mammals [67].

Receptor

Erythroid-lineage precursor cells express EPOR as a homodimeric transmembrane protein with a ligand-binding extracellular domain, and a cytoplasmic domain constitutively associated with the Janus-type tyrosine kinase JAK2 [68]. Upon ligand binding, EPOR undergoes a conformational change, triggering the activation and transphosphorylation of JAK2, which then phosphorylates multiple tyrosine residues in the EPOR cytoplasmic tail [69]. These p-Y residues serve as docking sites for assembly, activation, and propagation of mitogenic and prosurvival signals mediated by multiple effector pathways including STAT5, PI3-kinase/AKT, and Ras/MAPK [70]. Knockout of JAK2 in mice produces a phenotype similar to that observed for knockout of EPO [71] or EPOR [43], namely embryonic lethality at approximately E12.5 with BFU–E and some CFU–E present, but unable to undergo maturation [72, 73]. Thus, EPO, EPOR, and JAK2 form a linear pathway essential for erythroid expansion and maturation, but appear to have no role in lineage specification.

EPOR signaling is negatively regulated through interaction with the SH2-domain containing protein tyrosine phosphatases, primarily SHP1, which binds to p-Y429 of the Epo R, and can downregulate the activity of JAK2 [74]. The significance of this interaction is suggested both by cell culture experiments showing EPO hyper-responsiveness when Y429 is mutated, and by clinical studies of patients with familial erythrocytosis type 1, in which truncation mutations (lacking Y429) of the c-terminal portion of the receptor have been described [75–77]. Acquired mutations in the JAK2 kinase have been discovered to play a causal role in myeloproliferative disorders, most prominently in the chronic red cell disorder polycythemia vera [78–81]. The most common mutation, JAK2 Val617Phe, occurs in the autoinhibitory domain of JAK2 and results in a constitutively active kinase that confers cytokine hypersensitivity and cytokine-independent growth to hematopoietic cells [82]. Additional negative regulatory circuits exist, most notably interaction between EPOR and

CIS1, a member of the SOCS family (suppressors of cytokine signaling). Other SOCS family members (SOCS-1, SOCS-3) may inhibit EPO stimulation through direct interactions with the JAK-2 kinase [70]. Novel strategies involving analysis of transcriptional changes in cells with normal or mutated EPOR exposed to cytokine stimulation have identified additional proteins/genes involved in mediating and regulating EPO responses [83].

Abnormal erythropoiesis

While the spectrum of abnormalities of EPO are legion, Figure 4 presents a brief sampling of disorders with increased (polycythemic) and decreased (anemic) red cell production, and describes the relative endogenous EPO concentrations observed. Polycythemia (also known as erythrocytosis) can be divided into those that are primary or secondary. In primary erythrocytosis, progenitor cells harbor genetic defects conferring either hypersensitivity to EPO or cell autonomous growth, e.g., truncation of EPOR or JAK2 mutations. Secondary erythrocytosis results largely from a normal physiologic response to tissue hypoxemia, and is seen in conditions where oxygen delivery to tissue is impaired (e.g., high altitude, cyanotic heart disease, and pulmonary disease). However, two types of familial erythrocytosis (mutations affecting pVHL or PDH2) have been described in which HIF degradation is reduced, resulting in abnormal oxygen-dependent regulation of EPO production. Finally, secondary erythrocytosis can occur as a result of 'recreational use' of EPO (usually in the context of a particular yearly cycling event in France) or as a result of blood doping, i.e., the collection and subsequent transfusion of one's own red cells (*Ibid*, cycling event in France). In such cases, endogenous EPO concentrations will be appropriately low.

Anemias can be divided into those with reduced red cell production (proliferative defects), those with red cell maturation defects (ineffective erythropoiesis), and those with accelerated loss or destruction of red cells. EPO deficiency is the cause of anemia in the context of significant renal disease, i.e., due to loss or dysfunction of cells responsible for the bulk of EPO synthesis. However, in other hypoproliferative anemias, such as anemia of chronic disease [84], anemia associated with malignancy [2], and anemia of aging, EPO values may be within or above the normal range yet fail to adequately stimulate red cell production. One of the limitations on EPO bioactivity in the setting of chronic disease is the availability of iron, which is regulated by the 25-amino acid peptide hepcidin, which in turn controls degradation of the cellular iron export channel protein, ferroportin [85, 86]. Hepcidin production by the liver is responsive to proinflammatory cytokines, particularly interleukin (IL)-6, and increased hepcidin concentrations in the context of infection or inflammation restrict iron availability, suppressing erythropoiesis [87]. The role played by hepcidin in the regulation of iron availability in normal and disordered erythropoiesis is an area of active investigation.

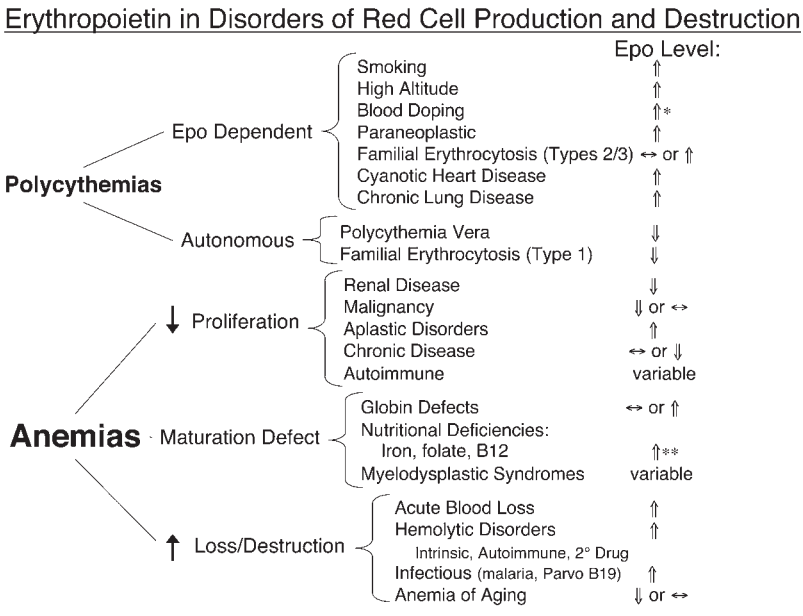

Figure 4. Erythropoietin in selected polycythemias and anemias. The increased red cell mass seen in polycythemia can be EPO dependent or cell autonomous at the level of erythroid progenitors. In many instances, increased EPO production is a normal response to tissue hypoxemia, such as that seen in individuals living at high altitude or those with cyanosis due to cardiac or pulmonary disease. Increased concentrations of EPO can occur in cancer (paraneoplastic) when produced by tumor cells, most notably in renal cell carcinoma. Mutations in pVHL or PDH2 (see text) affect regulation of EPO production, responsible for two types of familial erythrocytosis. Mutations affecting EPO signal transduction (in EPOR or the JAK2 kinase) result in erythrocytosis in the absence of increased amounts of EPO. EPO concentrations in anemic conditions can be quite variable. Robust increases in EPO concentrations are expected in normal individuals after blood cell loss or destruction. In the context of chronic disease, malignancy, and autoimmune disorders, EPO may be inappropriately low for the degree of anemia observed. In addition, the response of developing erythroid cells to EPO in these conditions may be blunted. Reduced EPO production may result from the effect of inflammatory cytokines on transcription of the *EPO* gene [106], while reduced EPO responsiveness may be a consequence of cytotoxic effects of the same cytokines on progenitor cells, as well as functional iron deficiency secondary to increased amounts of hepcidin. Increased EPO concentrations in hypoproliferative disorders may indicate marrow failure. * Blood doping, here defined as any method to boost the O_2-carrying capacity of blood. If done by transfusion, EPO concentration will be decreased; if stimulated by EPO, rHuEPO will be increased, with endogenous EPO suppressed. ** The relationship between iron regulation and regulation of EPO production is complex, with iron chelation activating HIF [107], possibly through effects on iron-dependent activity of PDH2. HIF in turn regulates hepcidin and ferroportin production, in addition to EPO [108].

A bright horizon or dark clouds?

In many nonclinical models, EPO has been shown to have a protective effect against ischemic or cytotoxic insults. Although the mechanism(s) of this effect remains unknown, it has been suggested that signaling through EPOR blocks apoptotic cell death [88, 89] in a fashion similar to that observed in erythroid progenitor cells [90, 91]. EPO signaling resulting in tissue protection appears

to differ from the canonical model presented above, as the functional receptor in (many) nonerythroid cells is a heterodimer of EPOR and the common beta cytokine receptor subunit [92]. That the observed cytoprotective effects of EPO administration can be distinguished from effects on red cell production, has been shown through use of EPO derivatives (such as carbamylated or desialylated EPO) that remain cytoprotective, but do not stimulate red cell production [93–96]. Additional evidence for nonerythroid roles for EPO/EPOR comes from the study of knockout mice, demonstrating a role for EPO/EPOR signaling in brain [97] and cardiac development [98]. Studies with conditional knockout of EPOR or with 'erythroid rescued' EPOR-knockout animals have demonstrated that an intact EPO/EPOR pathway mediates neuroprotection and cardioprotection in response to a variety of insults [99]. These nonclinical studies represent the bright horizon – and provide the basis for a series of ongoing clinical studies exploring the potential of EPO and derivatives as tissue protective agents in a variety of neurologic and cardiovascular disorders, and in a critical care setting.

Despite the bright outlook, there are clouds on the horizon in the form of recent clinical studies that highlight known or potential side effects of EPO therapy, namely an increased risk of thrombosis in many patient groups, and a question of tumor promotion when recombinant human EPO (rHuEPO) is given to patients with certain types of cancer. Two published meta-analyses, one involving patients with kidney disease [100] and the other patients with cancer [101] highlight the risk of increased thrombosis in patients treated with rHuEPO, and emphasize that risk increases with increasing target hematocrit, concomitant therapy with other known thrombogenic agents, or the presence of an underlying prothrombotic condition. Perhaps even more concerning are reports from placebo-controlled studies in patients with head-and-neck cancer [102], breast cancer [103], lymphoid malignancies, and nonsmall cell lung cancer [104] linking rHuEPO therapy with decreased survival, disease progression, or both. These published results, as well as reports of safety concerns from additional studies, have led to updated recommendations on the clinical use of rHuEPO [105]. Equally important, these findings point to the need for a more thorough investigation of the relationship between the cytoprotective effects of EPO and the possibility of enhanced tumor progression.

During the past 5 years, developmental biologists have begun unraveling the intricacies of erythropoiesis, as evidenced by the many reports on ontogeny ranging from defining the roles of the hemangioblast in early development to specific requirements for yolk sac and AGM in primitive *versus* definitive erythropoiesis. These ongoing studies are providing new insights into the precise regulation of stage-specific erythroid differentiation, and may reveal additional pathways for manipulation of red cell production without the side effects of thrombosis or tumor promotion. Insights into the cellular and molecular consequences of EPO signaling in nonhematopoietic cells should provide information as to the extent and risk of EPO protective effects for selected neurologic and cardiovascular disorders.

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We dedicate this chapter to Dr. Ernest Beutler, an outstanding hematologist/scientist and mentor. While Dr. Beutler had broad scientific interests, he was particularly fond of red cells and was a pioneer in elucidating the biochemical and genetic basis for a number of red cell metabolic disorders. Through his enthusiasm and insights, we have also come to appreciate the elegance, simplicity and medical import of the erythropoietic system.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Regulation of endogenous erythropoietin production

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Hypoxic regulation of erythropoietin

The principal function of the red cell is to convey oxygen from the lungs to the tissues through the oxygen transport molecule, haemoglobin, a concept finally established in the 19th Century after major advances in biology and chemistry over the preceding two centuries. The description of the blood circulation by William Harvey (1578–1657) in *De Motu Cordis et Sanguinis in Animalibus* in 1628 framed the question as to the purpose of moving such large volumes of fluid around the body. Richard Lower (1631–1691) working in Oxford with Robert Hooke (1635–1702) noted that whereas the blood leaving the heart for the lungs was blue, that returning from the lungs to the heart was red, concluding that 'Nitrous spirit of the air, vital to life is mixed with the blood during transit through the lungs'. After the first consistent measurements of oxygen in blood, by Gustav Magnus, the role of the blood circulation in delivering oxygen to the tissues was confirmed by showing that there was more oxygen in arterial than venous blood [1]. Finally, a specific role for erythrocytes in oxygen transport was established by the demonstration of reversible binding of oxygen to the pigmented haemoglobin that accounted for the colour change [2, 3].

The idea of hormonal regulation of erythropoiesis, the production of new erythrocytes, was first formulated by Carnot and Deflandre in 1906 [4] only a few years after the concept of hormones was first suggested by Henri Brown-Sequard in 1889. However, Allan Erslev (1919–2003) is generally credited with providing definitive proof of the existence of erythropoietin in 1953, by transfusing large quantities of plasma from anaemic rabbits into normal animals, which responded by generating a consistent reticulocytosis, followed by a major increase in hematocrit [5]. With remarkable foresight, he also postulated "Conceivably isolation and purification of this factor would provide an agent useful in the treatment of conditions associated with erythropoietic depression, such as chronic infection and chronic renal disease." Much of the early work on the effects of reduced oxygen levels (hypoxia) on erythropoiesis was undertaken at altitude, with the earliest description generally attributed to the Jesuit priest, Father Joseph De Acosta. Both Journadet and Bert, considered the pioneer of altitude medicine, described an association between alti-

tude and polycythaemia. Induction of erythropoiesis was noted to be both rapid and sensitive to small changes in altitude [6]. Subsequently, after the recognition of erythropoietin (EPO) as the regulatory hormone, measurement of EPO concentrations revealed an extremely rapid response to ascent, with endogenous EPO peaking at 1–3 days after arrival at a new altitude and decreasing close to baseline by day 10 [7].

Renal oxygenation and erythropoietin

The kidneys are the main source of circulating EPO in adults, as initially suggested by the lack of an EPO response to haemorrhage in nephrectomised rabbits and rats [8], and subsequently confirmed through the demonstration of EPO activity in isolated serum-free perfused kidneys from hypoxic rabbits [9]. Further confirmation was by the isolation of EPO mRNA from the kidneys of hypoxic rodents [10]. More sensitive analyses of mRNA demonstrated that the liver, spleen, lung, bone marrow, and brain also express lesser amounts of EPO mRNA [11], although the contribution to EPO production and the biologic role of gene expression at these sites is unclear. Within the kidneys, the EPO-producing cells reside within the interstitium of the renal cortex, and co-localise with fibroblast markers, defining the interstitial fibroblast as the source of renal EPO [12, 13].

Given that the teleological purpose of EPO is to maintain the blood haemoglobin concentration in the normal range, and that the main function of haemoglobin is to carry and deliver oxygen from the lungs to the respiring tissues, it is fitting that the sensed parameter in the feedback loop is tissue oxygenation. Given that the kidney, however, has no direct role in erythropoiesis or oxygen transport, it is not immediately apparent why the kidney is so well suited to sensing oxygen.

A potential answer to this paradox lies in the unusual anatomy of the renal circulation, in which the filtering glomeruli and reabsorbing tubules are supplied in series by the same network of vessels. Given that most of the glomerular filtrate is reabsorbed, the renal oxygen demand for tubular reabsorption will vary in proportion with the glomerular filtration rate. Thus, reductions in renal perfusion are predicted to result in a parallel reduction in oxygen supply and oxygen demand (proportional to filtration/reabsorption) so that renal oxygen balance is little affected by the rate of blood flow and more accurately reflects changes in oxygen content of the blood (as determined by haemoglobin concentration). A second interesting feature of the renal vascular anatomy is the tendency for arteries and veins to run countercurrent. This set up allows direct ‘shunt’ diffusion of oxygen from artery to vein so that the tips of the countercurrent loops are particularly hypoxic, which is most marked in the renal medulla where the innermost regions are profoundly hypoxic even under basal physiologic conditions. Similar effects, however, are also observed in the renal cortex, where EPO is produced, with cortical oxygen tension decreasing with

increasing cortical depth. Interestingly, under basal conditions, EPO production is confined to a few cells near the corticomedullary junction, but as the haemoglobin concentration decreases in progressive anaemia, so the hypoxic regions of the kidney propagate outwards, and increasing numbers of peritubular fibroblasts are recruited to produce EPO in a 'march' from deep to superficial cortex (Fig. 1) [14]. The induction of EPO in this manner shows a strikingly high gain response, with even modest changes in haemoglobin leading to large changes in EPO over several orders of magnitude.

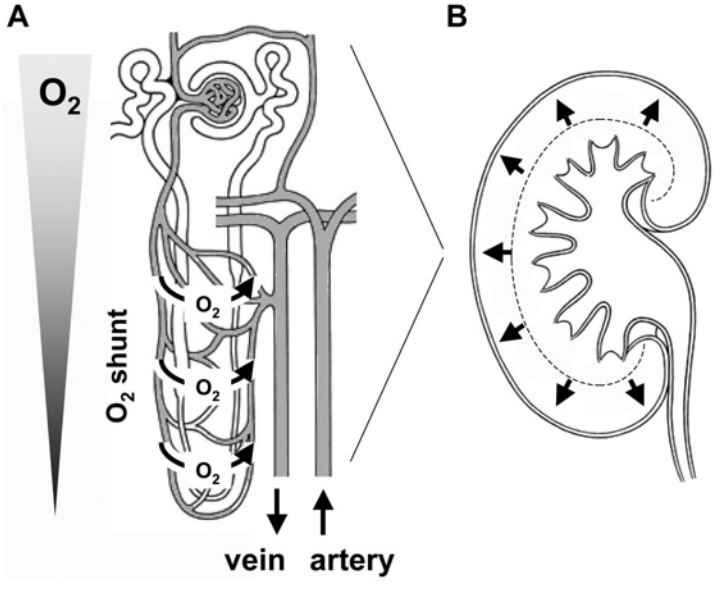

Figure 1. (A) The highly specialized microvascular anatomy of the kidney, illustrating the same network of vessels, in series, supplying both the filtering glomerulus and the reabsorbing tubule, of each nephron. Tubular reabsorption varies in proportion with glomerular filtration such that tubular oxygen requirements parallel blood flow, rendering renal oxygenation insensitive to changes in blood flow, while remaining sensitive to changes in oxygen content (and hence haemoglobin concentration) of the blood. Countercurrent tubular flow is paralleled by countercurrent blood flow, with consequent shunting of oxygen, generating an oxygen gradient within the kidney. (B) As the haemoglobin concentration decreases in progressive anaemia, so the hypoxic regions of the kidney propagate outwards, and increasing numbers of peritubular fibroblasts are recruited to produce EPO in a 'march' from deep to superficial cortex.

cis-acting regulatory elements of the erythropoietin gene

Identification of the *EPO* gene provided the opportunity to study the pathways controlling its expression by defining its *cis*-acting regulatory sequences (non-coding sequences at a gene locus that direct expression by interacting with transcription factors) (Fig. 2).

EPO contains five exons spanning a region of DNA of approximately 2.9 kb [15, 16]. Comparison of primate and mouse sequences showed strong homol-

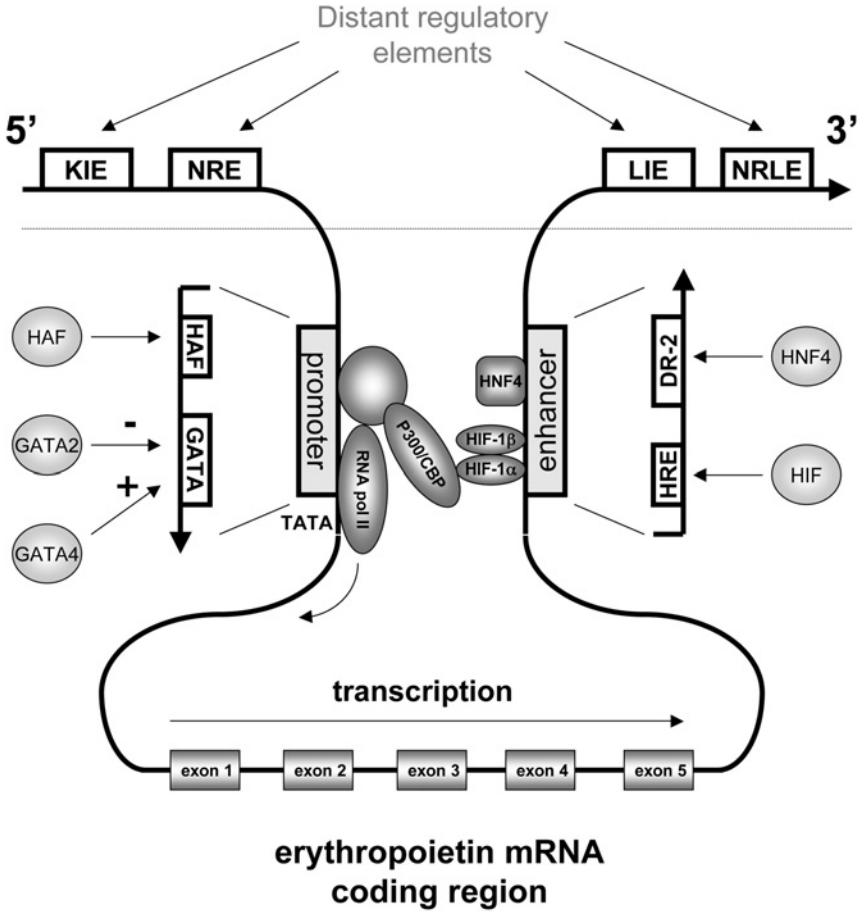

Figure 2. Regulatory elements of the *EPO* gene. This schematic representation of *EPO* shows the promoter region, the five exons and the 3' EPO enhancer. Additional *cis*-acting regulatory are required for tissue specific and developmental regulation. The kidney inducible element (KIE) confers expression in interstitial peritubular cells. The negative regulatory element (NRE) suppresses transcription. Two *cis*-acting regulatory elements 3' to the RPO-coding sequence, the liver-inducible element (LIE), and the negative regulatory liver element (NRLE) promote and enhance gene expression in the liver. At the promoter, 5' sequences contain a "GATA" motif that regulates opposing effects of diverse GATA factors. While the EPO promoter is a weak promoter, it does contribute to hypoxic regulation through a hypoxia-associated factor (HAF) binding site. However, the 3' enhancer containing a hypoxia-responsive element (HRE) that binds hypoxia-inducible factor (HIF) is responsible for most of the fold-induction of the *EPO* gene in response to hypoxia. In addition, the direct repeat of two steroid hormone receptor half-sites (DR2) confers regulation through binding of hepatic nuclear factor 4 (HNF-4).

ogy, not only in the coding sequence of *EPO*, but also within the 5' flanking region, the first intron, and the 3' flanking region, suggesting that one or more sequences within these regions might function in regulation. Both studies in transgenic mice and transfection studies in cultured cells have been used to

define important *cis*-acting sequences within *EPO* that lead to tissue-specific, developmental, and hypoxia-inducible control of *EPO* expression.

The first studies of EPO transgenic mice, containing a 4 kb DNA fragment that included 0.4 kb of the 5'-flanking sequence and 0.7 kb of the 3'-flanking sequence revealed anaemia inducible expression of the transgene in the liver, indicating the existence of at least one sequence that could interact with the hypoxia-signalling apparatus within these sequences. The widespread unregulated expression in other tissues that do not normally express EPO, however, indicated that additional, more distant, elements are required for correct tissue-specific and developmental regulation. The widespread anomalous expression of EPO was reduced in transgenes containing a further 6 kb of the 5'-flanking sequence, suggesting that this region contains one or more negative regulatory elements. In these mice, however, inducible EPO expression occurred in the liver, rather than kidney, in contrast to normal adult mice. Extension of the 5'-flanking sequence still further, identified a kidney inducible element that mediated inducible expression of the EPO transgene in interstitial fibroblasts of the kidney. These mice still manifest polycythaemia as a result of excessive hepatic EPO expression. Regulation of the transgene by physiologic and tissue specific signals only mirrored expression of the endogenous gene when the 3'-flanking sequence was extended to 7 kb defining a further negative regulatory element restricting hepatic expression. Thus, these studies have demonstrated the existence of distinct sequences that promote or restrict EPO expression in different tissues. Of particular interest, however, was evidence for local sequences that could mediate responses to anaemia in the liver. Mapping of a DNAse hypersensitivity site within this region suggested the possibility of a regulatory site lying 3' to the gene. Further analysis by transfection of EPO-producing hepatoma cell lines (HepG2 and Hep3b), identified a hypoxia-inducible control sequence termed the EPO 3' enhancer. In both the mouse and human *EPO*, this enhancer lies in the highly conserved region 120 bp 3' to the polyadenylation site. Analysis of the EPO 3' enhancer in hepatoma cells defined three functional sites. Critical for the regulation by hypoxia is a DNA motif, termed the hypoxia-responsive element (HRE) that was found to bind an inducible protein complex termed hypoxia-inducible factor (HIF), the transcription factor that is responsible for hypoxic regulation of EPO expression [17].

Two other sites were shown to be necessary for proper function of the enhancer, a second motif (CACA), 7 bp 3' to the HRE, and a third motif consisting of a direct repeat of two steroid hormone receptor half-sites separated by 2 bp (DR-2). In the setting of the EPO enhancer, this motif binds hepatic nuclear factor (HNF)-4. These multiple sites are typical of transcriptional enhancers and are presumed to enable cooperative assembly of transcriptional complexes. How these proteins cooperate with HIF bound to the core HRE is not fully understood, but they may modulate the response to hypoxia and contribute to the tissue specificity of EPO expression. For example, HNF-4, which is expressed in the liver and renal cortex, may contribute to developmental and

tissue specific regulation of endogenous EPO expression. In this respect, it is also interesting that binding of retinoic acid receptor (RXRα) to the DR-2 site during early embryonic development is superseded by HNF-4. This switch corresponds temporally with a change in the characteristics of EPO expression from responsiveness to retinoic acid to responsiveness to hypoxia [18].

DNA methylation may also have a role in regulation of endogenous *EPO* expression. The consensus HIF-binding site (G/ACGTG) contains a CpG dinucleotide known to be a potential target of cytosine methylation. Methylation at this site abolishes binding of HIF to the HRE, recruits inhibitory methyl-CpG-binding proteins, and abrogates hypoxic regulation of reporter gene expression [19, 20]. Overall, these studies define a specific point of interaction between the oxygen-sensitive signalling system and the *EPO* locus. Unexpectedly, however, transfection studies of the 3' EPO enhancer also demonstrated oxygen-regulated activity after introduction into a wide variety of cells, regardless of whether the cells produced EPO, or were derived from an EPO-producing organ. Thus, it became clear that the highly specific and sensitive response to hypoxia that was manifest in the EPO-producing tissues was, in fact, a general property of mammalian cells [21]. The biologic importance of this oxygen-sensing mechanism is further emphasised by the discovery that the HIF system is conserved across the entire animal kingdom, even in primitive species without specialised oxygen delivery systems, such as blood vessels.

EPO – a paradigm for widespread hypoxic gene regulation

The discovery that the oxygen-sensing mechanism operated in all cells, irrespective of their ability to express EPO, led to the realisation that the range of genes regulated by oxygen and HIF extended far beyond EPO, and was involved in the regulation of many biologic processes that both facilitate oxygen delivery and reduce oxygen demand (Fig. 3) [22]. Effective erythropoiesis requires not only stimulation of the bone marrow by EPO, but also coordinated provision of iron, supplied through its transporter, transferrin, and its cellular uptake mechanism, the transferrin receptor, and a range of other mechanisms, many of which are now recognised to be regulated by HIF.

Increased oxygen delivery is mediated not only through erythropoiesis, but also through changes in the vasculature. During localised hypoxia, vasomotor tone is controlled through HIF-mediated transcriptional regulation of genes encoding vasoactive factors such as endothelin 1, endothelial nitric-oxide synthase (eNOS), hemoxygenase-1 (HO-1), and atrial naturetic peptide (ANP), and also a range of ion channels that may operate to alter vascular tone and responsiveness.

Another important vascular response to hypoxia is the regulation of angiogenesis through angiogenic growth factors. Both vascular epithelial growth factor (VEGF) and its receptor Flt-1 are transcriptionally activated by HIF, and

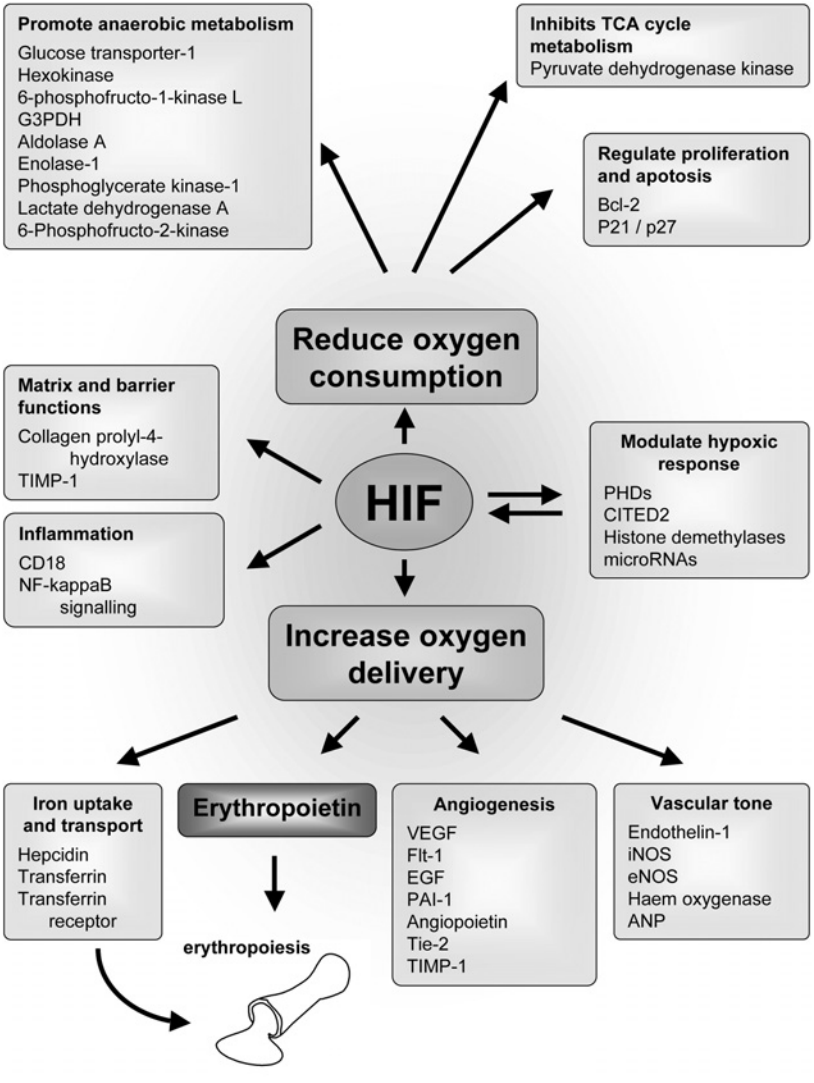

Figure 3. Transcriptional targets of HIF. A representation of the increasing number of genes regulated by the HIF oxygen-sensing transcriptional pathway is illustrated. Broadly these act to increase oxygen delivery systemically by promoting erythropoiesis and iron delivery, and at the tissue level by promoting angiogenesis and controlling vascular tone, in addition to reducing oxygen consumption by inhibiting TCA (tricarboxylic acid) cycle metabolism, promoting anaerobic glycolysis, and modulating cell proliferation and apoptosis. In addition it is now recognised that several components of the HIF regulatory pathway itself are also transcriptionally regulated by HIF, leading to autoregulatory feedback loops. Glyceraldehyde-3-phosphate dehydrogenase (G3PHD), B-cell CLL/lymphoma 2 (Bcl-2). vascular endothelial growth factor (VEGF), fms-related tyrosine kinase 1/VEGF receptor (Flt-1), epidermal growth factor (EGF), plasminogen activator inhibitor-1 (PAI-1), endothelium-specific receptor tyrosine kinase 2 (Tie-2), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), atrial natriuretic peptide (ANP), prolyl hydroxylase domain containing (PHD), CBP/p300 interacting transactivator with ED-rich tail (CITED2).

this alone is capable of initiating angiogenesis in quiescent vessels. However, for an efficient vasculature to be formed a more coordinated response is necessary, involving many other factors. For example, angiopoietin, its receptor Tie-2, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and balanced control of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (TIMP) all contribute to the endogenous angiogenic response. All respond to the HIF system. Activation of HIF therefore promotes a coordinated angiogenic response that directs the growth of new blood vessels into areas of impaired oxygenation to restore adequate oxygen delivery. This activation is of importance during the physiologic processes of growth and development, wound healing, and proliferation, as well as in pathological responses, resulting from neoplasia, ischaemia, and inflammation. Local oxygen tension is a balance between supply and demand, and the response to hypoxia and the HIF pathway includes measures that reduce oxygen utilisation. For example, HIF targets include enzymes at key checkpoints in the glycolytic pathway such as GLUT1 (glucose uptake), 6-phosphofructo-1-kinase L, and lactate dehydrogenase A. Hence, during conditions of oxygen deficiency under which oxidative phosphorylation by the tricarboxylic acid cycle cannot proceed, HIF coordinately upregulates the less efficient, oxygen-independent, glycolytic pathway, and facilitates conversion of the resultant pyruvate to lactate, for export to the liver (the "Pasteur" effect). In addition, in hypoxia HIF directly upregulates pyruvate dehydrogenase kinase (PDK), which phosphorylates and inactivates the pyruvate dehydrogenase enzyme complex that converts pyruvate to acetyl-coenzyme A, thereby inhibiting pyruvate metabolism by the tricarboxylic acid cycle.

Also important in limiting oxygen demand are cell-based decisions involving proliferation, apoptosis and protective responses such as autophagy, with key genes such as B-cell lymphoma-2 family members (Bcl-2), and the cell-cycle regulators p21 and p27 being directly or indirectly responsive to HIF.

Infection and inflammation often result in lowered oxygen tension. Myeloid cells, and other key effectors of the innate immune response, have evolved several HIF-dependent responses such as enhanced glycolysis, inhibition of NF-κB dependent apoptosis, and enhanced CD18 dependent diapedesis of neutrophils into hypoxic regions.

Hypoxia inducible-factor (HIF)

Hypoxia-inducible factor was first isolated by DNA affinity chromatography using the EPO 3' enhancer sequences and shown to be a heterodimeric transcription factor composed of α and β subunits [23]. The HIFβ subunit also functions as the aryl hydrocarbon nuclear receptor translocator (ARNT) in other transcription pathways, while HIFα subunits are specific to the hypoxic response and are highly regulated by the availability of oxygen [23–25]. Overall, three HIFα subunits have been identified, though HIF-1α and HIF-2α

are the best studied to date. Both HIF-1α and HIF-2α have a similar domain structure and contain, in their *N*-terminal regions, both a basic-helix-loop-helix (bHLH) motif, common to many transcription factors, and a PAS (Per AHR/ARNT Sim) domain, defined by its presence in the *Drosophila* Per and Sim proteins and in the mammalian ARNT and AHR proteins (Fig. 4) [23]. While this *N*-terminal portion of the HIF subunits is necessary for dimerisation and DNA binding, it is the *C*-terminal region of the HIFα subunits that is responsible for responsiveness to oxygen. In addition to oxygen-dependent degradation domains (ODDD) that regulate protein stability, *C*-terminal sequences of HIF-1α and HIF-2α also contain transactivating domains (TAD) that control the hypoxically inducible transactivating ability of the protein [26], in part through binding to p300/CBP [27]. Thus, the control of HIF by oxygen involves the regulation of two independent properties of HIFα subunits, stability and activity.

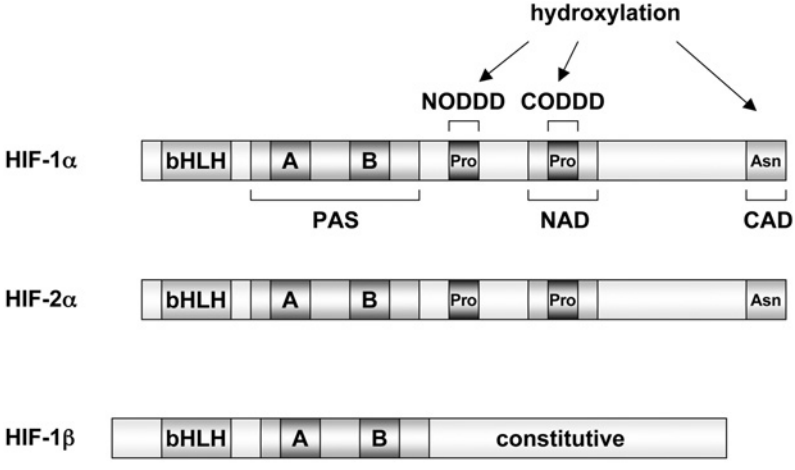

Figure 4. The domain structure of HIF-1α, HIF-2α, and HIF-1α/ARNT subunits. The figure shows the basic helix-loop-helix (bHLH) domains and the PAS domains responsible for dimerisation and DNA binding. The *C*-terminal portion of HIF-1α and HIF-2α contains the regulatory domains: the amino-terminal oxygen-dependent degradation domain (NODDD) and the carboxy- terminal oxygen-dependent degradation domain (CODDD), responsible for regulating HIFα stability and the amino-terminal activation domain (NAD) and the carboxy-terminal activation domain (CAD) involved in regulating transactivating ability. These regulatory properties involve enzymatic hydroxylation of specific residues in these regions. PAS domain = PER, AHR, ARNT, SIM domain; PER = periodic circadian protein, AHR = aryl-hydrocarbon receptor, ARNT = aryl-hydrocarbon receptor nuclear translocator, SIM = single-minded protein.

Differential functions of the HIF isoforms

The identification of multiple HIF isoforms raises questions about the existence and extent of distinct, *versus* overlapping, roles. At present the precise function of HIF-3α remains unknown, although a splice variant, inhibitory

PAS (iPAS) can antagonise responses to the other two HIFα isoforms [28]. To date, study has concentrated mostly on HIF-1α and HIF-2α. These isoforms have largely concordant, although not identical, mechanisms of regulation. However, differing patterns of organ expression and cellular distribution indicate differing roles for HIF-1α and HIF-2α. Originally, HIF-2α was thought to be largely restricted to the mouse endothelium, while HIF-1α had an almost ubiquitous distribution. Better-characterised patterns of expression have been reported by immunohistochemistry [29]. For example, in the hypoxic kidney HIF-2α is seen in the EPO-producing interstitial fibroblasts as well as capillary endothelial cells, whereas HIF-1α is the dominant form expressed in tubular epithelial cells [30].

Further evidence of distinct roles for HIF-1α and HIF-2α comes from the contrasting phenotypes seen in mouse knockout models. HIF-1α knockout mice have early embryonic lethality with enhanced mesenchymal cell death and defects in angiogenesis that involve both the yolk sac and embryonic tissues [31, 32]. By contrast, the phenotype of HIF-2α knockout mice is more variable, depending on the genetic background, with some homozygotes surviving into the perinatal period, and manifesting a range of abnormalities. Thus, defects in vascular remodelling, catecholamine production, fatty acid oxidation, lung maturation, and surfactant production, and an anaemia that can be rescued by exogenous EPO have variously been described in different reports of HIF-2α knockout mice [33–37].

HIF-1α and HIF-2α exhibit similar functional domain structures, containing DNA binding, dimerisation, and regulatory domains with a high degree of homology. Furthermore, each dimerises with HIF-1β and recognises identical DNA sequences. It was therefore initially suggested that HIF-1α and HIF-2α had unique roles in hypoxic responses as a function of their distinct expression patterns. However, with the development of microarray technology and the ability to inactivate each isoform in specific cells using small interfering RNA (siRNA), it has become apparent that HIF-1α and HIF-2α also differ in the range of target genes that each is able to activate [38, 39]. For example, lactate dehydrogenase A (LDHA), carbonic anhydrase-9 (CAIX), and many glycolytic genes, are exclusively regulated by HIF-1α in response to hypoxia [39–44]. Other HIF regulated targets, such as the Pou transcription factor Oct-4, cyclin D1, TWIST1, transforming growth factor-β (TGF-β) and EPO are dominantly or exclusively controlled by HIF-2α [43, 45–49]. Furthermore, both HIF-1α and HIF-2α are capable of regulating genes such as vascular endothelial growth factor (VEGF), facilitated glucose transporter-1 (GLUT-1), adipose differentiation-related protein, adrenomedulin (ADM) and N-myc downstream regulated 1 (NDRG-1).

Chromatin immunoprecipitation experiments have shown that both HIFα subunits can bind to the endogenous HRE of hypoxia-responsive genes irrespective of whether the gene is functionally regulated by HIF-1α, HIF-2α, or both factors, indicating that selective HIF target gene activation is not based on the unique DNA-binding abilities of these factors [50, 51]. In contrast,

domain-exchange experiments have demonstrated *C*-terminal regions of each isoform that are required to confer target gene specificity.

Several other transcription factors are known to physically interact with HIF-2α, but not HIF-1α and have been proposed to cooperate with HIF-2α in activating a number of target genes [52]. For example, HIF-2α interacts, through its *C*-terminus, with Ets-1 and synergistically activates expression of VEGF receptor-2 (VEGFR-2 or Flk-1) [52]. Elk-1, another Ets family member, cooperates with HIF-2α to activate a number of HIF-2α-specific targets genes, including *EPO* [50, 53]. Furthermore, several other HIF-2α-specific targets contain Ets-binding sites adjacent to HREs, suggesting that this may be a common mechanism for regulating target gene selectivity by HIF-2α [53].

Regulation of HIF by oxygen

Despite evidence for disparate roles of HIF-1α and HIF-2α, the mechanisms regulating the abundance and activity of the two isoforms in response to hypoxia are largely concordant, and this is reflected in similar domain structures [54] (Fig. 4). The amounts of HIF-1α and HIF-2α are regulated by proteolytic degradation, dependent upon distinct *N*-terminal and *C*-terminal oxygen-dependent degradation domains (NODDD and CODDD), located in the central region of the molecule [24–26, 55, 56]. In addition, both isoforms also possess two transactivation domains, required for recruiting cofactors that mediate transcription (such as the coactivator p300 [27]) – an *N*-terminal activation domain (NAD) that overlaps with the CODDD, and a *C*-terminal activation domain (CAD).

Both the proteolytic destruction and the transactivation domains are under oxygen-dependent regulation and in each case oxygen-dependence is conferred by enzymatic hydroxylation of specific amino-acid residues (Fig. 5). In the case of the NODDD and the CODDD, hydroxylation of PRO residues permits specific binding to an E3 ubiquitin ligase, which then targets the molecule for rapid degradation by the ubiquitin-proteasome pathway [57–59]. The apparent K_mO_2 (concentration of oxygen that supports a half maximal initial rate of catalysis) is reported to be 230–250 μM, well above the oxygen concentration in tissues (in the range 10–30 μM) [60]. Consequently, oxygen is rate-limiting for HIFα hydroxylation, such that the reaction rate varies with oxygen concentration over the physiologic range of tissue oxygen tensions. Because this step is rate limiting for the whole degradation pathway, this allows HIFα levels to reflect oxygen concentration in a graded manner, essential for the system to act as an oxygen sensor.

In a similar process, under oxygenated conditions, hydroxylation of an asparaginyl residue within the CAD blocks its interaction with the CH1 domain of p300, impeding its ability to initiate transcription [61]. Additionally, evidence has been reported for other levels of oxygen-dependent control. For example, oxygen-dependent regulation of HIF-1α nuclear/cytoplasmic locali-

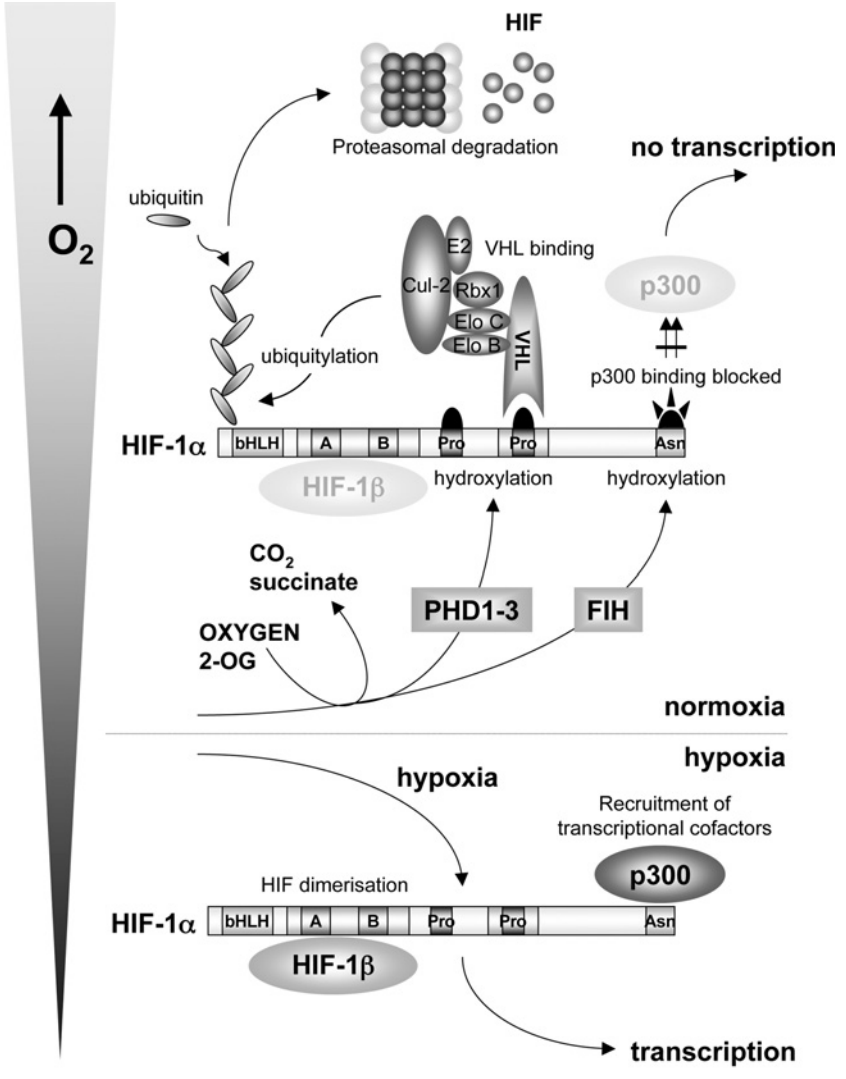

Figure 5. Oxygen-dependent regulation of HIF signalling. Under hypoxic conditions HIFα subunits dimerise with HIF-1α, bind DNA sequences containing hypoxia response elements (HREs) and recruit transcriptional cofactors such as p300 to effect transcription of target genes. When oxygen is available, enzymatic hydroxylation of residues within the HIFα subunit is effected by the PHD and FIH enzymes. Hydroxylation of a specific ASP residue within the CAD blocks recruitment of the cofactor p300, while hydroxylation of specific PRO residues in the NODDD and CODDD allows binding of the VHL E3 ubiquitin ligase complex. This tags the HIFα subunit with a multiubiquitin chain that targets it for proteasomal destruction.

sation has been observed in a number of systems [62, 63]. For HIF-3α prolyl hydroxylase-dependent proteolytic regulation that involves a region homologous to the CODDD is conserved [64]. Interestingly, the alternative splicing

process that generates the inhibitory protein IPAS is also reported to be regulated by hypoxia, though the mechanism is currently unknown [28, 65].

In addition, a number of non-oxygen regulated controls of HIF stability and activity have been reported though it is unclear what effect they have on the physiologic regulation of EPO. In addition to ubiquitylation, HIFα subunits can be sumoylated, though it unclear whether this promotes stability or instability. Mice that are deficient in Sumo-specific protease-1 after targeted inactivation develop anaemia due to failure to stabilise HIFα. Enzymatic hydroxylation of residues within HIFα subunit proved to be an unprecedented mechanism of signal transduction, although such modifications are well established, and have structural significance in the post-translational processing of collagen molecules [66]. To date, all prolyl and asparaginyl hydroxylases, including the HIF-modifying enzymes, have been found to be members of the super-family of 2-oxoglutarate dependent dioxygenases [67]. In addition to oxygen, these enzymes also require 2-oxoglutarate, as a cosubstrate, and nonheme iron (Fe^{2+}), as a cofactor. Furthermore, ascorbate (vitamin C) is required for full enzymatic activity, possibly to maintain the catalytic iron centre in the reduced state, or to facilitate the availability of Fe^{2+} in a more general way. The ease with which iron in these enzymes can be removed by metal chelators, or substituted by other transition metals, explains the classical observations that metal ions, such as Co^{2+}, and iron chelators can induce EPO. It also raises important questions as to what extent HIF-hydroxylase activity is affected by physiologic or pathologic variation in iron or ascorbate availability. Experiments in tissue culture suggest that such effects could be important, particularly in cancer [68, 69].

In mammalian cells, three closely related hydroxylases termed prolyl hydroxylase domain PHD1, 2, and 3 catalyse hydroxylation of the prolyl residues within the NODDD and CODDD [70, 71], and one asparaginyl hydroxylase, termed factor inhibiting HIF (FIH), is responsible for modifying the CAD [72, 73]. As with HIFα isoforms, the presence of multiple HIF hydroxylases raises questions about their possible redundancy and diverse roles. Although all three PHDs contribute to the regulation of HIF, inactivation of each PHD individually using siRNA, has shown that loss of PHD2 alone is sufficient to increase HIF-1α levels in oxygenated cells, leading to the proposal that PHD2 is the most important isoform in oxygen sensing [74]. This likely reflects the relative high abundance of PHD2, which is the most abundant isoform in most cell types. In support of the importance of PHD2 is the embryonic lethality seen in PHD2 knockout mice, while those lacking PHD1 and PHD3 survive relatively normally [75]. However, these enzymes show differential patterns of organ expression, intracellular localisation, inducibility by exogenous stimuli, and substrate selectivity for HIF-1α *versus* HIF-2α, suggesting that each has distinct, if overlapping, functions in modulating responses to hypoxia [59, 76–81].

While the activity of the HIF hydroxylases depends directly on the ambient oxygen concentration, the levels of various hydroxylase enzymes themselves

are also under the control of oxygen, adding another layer of control in the response to hypoxia. Hypoxia leads to increased transcription of PHD2 and PHD3 mRNA, in a HIF-dependent manner that acts, by a negative feedback loop, to limit the increase in HIF observed during prolonged hypoxia [70, 74]. In addition, the stability of PHD1 and PHD3 decreases in hypoxic cells, as a result of increased expression of another E3 ubiquitin ligase, Siah2, that targets these isoforms for proteasomal degradation and acts to enhance HIFα stability [79]. Indeed Siah2$^{-/-}$ mice exhibit an impaired hypoxic EPO response. Furthermore, an additional negative-feedback mechanism acts to autoregulate transcriptional activity of the HIF DNA-binding complex. CITED2 (CBP/p300 interacting transactivator with ED-rich tail), itself a HIF regulated gene, binds p300/CBP, in hypoxia, inhibiting its recruitment to the HIF transcriptional complex, and limiting the ability of HIF to activate gene transcription [82].

Von Hippel-Lindau disease

While hydroxylation of prolyl residues within the HIFα isoforms marks them for destruction, it is the von Hippel-Lindau (VHL) tumour suppressor protein that provides the specific recognition component that links the hydroxylated proteins to the constitutive ubiquitination machinery, and accounts for the dramatic upregulation of the HIF system after VHL inactivation in certain types of cancer.

VHL disease is a tumour syndrome, inherited as an autosomal dominant trait, affecting 1 in 36,000 of the population, and associated with (often multiple) clear cell renal cell carcinoma; hemangioblastomas (especially of the cerebellum, spinal cord, and retina); pheochromocytoma; endolymphatic-sac tumours of the inner ear; and cysts of the kidney, pancreas, and epididymis. Tumours are associated with somatic loss or inactivation of the remaining wild-type allele in individuals heterozygote for germline mutation in the VHL tumour-suppressor gene, in accordance with the Knudson 'two-hit' hypothesis. Sporadic tumours are associated with somatic loss or inactivation of both alleles within the same cell. Tumours are characteristically highly vascular and overexpress both HIFα and the target gene *VEGF*. The *VHL* gene, located on chromosome 3p25–26, was first identified in 1993 [83], and shown to comprise three exons coding for a protein of 213 amino acids with a second isoform of 150 residues resulting from internal translational initiation at an in frame ATG at codon 54. pVHL, the product of the *VHL* gene, forms a larger multiprotein complex, including elongins B and C, CUL2, and Rbx1, and forms the specific recognition component of an E3 ubiquitin-ligase that covalently tags HIFα subunits with a polyubiquitin chain. The labelled protein is taken up by the 26S proteasome, where it is rapidly destroyed [84–88]. X-ray crystallography has demonstrated that the formation of two hydrogen bonds between the alcohol of the hydroxylated prolines on HIFα and a binding pocket within pVHL stabilises the interaction by at least three orders of magnitude,

permitting exquisite discrimination between the hydroxylated and nonhydroxylated forms of HIFα [89, 90]. Loss of functional pVHL in tumour cells blocks oxygen-dependent degradation of the HIFα subunits. Substantial evidence supports a causal role for the consequent upregulation of HIF and hypoxia pathways in the promotion of VHL-associated renal cell carcinoma.

Integration of hypoxic, metabolic, and redox signals

The involvement of two sub-families of 2-oxoglutarate dependent dioxygenases at discrete points in the oxygen-sensing pathway raises questions as to whether such enzymes are especially suited to physiologic oxygen-sensing, and whether their unusually extensive cosubstrate and cofactor requirements play a role in a shaping responses to hypoxia. Indeed the cosubstrate 2-oxoglutarate is an intermediate metabolite of the TCA cycle. Furthermore, both succinate (a product of the hydroxylase reaction) and fumarate are also products of TCA cycle enzymes, and can both compete with 2-oxoglutarate to inhibit PHD enzyme activity (Fig. 6) [91, 92]. It is possible, therefore, that in addition to sensing oxygen, these enzymes are also in some way acting as metabolic sensors, able to detect stresses on oxygen-requiring TCA cycle metabolism. Clinically, normoxic upregulation of HIF is seen in tumours lacking succinate dehydrogenase or fumarate hydratase activity.

Other factors that influence HIF-activation independently of oxygen concentrations include many growth factors and can be broadly grouped, according to their ability to induce common cellular signalling pathways, in particular those involving kinases, reactive oxygen species (ROS) or both. Such stimuli may pre-prime the cell to anticipate an increase in oxygen requirement consequent on the increased cell growth. More specifically, kinases such as p38 mitogen-activated protein kinase (p38), p42/p44 extracellular signal-regulated kinse 1 and 2, and phosphatidyliositol 3-kinase (PI3K) have all been reported to enhance normoxic HIF levels. In addition, phosphorylation of the tuberose sclerosis gene, *TSC2*, by AMP kinase (AMPK), consequent upon energy starvation, leads to inhibition of mammalian target of rapamycin (mTOR). In turn, mTOR affects HIFα levels independent of oxygen, and may provide another means of coupling nutrient availability to oxygen sensing [93].

The effect of hypoxia on levels of ROS is complex and somewhat controversial. However, ROS do have the potential to interfere with HIF hydroxylation by altering the redox state of the active iron-binding enzymatic site, leading to HIFα accumulation, as observed in $JunD^{-/-}$ cells [94]. A ROS of particular interest is nitric oxide (NO), which has been reported to variably stabilise or destabilise HIFα under normoxic conditions. These discrepancies may result from interactions of NO with mitochondrial respiration and consequence effects on intracellular oxygen concentrations, from temporal observations of a transient effect and/or the ability of NO to affect other transcription factors, such as GATA-2 that are involved in the regulation of HIF target genes such as *EPO*.

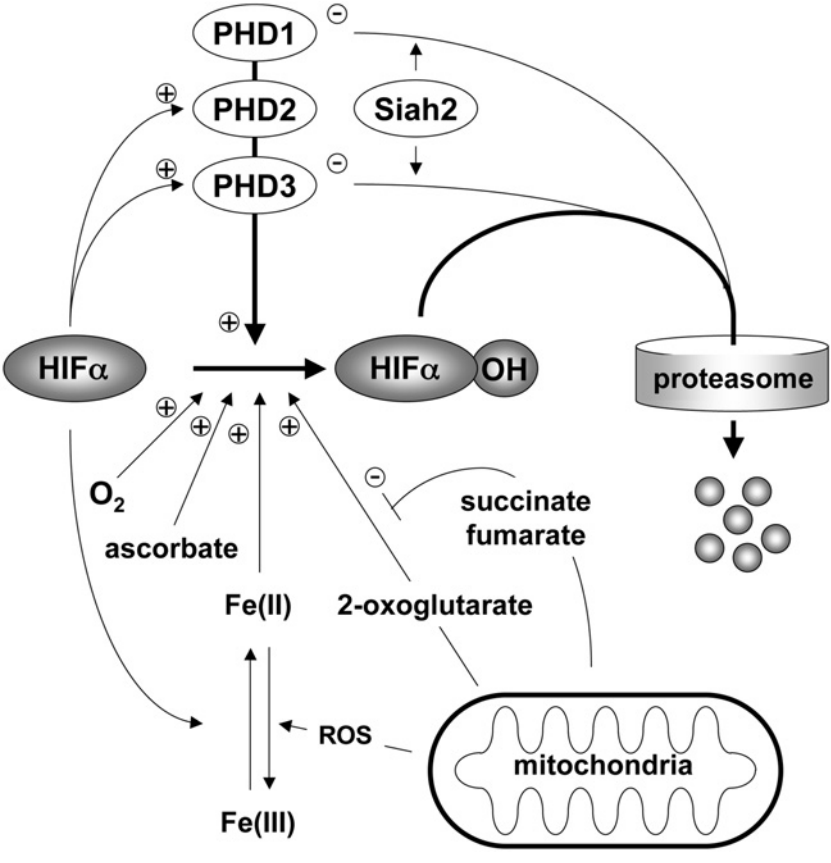

Figure 6. Integration of hypoxic, metabolic, and redox signals. In addition to oxygen, hydroxylation of HIFα subunits requires a number of cosubstrates, availability of which can modulate hydroxylase activity. While intracellular free-iron is tightly regulated, through HIF-dependent and HIF-independent mechanisms, reactive oxygen species (ROS) have the potential to interfere with the redox state of the active iron-binding enzymatic site, leading to HIFα accumulation by interfering with HIF hydroxylation. 2-Oxoglutarate, a tricarboxylic acid cycle (TCA) intermediate, is required for hydroxylation, and several other intermediates can antagonise this action, such that HIF also has the potential to respond to metabolic changes in the cell. Different isoforms of the HIF prolyl hydroxylases (PHDs) are variably transcriptionally activated by HIF, or hypoxically downregulated by Siah2 mediated proteasomal degradation, adding another layer of complexity.

Renal anaemia and erythropoietin regulation

Many factors likely contribute to the anaemia of chronic renal failure, including hematinic deficiency, lack of RPO, and accumulation of circulating inhibitors of erythropoiesis. It has been postulated that increased levels of uremic toxins may antagonise the bone marrow response to EPO. Indeed, it is known that dialysis alone improves anaemia in patients with renal failure.

It is clear, however, that in chronic renal impairment, the EPO response to a lowered hematocrit is dramatically blunted and that inappropriately low EPO concentrations are the main drive for anaemia. Given the very substantial capacity of the normal kidney to upregulate EPO production it seems unlikely that the failure of the kidney to generate an adequate EPO response to anaemia can be attributed to loss of renal mass alone. Several studies have shown that the failure to produce EPO in renal disease is relative rather than absolute. When subject to more severe hypoxic stimulation, individuals with renal disease can often generate an EPO response that would be sufficient to correct the anaemia if it were sustained. After renal injury, the number of fibroblast-like interstitial cells is increased in the kidney. The cells assume a myofibroblastoid phenotype and their potential for EPO expression is reduced [95]. The molecular mechanisms underlying this change in phenotype are unknown and despite much progress in understanding the molecular regulation of EPO production, the reason(s) why generation of EPO is inappropriately low in diseased kidneys remain unclear. The decrease in glomerular filtration rate (GFR) associated with chronic renal failure might be predicted to reduce the need for sodium reabsorption in the tubule, and hence oxygen consumption, leading to downregulation of the hypoxic drive for erythropoietin production. Owing to the portal nature of the renal circulation, however, glomerular sclerosis also reduces blood flow to the tubule, and intrarenal oxygen tensions in chronic renal failure are, in fact, lowered. Inappropriately low EPO concentrations have also been described in anaemias associated with chronic inflammatory conditions, such as acquired immunodeficiency syndrome (AIDS), and rheumatoid arthritis, as well as in cancer. Using animal and cell culture models, proinflammatory cytokines such as tissue necrosis factor-α (TNF-α) and interleukin-1 (IL-1) have been shown to suppress hypoxia induced EPO production, although the unexpected concomitant increase in HIF-1α activity indicates that the effects must be mediated by other transcription factors, possibly by GATA-2 and NF-κB, acting on the EPO promoter [96, 97].

Pharmacologic manipulation of the HIF response

The central role of HIF in a wide range of common pathologies, including cancer and ischaemia as well as renal anaemia has made it an attractive target for pharmacologic manipulation. The enzymatic basis of its regulation together with the absolute requirement for a number of co-factors and cosubstrates has identified the PHDs and FIH as key objectives for such a strategy. The experimental approaches of iron chelation or substitution are likely to prove too toxic for clinical use. Indeed in renal anaemia iron deficiency can often confound the anaemia. Approaches based on the use of 2-oxoglutarate analogues as competitive inhibitors and related compounds that are able to bind to the hydroxylases and hold greater promise. Existing knowledge of HIF pathways and of this class of enzyme suggests that obtaining sufficient specificity for

clinical use in anaemia therapy will be challenging [98]. Bioinformatic predictions suggest that there may be up to 60 2-oxoglutarate-dependent oxygenases encoded by the human genome, some of which are as yet uncharacterised and thus have the potential to generate unexpected off-target effects [99]. The pleiotropic actions of HIF also have the potential to generate unwanted effects, even with inhibitors that are entirely specific for the HIF hydroxylases. For example, HIF activation has the potential to increase both erythropoiesis and angiogenesis. Promotion of erythropoiesis, leading to polycythaemia, may provide an unwanted side-effect of treatments aimed at supporting angiogenesis and *vice versa*. However, dissociating these actions may simply be a matter of delivering the drug to the required organ. For example, systemic hypoxia at altitude predominantly affects erythropoiesis, with little in the way of systemic angiogenesis, whereas localised hypoxia, in healing or neoplastic in tissues incapable of expressing EPO, produces localised angiogenesis. In addition, drugs that preferentially target different HIF hydroxylases or different HIFα isoforms could further aid specificity.

Summary

Analysis of pathways regulating *EPO* expression, in accordance with oxygen availability, have revealed unprecedented signal systems involving the post-translational hydroxylation of specific prolyl and asparaginyl residues in HIF, the master regulator of transcriptional responses to hypoxia. These pathways are conserved throughout the animal kingdom and mediate diverse cellular and systemic responses to hypoxic challenge. Therapeutic manipulation has the potential to augment the natural cellular response to hypoxia in a coordinated way, and may be of benefit in ischaemic/hypoxic diseases including anaemia.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Biology of erythropoietin

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Introduction

Recombinant erythropoietin is a 165-amino acid glycoprotein hormone with a molecular weight of 30.4 kDa that contains 39% carbohydrate [1, 2] and has the same biologic function as the endogenous product of a human gene located on chromosome 7q22 [3, 4]. Both the endogenous material (eEPO), and recombinant human glycoprotein (rHuEPO) bind a single receptor (EPOR) [5–7]. Although data from one group challenge this concept by suggesting signaling through a heterodimeric receptor including the beta-common cytokine receptor [8], these data have yet to be confirmed by other groups. For some years it had been widely accepted that EPOR was expressed broadly within the body in both normal and diseased tissues (e.g., [9]), but as discussed elsewhere in this book, methodological flaws in almost all of the published data draw this conclusion into doubt. To date, it is possible only to conclude with certainty that EPOR is expressed at effective levels in erythroid tissues in the bone marrow and spleen, so our discussion will focus only on the function of EPOR in that context.

Manipulated overexpression or deletion of the *EPO* gene in 'transgenic' or 'knockout' mice provided insight into the physiologic role of EPO. EPO transgenic mice have high hematocrits (80%), increased blood volume, enlarged hearts, reduced exercise capacity, and premature death [10]. It is likely that the latter changes are compensatory in nature and not the result of a direct action of EPO in cardiovascular tissues. Both EPO and EPOR knockout mice lack embryonic erythropoiesis, have heart problems, and die at approximately gestational day 13.5 [11–13]. The close similarity between the EPO and EPOR knockout phenotypes is good evidence that only a single ligand exists for EPOR and only a single receptor exists for EPO. EPOR knockout mice can be rescued by expressing human EPOR within the hematopoietic system, thus suggesting EPOR is not required for normal non-hematopoietic tissue development and confirming the role of EPO and EPOR in erythropoiesis [14]. However, some brain-specific EPOR knockout studies have suggested EPOR may play a role in neural progenitor proliferation, survival and migration [15, 16], although the functional significance of these findings are unclear given the rescued mice appear normal.

Control of EPO production

The control of EPO production *in vivo* through the HIF-PH pathway has provided additional pharmacologic targets for novel therapeutic options. This finding assumes the plieotropic nature of HIF-1α stabilization can be harnessed; a significant proportion of the genome is controlled by this axis, but early signs are encouraging [17].

Pharmacologic stabilization of HIF complexes has been shown to increase the production of endogenous eEPO [17]. How this will differ from the pharmacologic administration of rHuEPO is not fully understood. For example, though eEPO has an identical amino acid sequence to rHuEPO [2] eEPO collected from urine contains more sulphate and is more negatively charged [18, 19]. How serum eEPO activated through the HIF-PH pathway corresponds to rHuEPO or urinary eEPO is unknown. Therefore, the role of HIF stabilizers in anemia treatment will require substantial investigation not only because the EPO which is liberated may have different clinical effects than rHuEpo (which we know a great deal about), but also because a large number of proteins potentially regulated by HIF could influence the pharmacologic effects of the eEPO.

A number of properties of the EPO molecule are known to affect its *in vivo* potency. However, two general attributes explain the performance of all erythropoiesis-stimulating agents (ESA) described to date: interactions with the receptor, and clearance from the body.

The first of these, receptor interaction, can be summarized by the affinity of the molecule for EPOR. The receptor probably exists on the surface of responsive cells as a preformed homodimer [20]. Two regions of EPO have been shown to interact with the receptor, one with high affinity, the other relatively low [21]. Direct modifications in these regions of EPO will obviously affect the affinity of the ligand for the receptor but destruction of either one will effectively neutralize activity [21]. The interaction between EPO and EPOR is highly dynamic. Receptor is cycled to and from the cell surface relatively quickly [22]. The physicochemical properties (e.g., charge, size, hydrophobicity) of the ESA as well as the concentration outside the cell will determine the probability of the ligand being bound to the receptor (Kd) at equilibrium although equilibrium is a consequence of both the rate of association (K_{on}) and the rate of dissociation (K_{off}) [23]. To date, all studied EPO analogs display only quantitative variations in these constants; none has been shown to present a qualitatively new type of interaction with the receptor. Should an EPO analog be bound to the receptor when the complex is internalized then its fate may be determined by a number of constants that contribute to the probability of the EPO analog being recycled or not [23]. EPOR activators described to date appear to be full agonists and their ability to transduce a signal through the receptor is dominated by affinity. An interesting class of agonists is represented by antibodies selected for their ability to activate EPOR [24]. These ‘agonistic antibodies’ show a unique bell-shaped dose response curve. It has been suggested that at lower concentrations, these agents work as conventional ago-

nists by inducing the appropriate activation of EPOR dimers. However, because each arm of the antibody F_{ab} region can itself bind a receptor, at high molar ratios of antibody to receptor, two copies of the antibody can potentially bind to the EPOR dimer while neither of them binds to both receptors to transduce a signal. The agents would, therefore, become self-antagonistic at higher concentrations. This curious observation has yet to be exploited to develop a therapeutic but a number of other modifications have been introduced to create analogs and derivatives of EPO, including hyperglycosylation (to different degrees), PEGylation, glycopegylation, Fc-conjugation, and albumin-conjugation. These changes tend to reduce the affinity of the ligand for the receptor, probably by simple stearic hindrance. These modifications in general slow the clearance of EPO from the body.

The clearance of EPO from plasma is not well understood. Various mechanisms and clearance organs have been suggested, but no explanation to date is fully satisfactory. What is known is that the modifications cited have extended the *in vivo* residence time of EPO analogs and that in general has led to the creation of more 'potent' molecules, i.e., more hemoglobin response per molar equivalent administered, despite the parallel loss of affinity for the receptor. The empirical observation made from these efforts is that persistence *in vivo* is a far more significant determinant of *in vivo* activity than is affinity for the receptor; though obviously a molecule that does not bind to the receptor will not activate it. This relatively simple observation needs to be borne in mind in any assessment of ESA 'potency' – data collected *in vitro* might be swayed by the dominant effect of receptor affinity in that setting, whereas data collected *in vivo* tend to be weighted in favor of persistence in the body.

Erythropoiesis

The major function of red blood cells (erythrocytes) is to transport oxygen to the tissues of the body. The erythrocyte component represents about 40% to 45% of the blood by volume. A normal adult may have 5 L of blood and an erythrocyte count of $5 \times 10^6/\mu L$. The red-cell lifespan in such a normal adult is approximately 100–120 days. To maintain a stable number of erythrocytes, replacing those lost by normal aging alone, requires replacement of 1% of the erythrocytes per day, i.e., production of 2.5×10^{11} cells per day or more than 10^{10} cells per hour. This prodigious rate of production is maintained in most circumstances for the lifetime of the individual. Thus, erythropoiesis is a highly dynamic process. EPO has a pivotal role in this process.

As discussed elsewhere in this book, the cellular target of EPO in erythropoiesis was traditionally referred to as the erythropoietin responsive cell (ERC). This population is now known to represent progenitor cells in late blast-forming unit – erythroid (BFU–E) stage through the colony-forming unit – erythroid (CFU–E) stage and the morphologically recognizable erythroblasts. EPOR is, however, absent on reticulocytes and mature erythrocytes [25].

$EPOR^+$ progenitor cells have somewhat different cytokine dependencies in culture, with CFU–E requiring the addition of only EPO to grow and BFU–E requiring EPO and more primitive-acting cytokines previously known collectively as burst-promoting activities (BPA). The BPA are now molecularly identified as cytokines such as stem cell factor (SCF), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6 among others, or occasionally mixtures of all or some of these.

Once EPO has bound and activated EPOR, a cascade of events is set in motion including activation of the dimerized receptor [26], and signal transduction through JAK2, Stat5, MAP kinase protein kinase, PI3 kinase (reviewed in [27–29] and protein kinase C [30, 31]. The actions of EPO include promotion of the survival of sensitive progenitors through prevention of apoptotic processes [32, 33], stimulation of proliferation [34, 35], and differentiation into large numbers of hemoglobinized cells. To maintain homeostasis and supply the necessary number of erythrocytes, the degree of proliferation required is quite remarkable, and occurs in parallel with the acquisition of features of the specialized functions of erythrocytes – accumulation of hemoglobin and disposal of the cell nucleus.

As discussed, EPO does not act alone to stimulate erythropoiesis in the bone marrow. A host of other agents have been shown to affect EPO-driven erythropoiesis. The hierarchy of cell populations leading to erythrocyte production includes successively more specialized cell types. Among the primitive-acting factors, the product of the mouse Steel locus, which is known as SCF and is the functional ligand for the *c-kit* cell-surface receptor, is one of the better understood factors. In mice anemic because of defects in either SCF or *c-kit*, administration of exogenous EPO still increases hemoglobin concentration [36, 37], although to a modest degree. This result would suggest that the few CFU–E that survive functional SCF deficiency *in vivo* can still respond normally to exogenous EPO, though their numbers are reduced by limitations imposed on the cellular pathway at more primitive levels. The interaction of SCF and EPO, which is of obvious significance in these earlier populations, has been elegantly examined at the molecular level and shown to result from a subtle interplay of survival, proliferation, and differentiation signals [38].

These effects at the cellular level lead consistently to increases in hemoglobin concentration in response to increases in circulating EPO, whether exogenous rHuEPO or eEPO produced in response to hypoxia (or a pharmacologic mimic of hypoxia). The relationship between serum concentrations of EPO and response is relatively straightforward (Fig. 1). A significant temporal mismatch exists between the residence time of rHuEPO ($t_{1/2}$ = approximately 8 h), the lifespan of reticulocytes (approximately 5 days), and the lifespan of erythrocytes (approximately 120 days). A more thorough discussion of the pharmacokinetics of EPO is to be found in other chapters in this book, but in summary, an increased EPO serum concentration of relatively short duration (measured in hours or days) can produce a very large number of reticulocytes (see calculations earlier). The flux through the reticulocyte compartment is huge under

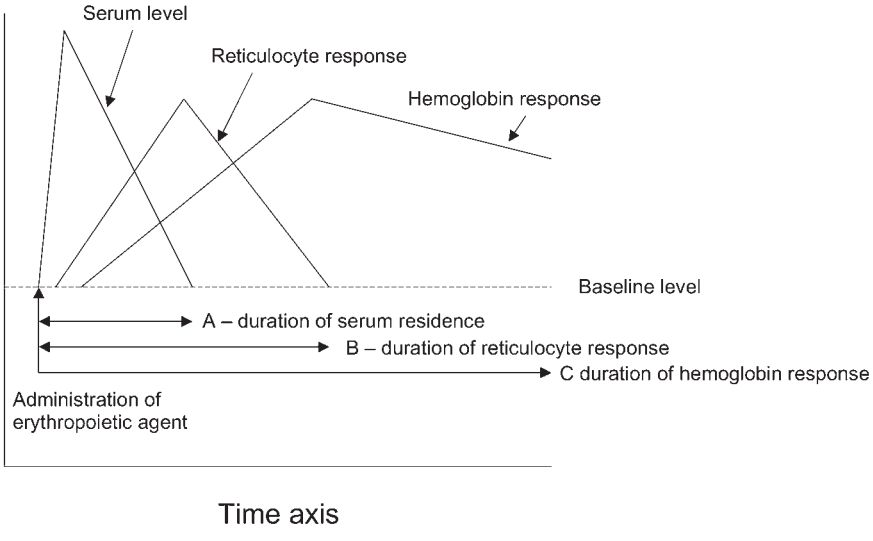

Figure 1. The relationship between residence time of EPO, or any other erythropoietic agent, reticulocyte response and hemoglobin response. The disproportionate effect on hemoglobin from increased amounts of an erythropoietic agent. The duration A is controlled largely by three factors: the dose, the route of injection that controls the gradient of the upward slope, and the rate of clearance that controls the gradient of the downward slope. The half-life of rHuEPO is approximately 8 h. The duration B of the reticulocyte response is determined by the duration of A – the time when serum concentrations of the erythropoietic agent are above baseline. Once serum levels of the erythropoietic agent return to baseline, reticulocyte numbers will begin to decline at a rate proportional to their half-life in the circulation. Reticulocyte lifespan is of the order of 5 days. The duration C is controlled again by the duration B. During the time supranormal numbers of reticulocytes are being produced the rate of production of hemoglobin exceeds the rate of loss, so hemoglobin concentration will be increasing. When reticulocyte numbers return to baseline, the hemoglobin curve will inflect and hemoglobin will be lost at a rate proportional to the lifespan of erythrocytes. Normal erythrocyte lifespan in humans is around 120 days. Overall, an erythropoietic agent with a half-life measured in hours can influence hemoglobin concentrations on a timescale measured in months. Thus changes in the duration A, influenced by dose, route of administration and rate of clearance, will have a disproportionate effect on the duration C.

conditions of accelerated erythropoiesis and delivers a large number of erythrocytes to the circulation. The erythrocytes formed, however, live a comparatively long time (on the order of 3 months). This process, in effect, leads to a disproportion between the duration of accelerated erythropoiesis (sustained by increased amounts of EPO and measured in hours) and the longevity of the hemoglobin response (measured in months). This information is of little value beyond curiosity until new therapeutic derivatives and analogs of EPO or novel dosing paradigms are considered, where an increase in circulating half-life of an erythroid molecule will have a large effect on hemoglobin – on a scale disproportionate to the increased residence time of the EPO analog (Fig. 1).

The body uses short-lived eEPO to sustain a normal blood hemoglobin concentration between 14 and 15 g/dL (in men) by maintaining an eEPO concentration of approximately 20 mU/mL in plasma. Pharmacologic dosing with an

ESA results in concentrations many fold higher than this (e.g., EPO C_{max} is approximately 1,000 mU/mL after a subcutaneously administered dose of 450 U/kg [39] and remains >20 mU/mL for approximately 2 days in humans and perhaps 4 days in rats). Ignoring for a moment disease states where response may be impaired, administration of any of these agents yields serum concentrations many fold higher than the baseline level and effectively maximizes erythroid response while [ESA] > MEC (minimum effective concentration).The essential concept that emerges from animal models of ESA dosing and its relationship to hemoglobin response is the concept of percentage cycle time coverage (%CT, cycle time being the interval between doses). On any dosing schedule, ESA plasma concentrations above the MEC will provide a stimulatory signal to erythropoiesis (accelerated erythropoiesis is 'on'). When plasma concentrations decrease below MEC, hemoglobin is in free fall (erythropoiesis is 'off', the rate of hemoglobin decline is controlled mainly by the lifespan of red blood cells). The proportion of the cycle time (%CT) when erythropoiesis in 'on' *versus* 'off' can then be related to hemoglobin response. The higher the percentage of a cycle that erythropoiesis is 'on' the greater will be the accumulation of hemoglobin over that period. Should the %CT approach 100, then obviously a maximal rate of rise in hemoglobin will be noted, until that is, something else constrains response (iron availability, blood viscosity, etc.). A lower %CT will yield a lower average rate of hemoglobin increase. At some point, any dose and schedule of ESA administration will result in a more or less stable hemoglobin concentration when loss from the compartment that occurs throughout the entire cycle is balanced by input during the period where [ESA] > MEC and red blood cells are being added to the circulating pool. Hemoglobin concentration is relatively well buffered from changes related to ESA dosing, unlike reticulocyte measurements that can wax and wane even on an ESA dose schedule that can be shown to maintain stable hemoglobin concentrations; high shortly after dosing (where [ESA] > MEC and zero later in the cycle (where [ESA] < MEC) (Fig. 2).

Consider the example of weekly dosing, an interval of 7 days between consecutive ESA administrations. In rodents, data obtained with a number of ESAs suggest approximately 40%CT is required, i.e., approximately 2.8 days of [ESA] > MEC is required to sustain a 1 g/dL increase in hemoglobin concentration. With knowledge of the clearance profile of any ESA, it is relatively straightforward to calculate the dose to give 2.8 days coverage and thus the desired dose to sustain hemoglobin concentration on a weekly schedule. The 40%CT can also be used to calculate the dose to sustain an interval of 3 days ([ESA] > MEC for 28.8 h), 2 weeks ([ESA] > MEC for 5.6 days), or any other interval.

Though this model is generally applicable, it is also oversimplified. MEC will be different for each different ESA – reflecting, among other things, affinity for the receptor. MEC also changes in disease settings where response may be constrained by multiple factors, it changes from one species to another, it may be a simple single concentration level, or may be a range through which

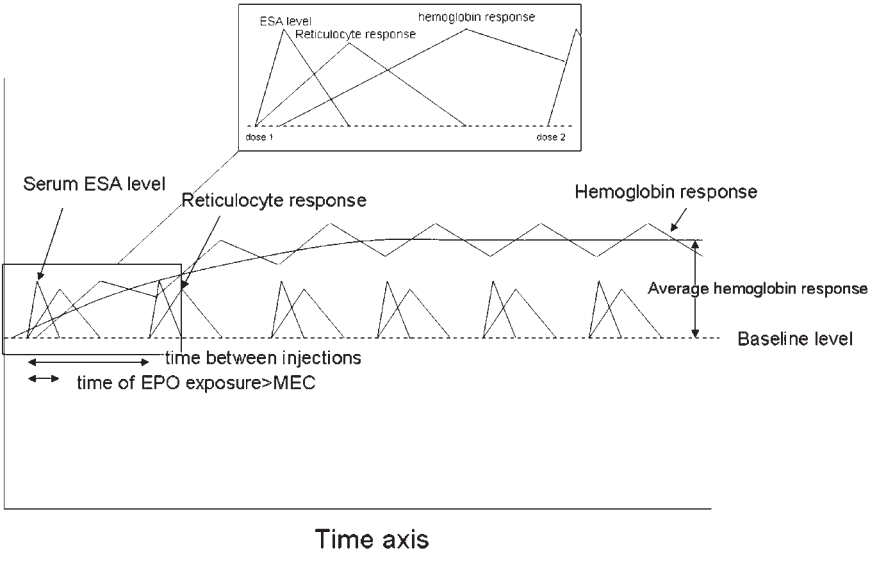

Figure 2. The relationship between exposure to ESA and stable hemoglobin response. As indicated in Figure 1, the duration of ESA exposure can be a small fraction of the time over which a hemoglobin effect can be detected. Further exploring this relationship reveals that to sustain a stable hemoglobin concentration by repeated ESA administration, the major determinant of the hemoglobin concentration attained is the fraction of the interval between administrations where ESA serum levels are above a threshold value. Irrespective of the clearance, the intrinsic potency of the ESA or the interval between administrations it is possible to calculate the percentage of the cycle time (%CT) – the fraction of the interval between administrations – that drives efficacy to a set level.

submaximal response may be obtained, in addition %CT will vary with red blood cell lifespan that is, in turn, dependent on species, disease, and numerous other variables. In general, this concept is broadly applicable to a number of ESAs and forms the basis of how ESA pharmacodynamics can be explained.

One interesting aspect of the %CT model is that essentially any ESA can sustain almost any dosing interval. A short-lived ESA, such as eEPO or rHuEPO, will require doses that increase logarithmically as dosing interval extends beyond their practically useful range. Longer acting analogs, such as darbepoetin alfa and PEGylated epoetin beta, may not suffer this logarithmic dose increase quite so soon, but in turn the maximum dose interval they can sustain will be constrained by the acceptability of their short-term effects.

Overall, much of the machinery involved in translating an increase of either endogenous or recombinant EPO into an increase in circulating hemoglobin concentration has been defined. At the opposite end of the response is the effect of EPO withdrawal. In addition to the predictable cessation of erythropoiesis when EPO is in short supply, the precipitous decrease in circulating EPO concentrations associated with a return from altitude, the return from space flight, and, perhaps, the end of a course of ESA treatment in the clinic, is associated with an accelerated rate of hemoglobin loss from the blood. The

seminal work in this area has led to the proposal of a mechanism of neocytolysis, or the premature destruction of newly formed erythrocytes after interaction with endothelial cells or macrophages [40–42]. The precise role a mechanism such as neocytolysis may play in therapeutic intervention with rHuEPO has to be defined.

Assays

A number of assays were developed to detect erythropoietic activity in body fluids and to assess the potency of manufactured erythropoietic agents. In the early part of the 20th Century possibly the first bioassay of EPO was devised by Carnot when he transferred serum from anemic to normal rabbits and counted red cell number. Although preceded by measurements more or less proximal to erythropoiesis, the seminal work of Cotes illustrated the first viable technique for the quantitative estimate of EPO activity in body fluids [43]. This assay, which was later slightly modified to assess potency and well as quantity, exploited the normal physiologic response to polycythemia.

Rodents can be made polycythemic by either blood transfusion or they can be preconditioned at low oxygen tension. Under either condition the resulting plethora suppresses EPO production and therefore ongoing erythropoiesis. Under this condition, the animal becomes very sensitive to the administration of exogenous ESA and its effect can be readily measured by the incorporation of administered radioactive iron into the hemoglobin newly synthesized in response to ESA administration. This type of assay was the mainstay of rHuEPO potency assays for most of the 1990s, indeed it was even modified to account for longer acting analogs such as darbepoetin alfa [44]. This assay is perhaps still the gold standard assay for EPO activity for a number of reasons. Other assays such as single timepoint reticulocyte assays and *in vitro* cell proliferation assays (both cell lines and primary tissues) are also employed but a number of measurements from different systems need to be combined to approach the usefulness of the ^{59}Fe incorporation assay (Tab. 1).

The unit of EPO activity

Historically, assessing the activity of erythropoietin purified from an array of sources required comparison with a standard preparation, to assure comparability across investigators, sites, and studies. Preceding the adoption of a standard preparation one comparator was the result obtained in an iron incorporation assay with 5 μM cobalt chloride [45] – the so-called ‘cobalt unit’. The mechanism by which cobalt salts such as this can indirectly stabilize HIF-1α and upregulate *EPO* gene expression is described [46]. Other standards were a dried extract of rabbit plasma and a preparation of anemic sheep plasma (adopted as the first International Reference Standard; Standard A in 1961

Table 1. A comparison of assays of EPO performance

	Example format	Detects	Pros	Cons
In vitro assays				No PK component. Tend to emphasize differences in affinity for EPOR.
Immunoassay	EPO ELISA	EPO core peptide	Sensitive, specific, inexpensive	Does not measure activity. Variably applicable to different analogs but can only be used for ESA-like analogs Cannot distinguish eEPO from other ESAs
Affinity	BIACORE	Affinity for receptor	Sensitive, specific	Expensive Does not measure activity. For analogs, can read out as the inverse of *in vivo* activity
Cell line proliferation	UT/7 EPO	Proliferation or other measure of response (e.g., respiration, reporter genes)	Sensitive, can be selective, simple, inexpensive, acceptable biological relevance	Can read out as inverse of *in vivo* activity
Primary cell proliferation	Spleen cells BFU–E, CFU–E	Proliferation, or clonal growth	Good biological relevance. Can be performed with human tissue.	Can read out as inverse of *in vivo* activity. Clonal assays somewhat subjective. Labor intensive. Subject to inter-donor variability
In vivo assays				Animal use, labor intensive
^{59}Fe incorporation	Ex-hypoxic (polycythemic) mouse assay	ESA or non-ESA potentcy and short-term PK	Integrates activity over exposure time i.e., includes both potency and PK (requires adaptation for longer lived molecules. Sensitive, reproducible (low n).	Radioactive basis

(continued on next page)

Table 1. (Continued)

	Example format	Detects	Pros	Cons
In vivo assays				Animal use, labor intensive
Reticulocyte assay	Single dose ESA, single timepoint reticulocyte count	ESA or non-ESA potency and to a lesser degree, PK	High biological relevance Sensitive.	Highly variable, requires high n.
Single timepoint hemoglobin	Single dose ESA, single timepoint erythrocyte count	ESA potency and to a lesser degree, PK	High biological relevance	Highly variable, requires high n. Insensitive
Hemoglobin maintenance assay	Weekly administration of ESA, repeated hemoglobin measures over several weeks to months	ESA potency and short-term PK	Highly sensitive	Long-term assay, significant risk of immune reaction; hence highly variable, highest n

eEPO = endogenous EPO; EPOR = EPO receptor; ESA = erythropoiesis-stimulating agent; PK = pharmacokinetics

[47]). This material was assigned the activity of 10 units per ampoule, the unit being roughly equivalent to the 'cobalt unit'. Subsequent to this, the first standard adopted by the WHO in 1964 (the Second International Reference Standard) was purified from human urine and named Standard B [48] with an assigned activity of 10 units per ampoule. 'Activity' defined by this unit came from some form of iron incorporation assay. In the standard application of this assay, the radioactive readout represents an index of the accumulated hemoglobin synthesis across the time interval between administration of isotope and sampling of the blood. It therefore comprises both a pharmacokinetic and a pharmacodynamic component – integrating the persistence of the agent across the time interval (pharmacokinetic) with the stimulatory effects (pharmacodynamic), yet these components cannot be easily deconvoluted. A highly potent analog that is rapidly cleared from the body might read out the same as a modestly potent material which persisted for the duration of the assay. It became apparent that even though this type of assay was suitable for the level of heterogeneity found in different EPO preparations it was not readily applicable to unnatural or engineered forms such as darbepoetin alfa. In considering in some detail how activity of epoetin alfa and darbepoetin alfa might be compared Sasu et al. [49] concluded that the EPO 'unit' is not a legitimate measure of the activity of engineered derivatives. The usefulness of the standard preparation remains however, as more pharmaceutical preparations of erythropoietin become available under emerging regulatory guidelines for follow-on or biosimilar biologics. The International Standard has been updated with The First International Standard for Recombinant Derived Erythropoietin in 1990 and The Second International Standard for Recombinant Derived Erythropoietin in 2003.

Pharmacology – disease rationale

The administration of rHuEPO in a situation where every other constituent of the biologic response is in excess and EPO is the only limiting factor in erythropoiesis will yield the most marked response. Such a situation is extremely rare. Even in the case of renal disease where compromised endogenous EPO production is an issue, other factors such as uremia, general inflammation, or iron deficiency can limit the effectiveness of injected rHuEPO by placing other constraints on the response [50]. These constraints may be the demonstrated inhibition of erythropoiesis by tumor necrosis factor (TNF), IL-1, and interferon, the proinflammatory cytokines [51], or by limited supply of iron for hemoglobin synthesis. Recognizing that such a clear-cut situation is not likely to present itself, the effectiveness of administered rHuEPO will depend on a milieu of negative influences including those above in addition to anti-EPO antibodies and soluble EPOR. The latter two factors have direct consequences for rHuEPO therapy. Antibodies to EPO that are capable of neutralizing the action of administered rHuEPO have been noted in normal patients [52] and

also in diseases such as systemic lupus erythematosus and HIV infection [53–55]. Antibodies may be raised in response to administered rHuEPO with serious consequences that may include refractoriness to both rHuEPO and eEPO and a long-term dependence on blood transfusions for survival. Soluble EPOR is a truncated form of the full-length membrane-bound version that has been suggested to act as a competitive inhibitor of the cell-borne form of the receptor and can limit the response to injected rHuEPO [56], although this has not been well established.

Interaction with iron

Iron is a vital component of hemoglobin and limitations on its supply can compromise the ability of erythropoietic cells to synthesize hemoglobin. Paradoxically, iron is also a poisonous metal for which most mammals, including humans, have highly evolved strategies to ameliorate its toxicities. Iron deficiency is commonly discussed, although the body's treatment of iron as a toxin can lead to its deposition as relatively inert forms (such as ferritin or hemosiderin) in various tissues [57]. This iron maldistribution situation, where the body load of iron may be excessive yet the presentation is one of functional iron deficiency, is typical of anemia of chronic disorders, the anemia of cancer, and perhaps other anemias with an inflammatory component. When deficient, and depending upon the reason for the deficiency, supplementation with oral or intravenous iron may be relatively straightforward. The move from oral administration of simple salts such as ferrous sulfate towards more complex intravenous forms of iron resulted from a series of clinical studies, although agreement is not complete on whether one is more beneficial than the other [58–61]. Emerging data suggest that when hepcidin, the key regulator of iron metabolism, is increased absorption of iron from the diet may be compromised. Assays for the detection of hepcidin are in their infancy and it remains to be seen whether circulating hepcidin affects the effectiveness of oral iron supplementation in some cases. EPO has not been shown to have a direct role in mobilizing iron from storage organs, but can act indirectly by stimulating hemoglobin synthesis and drawing iron from storage pools or perhaps other mechanisms [62]. Although iron may often be a limiting step in erythropoiesis, it may be in part corrected by supplementation or mobilization from existing body stores.

The use of EPO in different species

EPO from mammalian species as diverse as human, hamster, rat, mouse, cat, lion, dog, horse, sheep, dolphin, and pig has been cloned and sequenced and found to be remarkably conserved with a minimum identity of 79.8% between any two of these species [63, 64]. This finding has lead to the experimental use

of rHuEPO in cross-species settings, for example in anemic FLV-infected cats [65], for idiopathic anemia in dolphins [66], in blood doping of racehorses [67], in transient brain ischemia in gerbils [68], for subarachnoid hemorrhage in rabbits [69], and to study erythrokinetics in sheep [70]. Even with the high degree of conservation across species, the administration of rHuEPO can raise antibodies that may cross-neutralize eEPO (e.g., in horses [71]). Despite the precise species match between recombinant and endogenous forms of EPO, instances of cross-neutralizing antibodies have been reported in humans receiving some forms of rHuEPO, but not with other forms bearing an identical amino acid sequence. It may be the case that although these antibodies bind to similar epitopes on the protein backbone of rHuEPO and endogenous EPO, their induction is dependent on other factors such as autoimmune disease or manufacturing or formulation methods resulting in protein aggregation or degradation.

Rationale for rHuEPO treatment in disease

Anemia of renal failure

As the kidney represents the major site of EPO production in the adult, the progressive destruction of this organ might be intuitively linked to anemia of an EPO-limited nature. Indeed, since shortly after the gene was cloned in 1983, the anemia of renal failure has been successfully treated with rHuEPO [72, 73]. In fact, rHuEPO represents a routine treatment for patients receiving regular hemodialysis or peritoneal dialysis and for many patients not receiving dialysis (i.e., predialysis patients) [74, 75]. Biologically, this use represents a form of hormone supplementation therapy, although the benefits to the patient extend beyond the reduced need for transfusions and the associated risks of iron overload, incompatibility reactions, and viral infections; and include the benefits of increased exercise tolerance, cognitive and psychomotor changes, and relief from hyperdynamic cardiac states [76–78]. In total, these changes produce a significant impact on the quality of life of patients [79]. Beyond the surprisingly wide ramifications of correction of anemia in these patients, the biologic basis for rHuEPO therapy in patients with renal disease remains replacement of a relative hormone deficiency (in contrast to absolute deficiency) secondary to failure of the organ that produces the hormone. Other uses for rHuEPO, for example in anemias associated with chemotherapy treatment have led to extensive use outside the dialysis clinic.

Anemia in oncology

Anemia in patients with cancer results from a complex interaction of various factors [80] that make treatment somewhat less predictable than the hormone

replacement-like use in renal failure states. Included in the list of contributing factors are hemodilution, bleeding, hypersplenism (and hemophagocytosis), hemolysis, nutritional deficiencies, marrow damage, chemotherapy and radiotherapy, and the anemia of cancer itself (included in the broader classification of anemia of chronic disease) [81–84].

Addressing any one of these factors alone represents a significant challenge, yet the perceived benefits of managing anemia in patients with cancer has led to a number of successful therapeutic strategies. Perhaps the most obvious rationale for rHuEPO use in anemic patients with cancer is in patients with documented low endogenous serum EPO concentrations. Concentrations of eEPO are inherently variable and use of the data is further confounded by a phenomenon widely documented in anemia of chronic disease where the absolute EPO concentration is within the normal range and is low only when considered relative to the degree of anemia – the so-called 'blunted' EPO production [85]. For the most part, studies of endogenous EPO concentrations in animals have not illustrated principles to be applied to clinical practice. For example, inappropriately low EPO concentrations in tumor-bearing mice did not confirm the earlier observations made in tumor-bearing rats [86, 87]. Later work, however, showed that eEPO concentrations were predictable and consistent with the degree of anemia [88]. In addition, the effects of chemotherapy on eEPO concentrations have been variously reported to increase, decrease, or remain unchanged [89–91].

Patients with cancer frequently have circulating eEPO amounts that are lower than would be expected for the degree of anemia. In early studies in patients with cancer, inappropriately low eEPO concentrations were reported, yet only in association with other problems such as infection or inflammation [92], while other studies showed normal levels [93, 94]. Blunted EPO production, however, has been seen in more recent work in patients with cancer without complications, in contrast to patients with iron-deficiency anemia [95–99]. Overall, a lower endogenous serum EPO concentration than might be expected with the degree of anemia has suggested that rHuEPO use is warranted in anemic cancer patients even before they receive chemotherapy.

Chemotherapy- or radiotherapy-induced anemia is the most frequently encountered anemia in patients with cancer [100]. Chemotherapy or radiotherapy cause systemic changes outside the bone marrow that result in anemia, e.g., hemolysis, blood loss, nutritional deficiency, damage to the erythroid populations in the bone marrow, or other conditions may reduce hemoglobin concentrations [101]. The eEPO concentration in such patients can be variable depending on a host of factors including, but not limited to, tumor type and chemotherapy agent. Most patients (more than 50%) respond to administered rHuEPO [102]. Whether this responsiveness results from the correction of low eEPO values or a heightened response from supernormal circulating EPO values (combining both endogenous and exogenous material) is unclear. The observation remains that the proportion of patients responding to rHuEPO after chemotherapy increases with increasing dose. In the case of limited marrow damage, the

problem can be overcome by rHuEPO administration, although when marrow damage becomes extensive, it is unlikely that rHuEPO will be effective.

Other anemias

Although the most appealing indication for a recombinant protein therapeutic may be where amounts of the endogenous prototype are limited, the discussion earlier suggests that, in cancer at least, an eEPO-deficit condition is not the only time when using rHuEPO may be useful. In general in those cases where endogenous serum EPO concentrations are reduced, administration of rHuEPO is most effective. Thus, the rationale for rHuEPO therapy in iron-deficiency anemia due to limited dietary iron intake, for example, is weak. Anemic conditions range from an apparent EPO-deficit condition documented in neonates through premature erythrocyte destruction noted in elderly patients. Despite the different cause of these two types of anemia, rHuEPO appears to be effective in both. In contrast to these anemias are the hemoglobinopathies, where an rHuEPO-induced increase in hematocrit, without the accompanying conversion of hemoglobin type from diseased to fetal (e.g., with hydroxyurea therapy), would be of limited benefit. Anemia in the various intensive care patient populations responds, in general, quite well to rHuEPO and appears to have many features in common with anemia of chronic disease. Similarly, the anemia noted in patients with AIDS, despite the multifactorial causation, responds predictably to rHuEPO.

A variety of anemias exist, many of which will respond well to rHuEPO administration, but other cases exist where rHuEPO may not be a good therapeutic choice. In anemic conditions where eEPO concentrations are low or even normal, rHuEPO has a good chance of being effective. In other conditions where EPO concentrations are supernormal, the administration of rHuEPO can help where it might be reasoned that EPO is not the only limiting factor in the development of anemia, but that supplementing EPO can make up for the limitation elsewhere in erythropoiesis [36].

Non-anemic conditions

There exists a widespread literature on the effects of EPO in nonerythropoietic tissues. For these effects to be direct EPOR must be present in the target tissue. Recent studies illustrate very clearly that the presence of mRNA for EPOR is not sufficient to guarantee that functional receptor is expressed or functional [103–106]. Neither it turns out is antibody mediated detection of EPOR with the most commonly used reagents a reliable method to detect the receptor. However, it remains a point for discussion whether a direct action of ESAs via tissue borne EPOR is the only way in which an ESA could result in a biologically useful signal in target tissues. A shift in oxygen saturation asso-

ciated with a hemoglobin response, changed ferrokinetics in response to ESA administration, and changes in blood pressure or coagulation parameters are examples of effects that might be mediated indirectly by ESAs.

Unfortunately, many of the studies purporting to show an effect of rHuEPO in tissue protection, angiogenesis, nerve regeneration, stroke, etc., have to be re-examined in the light of defective methods used to detect EPOR expression and function. If, as would appear to be the case, these tissues do not express physiologically relevant, functional amounts of EPOR, then the findings cannot be interpreted as being direct since there is no tenable mechanism of action.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Erythropoiesis – genetic abnormalities

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Introduction

We review genetic mechanisms altering erythropoiesis that lead to either an increased red cell mass (polycythemia/erythrocytosis) or decreased red cell mass (anemia). Since polycythemia is often and inconsistently referred to by an alternative term, erythrocytosis, and, as no consensus on usage has been reached, we refer to the individual entities by the term used in the original description. Anemias are far more common than polycythemic states, and most are due to acquired nutritional, autoimmune, and toxic causes; or to inherited globin, cytoskeleton, and red cell enzyme mutations. These causes result in hemolytic, microcytic/hypochromic, hypoproliferative, or inefficient erythropoiesis anemia phenotypes. In this review, we concentrate on the few well-delineated germline or somatic mutations disturbing normal control of erythropoiesis that cause a disease phenotype either in humans or in mouse.

Erythropoiesis

Although regulation of erythropoiesis is described in other chapters of this book, for better understanding of this chapter we provide the pertinent details that are relevant for delineation of polycythemias and anemias. Erythrocyte production, i.e., erythropoiesis, is a tightly regulated system, but the details of its regulation are not fully elucidated. Much remains to be learned from uncovering the molecular basis of many congenital and acquired mutations that disrupt the control of erythropoiesis.

Erythropoiesis can be viewed as composed of three stages. In the initial stage, commitment of pluripotent hematopoietic progenitors to committed erythroid precursors takes place. The second stage is characterized by expansion of erythroid progenitors that is largely regulated by erythropoietin (Epo) and is made effective by the appearance of its receptor (EpoR) on surface of these progenitors. The expression of EpoR peaks in early erythroblasts and then declines. The terminal stage consists of enucleation and removal of remnants of organelles and nucleotides that may be toxic to mature circulating erythro-

cytes. Defined defects of erythropoiesis leading to polycythemia occur at the second stage of erythropoiesis and are generally those enhancing proliferation and or delaying apoptosis.

Prevention of apoptosis is a well-recognized mechanism assuring productive early erythropoiesis and preventing anemia; however, the importance of proapoptotic processes for productive terminal erythropoiesis is newly recognized. Whether a congenital imbalance of proapoptotic and antiapoptotic processes can contribute to polycythemia remains to be determined.

The role of microRNAS (miRNAs) in regulation of erythropoiesis is being defined. Some miRNAs are mainly expressed in early stages of erythropoiesis, others in late stages, and some have biphasic expression during erythroid differentiation; and some appear to have erythroid specific expression. miRNAs exact role in erythropoiesis and their molecular targets are currently being defined at many laboratories [1].

Polycythemias

Classification

Polycythemia can be classified as primary polycythemia, secondary polycythemia, or polycythemia due to abnormal hypoxia sensing, of which one such entity (Chuvash polycythemia) has features of both primary and secondary polycythemia. Primary polycythemias are caused by intrinsic defects in the erythroid precursors that result in an excessive inappropriate response to normal stimulators of erythropoiesis. In contrast, in secondary polycythemias, the cytokine responsiveness of erythroid progenitors is normal. Secondary polycythemias are driven by hormonal factors (predominantly Epo but also insulin-like growth factor-1, angiotensin 2, and cobalt) extrinsic to the erythroid progenitor cells, and the increased erythrocyte mass represents a physiologic response to tissue hypoxia or abnormal autonomous Epo production [2]. Secondary polycythemias may result from an appropriate physiologic response to tissue hypoxia; for example, tissue hypoxia due to pulmonary or cardiac disease of from high affinity hemoglobins, which release less oxygen per gram of hemoglobin, results in an increased compensatory erythropoiesis that is an appropriate physiologic response to accommodate for decreased oxygen delivery. Secondary polycythemias resulting from increased amounts of Epo secreted from tumors or from dysregulated control of Epo production are inappropriate physiologic responses.

Polycythemias due to abnormal hypoxia sensing may blur the distinction between primary and secondary polycythemias. Increased amounts of Epo (or inappropriately normal Epo in conjunction with concomitant increase of red cell mass) resulting from abnormal hypoxia sensing are characteristic of these polycythemias. Yet when erythroid progenitors responses to Epo were studied

in Chuvash polycythemia, the erythroid progenitors were also hypersensitive to Epo.

Both primary and secondary polycythemias can be either acquired or congenital, but polycythemia due to abnormal hypoxia sensing is usually congenital. Acquired conditions that lead to increased Epo production, such as chronic hypoxia and a variety of tumors, are the most common causes of secondary polycythemias. Congenital cyanotic heart or lung disorders leading to tissue hypoxia and increased amounts of Epo are examples of secondary congenital polycythemias. These types of polycythemia are not addressed in this review, which focuses only on polycythemias due to known genetic mechanisms. Hypoxemia from pulmonary or heart disease or from exposure to extreme high altitude, however, leads in some but not all of the affected individuals to an appropriate increase of red cell mass. The individual variation of erythropoietic responses to a hypoxic stimulus suggests that there is a genetically determined pleomorphism underlying the erythropoietic response to hypoxia.

Primary polycythemias

Primary polycythemias result from intrinsic defects in red blood cell precursors that cause increased responsiveness to Epo. The most common type of primary polycythemia is polycythemia vera, which is due to an acquired somatic mutation. An inherited primary polycythemia is primary familial and congenital polycythemia (PFCP).

Polycythemia vera (PV), an acquired primary polycythemia, is characterized by clonal expansion of hematopoietic precursors, and is the most common type of primary polycythemia [3]. Hematopoiesis in PV is characterized by accumulation of phenotypically normal erythrocytes with variable overproduction of platelets and myeloid leukocytes. *In vitro*, PV erythroid progenitors (BFU–Es) develop in the absence of exogenously added Epo [4]; a hallmark of PV. A single nucleotide substitution (G1849T) generates *JAK2 V617F* [5] that is present in the bone marrow and peripheral blood myeloid cells of >95% of patients with PV. This mutation leads to loss of function of the inhibitory auto regulatory pseudokinase domain [5], which results in augmented or constitutively active JAK2 activity that explains many of the *in vitro* defined characteristic abnormalities of PV erythroid progenitors [5]. Most patients with PV have at least some progenitors homozygous for *JAK2 V617F* mutation as a consequence of uniparenteral disomy [6]. However, *JAK2 V617F* is not specific for PV as it is also found in approximately 50% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) [7, 8]. *JAK2 V617F* also has been identified in a small proportion of patients with chronic neutrophilic leukemia, acute megakaryocytic leukemia, and in most patients with refractory anemia with ringed sideroblasts and thrombocytosis (RARS-T) [7,

8]. The nonspecificity of the mutation, the absence of *JAK2 V617F* in some patients, the existence of familial PV in which affected members can be either *JAK2 V617F*-negative or positive, the fact that only a proportion of clonal PV cells are *JAK2 V617F*-positive, and the existence of Epo-independent BFU–Es that are either *JAK2 V617F*-negative or positive demonstrate that this somatic mutation of *JAK2* is not the sole pathogenic process in PV [9–12]. Moreover, several mutations in exon 12 of JAK2 have been described in *JAK2 V617F*-negative patients [13, 14].

Evidence exists that the acquired augmented erythropoiesis in PV, which is caused by somatic mutations, often has a familial predisposition [15, 16], which suggests the existence of germ-line mutation(s) that either facilitate the somatic mutation(s) or contribute to the acquisition of somatic acquired mutations to result in familial clustering of PV. The molecular basis of these interactions remains to be defined.

PFCP is characterized by an autosomal dominant mode of inheritance, and less frequently, by the occurrence of sporadic cases [17, 18]. The clinical features of PFCP include the presence of isolated erythrocytosis, absence of predisposition to development of acute leukemia or other myeloproliferative disorders, absence of splenomegaly, normal white blood cell and platelet counts, low plasma Epo values, normal hemoglobin-oxygen dissociation curve (indicated by a normal P50), and hypersensitivity of erythroid progenitors to exogenous Epo in *in vitro* studies [17–20]. PFCP is generally thought to be a benign condition, but it has been reported to be associated with predisposition to cardiovascular problems, such as hypertension, coronary artery disease, and cerebrovascular events, that are not clearly related to an increased hematocrit. Association with cardiovascular disease however, has not been described in all series [21–23].

The distal cytoplasmic region of EpoR, in association with SHP-1, is required for down-regulation of Epo-mediated activation of JAK2/STAT5 proteins [24–26]. To date, nine mutations of *EPOR* have been convincingly linked with PFCP [27]. All of these mutations result in truncation of the EpoR cytoplasmic carboxyl terminal leading to loss of its negative regulatory domain, resulting in a gain-of-function of EpoR. Three additional missense *EPOR* mutations have been described in families with PFCP, but they have not been linked to PFCP or any other disease phenotype [28]. The absence of a polycythemic phenotype in some patients with *EPOR* mutation is suggestive of a role played by gene modifiers or epigenetic factors in phenotypic penetrance [29]. On the other hand, mutations of the *EPOR* were found in only 12% of subjects with a PFCP phenotype, suggesting that in a majority of PFCP families, mutations in genes other than *EPOR* result in defective Epo signaling and accumulation of erythrocytes [27, 30, 31]. In those sporadic cases with a proven *EPOR* gain-of-function mutation, a *de novo* occurrence of this mutation is typically demonstrated [27, 32], underscoring the likely deleterious impact of this mutation on survival.

Secondary polycythemias

Secondary congenital polycythemia results from conditions that lead to increased amounts of endogenous Epo. Genetic causes include hemoglobin variants with high affinity for oxygen, congenitally lower amount of erythrocyte 2, 3 biphosphoglycerate (2,3-BPG), and inherited methemoglobinemias. All these conditions are characterized by a left shift in the hemoglobin dissociation curve, which in turn leads to tissue hypoxia and physiologically appropriate increased amounts of Epo. Although these entities represent the first polycythemias with a delineated pathophysiology and molecular cause, in our experience, they are frequently misdiagnosed as PV and PFCP.

High-oxygen affinity hemoglobin mutants

An autosomal dominant polycythemia with a normal-to-increased Epo concentration is suggestive of a mutant hemoglobin with high affinity for oxygen [33]. The affinity of hemoglobin with oxygen is expressed as the P50, which is the partial pressure of oxygen in blood at which 50% of the hemoglobin is saturated with oxygen. An high-affinity hemoglobin reflected in the laboratory by an abnormally low P50 reflects an increased affinity of hemoglobin for oxygen and is supportive of a high-oxygen affinity hemoglobin variant or decreased 2,3-BPG level. If cooximeter equipment is unavailable, the P50 can be conveniently calculated from venous gas parameters [34].

Amino acid substitutions involving the hemoglobin $\alpha 1/\beta 2$ interface, the *C*-terminal residues of the β chain, or the 2, 3-BPG binding sites can all affect the affinity of hemoglobin for oxygen. Most mutations affecting oxygen affinity result in high-affinity hemoglobin variants that produce relative tissue hypoxia resulting in compensatory secondary appropriate polycythemia [33, 34]. Almost 100 hemoglobin variants are known to be associated with high affinity for oxygen. Establishing a correct diagnosis of a high-affinity hemoglobin variant is important as these patients have normal life expectancy and do not require phlebotomy.

2, 3-BPG deficiency

Congenitally low amounts of erythrocyte 2, 3-BPG can occur because of deficiency of the red cell enzyme 2, 3-BPG mutase [35, 36]. This condition is an extremely rare autosomal recessive one but should be suspected in the case of isolated polycythemia (without any feature of myeloproliferative disorders such as progressive increase of red blood cell mass, high platelet and granulocyte count, and splenomegaly), absence of a family history, and low P50 (signifying high-oxygen affinity). A mutant hemoglobin needs to be ruled out first. In both 2, 3-BPG deficiency and high-affinity hemoglobins, the red cells will have high oxygen affinity; however, unlike high-affinity hemoglobin, the oxygen affinity of the dialyzed hemolysate is normal and the level of 2, 3-BPG is very low.

Congenital methemoglobinemias

Three types of hereditary methemoglobinemias have been identified (reviewed in [36]). Two are inherited as autosomal recessive traits: cytochrome b5R deficiency and cytochrome b5 deficiency. The third type is an autosomal dominant disorder, hemoglobin M disease in which there is a mutation of one of the globin genes. All congenital methemoglobinemias are associated with suboptimal delivery of oxygen per red cell and this may result in compensatory secondary appropriate polycythemia. The resulting polycythemia is typically mild and therapy is not necessary as it would only decrease tissue oxygen delivery and lead to tissue hypoxia.

Polycythemia due to abnormal hypoxia sensing

Chuvash polycythemia

Chuvash polycythemia was the first recognized hereditary condition of augmented hypoxia-sensing. Chuvash polycythemia (CP), the only known endemic polycythemia, is an autosomal recessive hereditary polycythemia. The Chuvash people reside in the mid-Volga River region in Russia wherein CP affects hundreds of people, making it the most common congenital polycythemia [37]. In a study of five multiplex Chuvash families with CP, a homozygous mutation of the von Hippel Lindau (*VHL)* gene was found in the affected individuals [38, 39]. This mutation impairs the interaction of pVHL with both hypoxia inducible factor (HIF)-1α and HIF-2α, reducing the rate of ubiquitin-mediated destruction of HIF-1α. As a result, the amount of the HIF-1 and HIF-2 heterodimers increase and lead to increased expression of target genes including *EPO*, *VEGF*, and plasminogen activator inhibitor (*PAI*) among others [39, 40]. CP is associated with a predisposition to development of thrombosis, bleeding, cerebral vascular events, and increased mortality. It is characterized by an intact response to hypoxia despite increased basal expression of a broad range of hypoxia-regulated genes in normoxia. Unexpectedly, CP erythroid progenitors are hypersensitive to Epo [38, 39], thus CP shares the features of both primary as well as secondary polycythemia. Despite increased expression of HIF-1α and HIF-2α and VEGF in normoxia, patients with CP do not display predisposition to tumor formation. Imaging studies of 33 patients with CP revealed unsuspected cerebral ischemic lesions in 45% but no tumors characteristic of VHL syndrome [40].

Homozygosity for the *VHL 598C>T* has been reported to occur sporadically also in whites in the United States and Europe and in people of Southeast Asian (Indian subcontinent) ancestry [41–43]. A high prevalence of this disorder in the Italian island of Ischia has been reported [44]. To address the question of whether the *VHL 598C>T* substitution occurred in a single founder or resulted from recurrent mutational events, haplotype analysis was performed on subjects bearing the *VHL 598C>T* mutation and normal unrelated individuals from Chuvash, Asian, white, Hispanic, and African-

American ethnic groups [45]. These studies indicated that in most individuals, the *VHL 598C>T* mutation arose in a single ancestor between 12,000 and 51,000 years ago. However, a Turkish polycythemic family had a different haplotype indicating that the *VHL 598C>T* mutation in this family occurred independently [46].

In contrast, autosomal dominant mutations of the *VHL* gene cause VHL syndrome [47]. Patients heterozygotic for dominant *VHL* mutations are at increased risk of developing hemangioblastomas, renal cell carcinoma, pheochromocytoma, pancreatic endocrine tumors, and endolymphatic sac tumors when they acquire a somatic mutation in the normal *VHL* allele [47–50]. Some patients with VHL syndrome also develop acquired polycythemia [47]. The development of hemangioblastoma and renal cell carcinoma associated with VHL tumor predisposition syndrome has been proposed to be related to increased expression of HIF and possibly VEGF [51, 52]. The absence of a predisposition to tumorigenesis in patients with CP implies that deregulation of HIF-1 and VEGF may not be sufficient to cause predisposition towards tumor formation in VHL syndrome.

Non-Chuvash germline *VHL* mutations also cause polycythemia. Some patients with congenital polycythemia have proven to be compound heterozygotes for the Chuvash mutation, *VHL 598C>T*, and other *VHL* mutations including *562C>G*, *574C>T*, *388C>G*, and *311G>T* [27, 41, 42]. A Croatian boy was homozygous for *VHL 571C>G*, the first example of a homozygous *VHL* germline mutation other than *VHL 598C>T* causing polycythemia [27, 42]. Additionally, a polycythemic Portuguese girl was a compound heterozygote for *VHL 562C>G* and *VHL 253C>T* [27, 53].

A few cases of congenital polycythemia, known to have mutations of only one *VHL* allele, confound an obvious pathophysiological explanation. Two Ukrainian children with polycythemia were heterozygotes for *VHL 376G>T*, but the father with the same mutation was not polycythemic [41]. Peripheral blood erythroid progenitors from the children and father were hyper-responsive to recombinant human Epo in *in vitro* clonogenic assays in a way similar to what is seen in patients with CP. An English patient was a heterozygote for *VHL 598C>T* [43], although the inheritance of a deletion of a *VHL* allele or a *null VHL* allele in a trans position was not excluded in this patient. Subsequently, two patients with polycythemia who were heterozygous for VHL were described in whom a *null VHL* allele was more rigorously excluded [27, 46]; one of these patients also had ataxia-telangiectasia [53].

Proline hydroxylase and HIF-2α *mutations*

Two families, one with a proline hydroxylase (*PHD2)* mutation [54] and other with a *HIF-2α* mutation [55], were described. In both cases, heterozygotes for this mutation had a mild or borderline polycythemia that the authors referred to as erythrocytosis [54, 55]. Testing for Epo-hypersensitivity of erythroid progenitors was not reported. Because of the small family size, the possibility of a nonerythroid etiology for the erythrocytosis could not be excluded, but this

work further supports the concept that abnormalities in factors involved in the regulation of HIF signaling can result in polycythemia.

Based on their study of the *HIF-2α* mutation [55], Percy and colleagues concluded that HIF-2α may be sole and central to regulating amounts of Epo. While it is clear that HIF2 is the transcription factor that regulates hepatic Epo production, the kidney is the major site of Epo synthesis, HIF1α was identified by its binding to a 3' hypoxia responsive element of kidney *EPO* [56], and severe anemia develops after nephrectomy. In contrast, liver *EPO* is regulated by upstream nucleotide sequences [57] and only about 10% to 20% of Epo is produced by the liver (reviewed in [55]).

A potential alternative explanation for the phenotype described is that it may result predominantly from autonomous liver-generated Epo (possibly with suppressed renal Epo production) [58]. These investigators created a mouse with a conditional deletion of *Vhl* in epidermal keratinocytes, which caused cutaneous vasodilatation and increased expression of *Hif-1α* and *Hif-2α*. Although keratinocytes do not make Epo, the amount of Epo in these mice was increased, and they became polycythemic. Further studies of this epidermal *Vhl* knockout mouse revealed that the increased amounts of HIF-1 caused upregulation of inducible nitric oxide synthase, which in turn led to increased cutaneous nitric oxide (NO), a potent vasodilator. This NO-induced skin vasodilatation resulted in decreased perfusion of other organs, most notably the liver, with subsequent hypoxia-induced increased expression of hepatic *Hif-2α*, which in turn caused increased expression of the *Epo* gene. In follow-up experiments using mice with wild-type *Vhl*, the authors deleted the cutaneous genes for either *HIF-1α* or *HIF-2α* and crossbred these mice with those with the conditional skin *vhl* deletion. Unexpectedly, under conditions of normoxia, the loss of *HIF-1α* and *HIF-2α* had no effect on Epo levels. Under hypoxic conditions, however, the *HIF-1α* epidermal knockout mice did not display an appropriate increase in renal *EPO* gene transcription and were unable to mount an appropriate renal Epo response. These experiments show the importance of epidermal HIF in sensing environmental oxygen levels and regulating systemic hypoxic responses in mice, with physiologic regulation mediated primarily by HIF-1α while the pathologic loss of *Vhl* is mediated primarily by HIF-2α [58]. Whether this intriguing mechanism of regulation of the mouse *EPO* gene by a skin/NO pathway is present only in mice, or also operates in human *EPO* regulation remains to be proven.

Unexplained congenital polycythemias with increased or inappropriately normal amounts of erythropoietin

In our experience, most patients with congenital polycythemias with normal or increased amounts of endogenous Epo do not have *VHL* mutations, hemoglobinopathies, or 2,3-BPG deficiency, and the molecular basis of polycythemia in these cases remains to be elucidated. Proline hydroxylase and *HIF-2α* mutations have been excluded in some, but not in all of these patients. It is not clear why in some families, the polycythemia is dominantly inherited

[59], in others recessively, and in some it is sporadic, or why in families with the same mutation the phenotype differs. Lesions in genes linked to hypoxia independent regulation of HIF as well as oxygen-dependent gene regulation pathways are leading candidates for mutation screening in polycythemic patients with normal or increased concentrations of Epo without *VHL* or proline hydroxylase mutations.

Anemias

Classification

A review of the myriad of acquired and congenital causes of anemia is beyond the scope of this chapter. Anemia is caused by variety of genetic conditions, including red cell enzyme deficiencies, hemoglobinopathies, thalassemias, red cell membrane defects, iron disorders, B12 and folate deficiency, and sideroblastic anemias. This chapter focuses solely on a few examples of acquired and congenital, and experimental genetic lesions that result in dysregulated erythropoiesis. Some of the congenital molecular lesions leading to dysregulated erythropoiesis have unanticipated phenotypes and underscore the complexity of erythroid regulation.

Mutations leading to anemia, tend to be more clinically symptomatic and appear to be more numerous than those causing polycythemia. Mutations leading to anemias occur in all stages of erythropoiesis, including its early stage. Some mutations disturb both erythroid and nonerythroid regulatory events, as exemplified by the motheaten mouse. In other conditions, such as myelodysplastic syndrome, the exact molecular basis of the disease is still largely unknown.

Myelodysplastic syndromes

Myelodysplastic syndromes (MDS) is a group of acquired clonal hematopoietic disorders due to mostly uncharacterized somatic mutations associated with one or more cytopenias, of which anemia is most common. While some subtypes of MDS are associated with well-defined chromosomal abnormalities, the exact molecular consequences of these lesions largely remain to be elucidated [60]. One exception is in MDS with the 5q-chromosomal abnormality, in which dysregulation of ribosomal- and translation-related genes was described [61]. Somatic mutations of MDS are being defined using increasingly comprehensive single nucleotide polymorphisms (SNP) chip technologies [62, 63]. It is anticipated that uncovering the molecular basis of these mutations and their function will lead to better and more complete understanding of regulation of erythropoiesis.

Gas6 and erythropoiesis

Growth arrest-specific 6 (Gas6) protein is a secreted vitamin K-dependent protein that interacts with cell membranes and leads to intracellular signaling (through its receptor, tyrosine kinases), including activation of the PI3K and Akt pathways. Gas6 receptors Tyro3, Axl, and Mertk are expressed in hematopoietic tissue, megakaryocytes, myelomonocytic precursors, and bone marrow stromal cells. Gas6 has been shown to amplify the erythropoietic response to Epo using a mouse model of Gas6 knockout. These findings warrant further investigation of the therapeutic potential of Gas6 for the treatment of patients who are hyporesponsive or resistant to Epo [64]. Microarray data has shown that the *Gas6* gene is a HIF-1 target gene [65]. Moreover, Gas6 is known to downregulate the expression of inflammatory cytokines such as tumor necrosis factor (TNF)-α by macrophages [66]. Because apoptotic depletion of bone marrow progenitors by TNF-α has been implicated in the development of anemia [67], Gas6 might also play a beneficial role in erythropoiesis by reducing TNF-α production by macrophages.

Motheaten mouse

Deficiency of hematopoietic phosphatase (SHP-1) would be predicted to result in expansion of erythroid and other hematopoietic progenitors, and this phenotype has indeed been observed in motheaten mice. While viable motheaten mice that are deficient in SHP-1 have hyperproliferation of myeloid cells, widespread inflammatory lesions, and their myeloid and erythroid progenitors are hypersensitive to cytokines, they are nevertheless anemic [68]. The anemia in motheaten mice is independent of the presence of anti-erythrocyte antibodies, and is at least in part due to increased erythrocyte fragility and augmented oxidant stress by an incompletely defined mechanism [69].

GATA-1 mutation with anemia and other phenotypes

A hypochromic, microcytic anemia with elevated fetal hemoglobin, thalassemia, porphyria, and thrombocytopenia was described in a boy, and proved to be due to *GATA-1* mutation (*R216W)* that was also present in one maternal allele [70]; however, since the *GATA-1* gene is an X-chromosome encoded gene, the heterozygous mother was not affected. A bone marrow allograft corrected the anemia, thrombocytopenia, porphyria, and the thalassemia. This report is the first of association of anemia, porphyria with thalassemia, and thrombocytopenia due to a mutation of a trans-acting factor.

Bnip3L$^{-/-}$ mouse

Prevention of apoptosis is a well-recognized mechanism assuring productive early erythropoiesis and preventing anemia, but the importance of pro-apoptotic processes for productive terminal erythropoiesis is a more recent finding. Bnip3L is a proapoptotic Bcl-2 family member. Bnip3L$^{-/-}$ mice predictably developed expansion of erythroid precursors and splenomegaly. The mice unexpectedly develop a hemolytic anemia due, at least in part, to failure to tar-

get the mitochondria into autophagosomes for degradation during erythroid maturation, which results in increased oxidative erythrocyte stress and shortened red cell survival [71]. Thus, this mouse model demonstrates that both pro- and anti-apoptotic mechanisms at appropriate stages of erythropoiesis are essential for proper formation of a normal erythrocyte.

Other causes of dysregulated erythropoiesis
Many other examples exist of dysregulated erythropoiesis by well-defined mechanisms including podocalyxin [72, 73], DMT-1 defect in which the human mutation causes iron overload but also a hypochromic microcytic Epo-responsive anemia [74], as well as others, but is beyond the scope of this chapter.

Conclusion

Regulation of erythropoiesis is a complex and incompletely understood process. We conclude that elucidation of molecular basis of anemic and polycythemic phenotypes will lead to a better understanding of the regulation of erythropoiesis and eventually to the development of novel targeted therapies of human diseases.

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Erythropoiesis-stimulating agents

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Studies of erythropoiesis and the discovery and cloning of recombinant human erythropoietin

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Introduction

Patients who are anemic because of chronic kidney disease, cancer, arthritis, or chemotherapy or radiation therapy often report fatigue [1–6]. Before the introduction of recombinant human erythropoietin (rHuEPO), anemia and its sequelae fatigue were treated with red blood cell transfusion, androgen stimulation of red blood cell production, and/or iron supplementation, among other treatments [7]. While effective in increasing red blood cell counts, both transfusions and androgen therapy have inherent risks [8]. Transfusions of red blood cells can be complicated by blood-borne pathogens, iron overload, immunologic consequences, and lack of or delayed hemoglobin response. Transfusions often improve but do not correct anemia and usually must be given frequently, and androgen therapy can cause viralization or abnormal liver function. rHuEPO is an ideal therapy because it mimics the action of the endogenous hormone by stimulating the production of red blood cells. Patients with chronic kidney disease are unable to produce adequate amounts of endogenous erythropoietin (EPO) to stimulate red blood cell production. Patients with cancer often have damaged bone marrow, with or without the insult of chemotherapy, that does not completely respond to the endogenous hormone.

The cloning of the human *EPO* gene by Fu Kuen Lin and colleagues was a difficult and frustrating endeavor. This milestone and the subsequent creation and production of rHuEPO as a therapeutic option was a breakthrough that has enabled physicians to ameliorate anemia and its sequelae. Patients treated with rHuEPO report a return to more normal lives. rHuEPO is the standard of care for treatment of anemia in patients with chronic kidney failure or receiving chemotherapy, and in other disease settings.

This chapter is a literature review of the history of the early work in erythropoiesis and the discovery and cloning of EPO. Other chapters discuss its commercial production and the clinical uses of rHuEPO.

Early studies in erythropoiesis

Bright [9] is credited with being the first scientist to recognize that anemia was a complication of kidney disease, but Jourdanet [10] has been credited as the first scientist to observe the relationship between altitude and blood viscosity. Jourdanet noted the similarity of symptoms reported by patients with altitude sickness and the symptoms reported by patients who had experienced severe blood loss. Several years later, Viault [11, 12] expanded knowledge of red blood cells and the effect of altitude on them, and quantified the change in red cell counts as altitude increased. On a train trip from the city of Lima, Peru to the high-altitude tin mines of that country, he repeatedly sampled his blood, blood of willing fellow travelers, and blood of a dog, rooster, and llama. Viault noted an increase in his red blood cell count from $5 \times 10^6/mm^3$ to $8 \times 10^6/mm^3$ during the ascent to higher altitude.

Other early scientists continued studies in an attempt to understand the mechanism of erythropoiesis in rabbits [13] and immigrants to the high Alps [14, 15]. One theory proposed at the time to explain the polycythemia seen at high altitudes was that low oxygen pressure directly stimulated bone marrow to increase red blood cell production. This theory held for nearly 50 years.

In 1906, Carnot developed the concept of humoral regulation of erythropoiesis [16–18]. Serum from anemic rabbits was injected into normal rabbits, and caused an increase in the red blood cell counts of the normal rabbits. Carnot suggested that "hemopoietine" present in the serum of anemic rabbits was responsible for the increase in cell numbers. Many other investigators repeated these experiments in anemic rabbits or rabbits raised at high altitudes [19–23].

For almost 30 years, researchers continued to repeat Carnot's work. Because some investigators were successful and others were not, controversy continued about the mechanism of erythropoiesis. Finally, Erslev [24] modified Carnot's original study: He injected large amounts of plasma from anemic rabbits into normal rabbits and found that the number of nucleated red blood cells in the bone marrow, the number of peripheral reticulocytes, and the hematocrit of the normal rabbits increased. This study suggested that red blood cell production is mediated by a humoral factor in rabbits. Four years later, Jacobson et al. [25] demonstrated that this factor, EPO, was produced by the kidney. Progress was being made in understanding the relationship between oxygen supply and demand of the body and EPO and erythropoiesis; however, despite more than 100 years of research, nothing was known about the structure of EPO or the *EPO* gene. Some debate continued whether EPO was produced as an inactive precursor in the kidney that was activated in some other tissue or organ.

The role of kidney and bone marrow in erythropoiesis

In the fetus, the liver is the primary site of endogenous EPO production [26]. In the adult, EPO is produced primarily (i.e., >90%) in the adult kidney [25, 27]. The liver [28, 29] and the brain [30] both synthesize some EPO, but the amount produced by these tissues alone is insufficient to maintain adequate erythropoiesis. Thus, kidney disease causes anemia due to loss of the main source of EPO production.

An oxygen sensor within renal cells detects the oxygen content of the blood and the kidney regulates the amount of EPO released into the blood (Fig. 1). The hormone acts on red blood cell precursor cells in the bone marrow to stimulate their proliferation and maturation and to increase the number of red blood cells in the peripheral circulation. The feedback loop is completed when the kidney cells recognize the change in oxygen delivery secondary to the

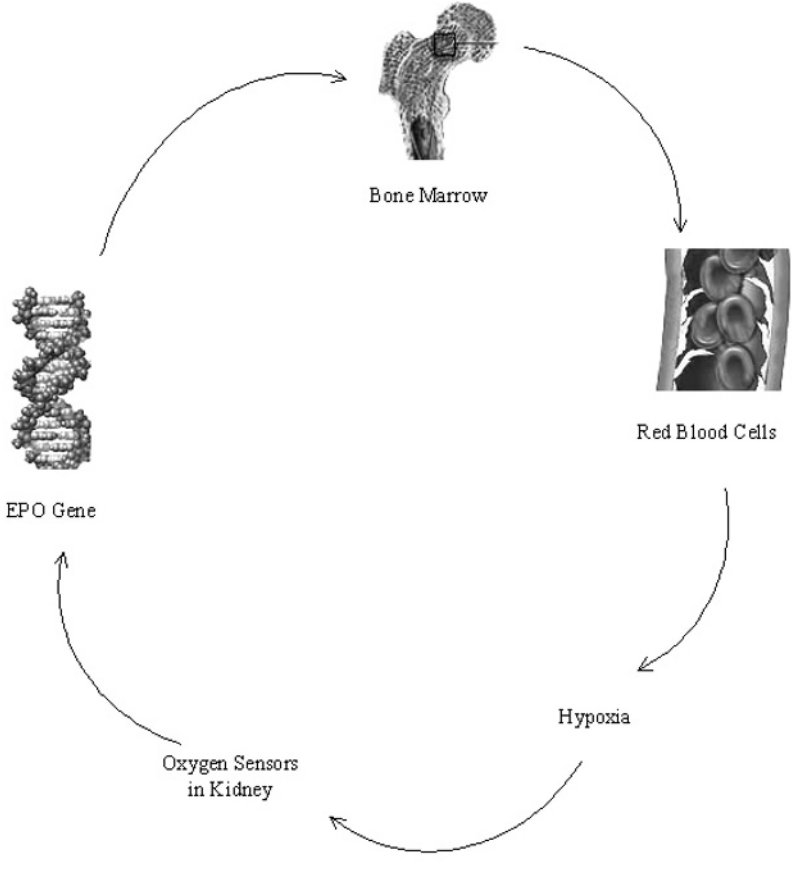

Figure 1. Relationship of kidney and bone marrow in production of red blood cells. (Figure courtesy of Amgen.)

change in the number of circulating red blood cells. Production of new red blood cell slows until the sensor cells recognize a need for increased erythropoiesis. Some EPO, albeit in trace amounts, is always detectable in the circulating blood, even in patients with total kidney failure, suggesting that a subset of cells provides a continuous output of EPO even when oxygen delivery is normal.

Isolation of EPO from urine

Even though the amounts of endogenous EPO increase under conditions of hypoxia or anemia, EPO represents a minor fraction of the total protein in the blood. A significant obstacle to the development of EPO as a therapeutic agent was the difficulty in isolating and purifying adequate amounts of the hormone to allow for its characterization. Several groups attempted to purify human [31, 32] and sheep [33, 34] EPO. The results were inconclusive, with the purity of the product questionable or produced in insufficient amounts to allow chemical characterization.

In 1977, Miyake, Kung, and Goldwasser succeeded in isolating and purifying milligram amounts of EPO from 1500 L of urine from patients with aplastic anemia [35]. Patients with aplastic anemia characteristically overproduce EPO, however the amounts present are still small. Large volumes of urine were essential for the recovery of a sufficient amount to purify EPO.

Miyake et al. [35], using a seven-step process that included ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography, produced a preparation with a potency of 70,400 units/mg protein, 21% yield, and a purification factor of 930. This method allowed the production of enough material to partially characterize the hormone. With a source of EPO now available, a strategy for the cloning and expression of the human gene was devised.

Cloning of the *EPO* gene

In the 1980s, the nucleic acid sequences for cynomalogous monkey and human EPO were finally isolated and characterized, each by a different method. Several hurdles needed to be overcome, including the limited amount of data about the primary structure of human or monkey EPO, lack of a known source of mRNA, no information about the genomic structure of the gene, lack of simple tests to confirm that the cloned sequence encoded the *EPO* gene, and controversy about the induction mechanism for EPO production due to hypoxia. The difficulty in cloning the gene was further complicated by an inability to determine which step in the process was responsible for the failure.

In 1981, Goldwasser presented the first 26 amino acids of the protein at a meeting. Sue and Sytkowski [36] published the sequence in a paper that

described the development of polyclonal antibodies to EPO. The Sue and Sytkowski paper was subsequently shown to have two errors, unknown at the time, in the amino acid sequence. Another group [37] published a putative amino acid sequence for the first 31 amino acids of EPO, but it was proved to be erroneous, as it had the same two errors reported by Sue and Sytkowski as well as three additional errors in the amino acid sequence, again unknown at the time of publication. Goldwasser had provided Lin and colleagues at Amgen Inc. with the sequence he and his colleagues had obtained for the first 26 amino acids of human EPO, but of course, none were aware at the time of the errors. They were aware of the possibility that the available peptide sequence may overlap the intron-exon boundary of the *EPO* gene thereby preventing successful cloning using oligonucleotide sequences based on the peptide sequence. Earlier, Goeddel et al. [38] had successfully sequenced, cloned, and produced recombinant human insulin. This protein has only 51 amino acids, compared with EPO's 165 amino acids, and required nearly 10 years of work to sequence. With newer biotechnology techniques, it was naively thought that the sequencing of EPO would be easy. Lin needed not only to isolate the gene with no knowledge of its structure or of a simple way of confirming that the gene was in hand, but also to express the gene in a suitable host cell to provide a product with the proper structure, including the carbohydrate and polypeptide components of the molecule.

Lin used many approaches, including the standard gene-cloning routes known at the time, all of which failed. He persisted and eventually succeeded only because he used a technique far more complex than any technique tried earlier. This novel approach involved the use of multiple sets of fully degenerate oligonucleotide probes to screen a human genomic library. Two small pools of oligonucleotides corresponding to short fragmented samples of EPO amino acid sequences were used. Both pools, one of 20 nucleotides and the other of 17 nucleotides, had low codon degeneracy. Because of the degeneracy of the genetic code, the same amino acid can be encoded by more than one codon; Lin and colleagues accounted for every possible codon that encoded these putative amino acid sequences necessitating 128 different probes in each pool. The probes were labeled with radioactive phosphorus to identify any matches of a single probe with the human genome. The gene library on which the probes were tested consisted of the total human genome, fragmented into pieces 10,000 to 20,000 nucleotides long. Lin and colleagues found that probes in both mixtures hybridized with four of the 1.5 million clones in a human fetal liver genomic library [39]. Analysis of these clones showed that at least one contained the entire coding region of the human gene for EPO and it was the basis for developing the expression system using transfected Chinese hamster ovary (CHO) cells. In parallel with this effort, cDNA from the kidneys of anemic monkeys was prepared using mixed probes based on the human EPO peptide sequences [40] and the monkey gene also was cloned.

After Lin and his colleagues successfully cloned the gene, Jacobs and colleagues [41] also cloned the human *EPO* gene using degenerate oligonu-

cleotides and a peptide sequence derived from urinary EPO supplied by Miyake. Cloning of additional *EPO* genes from other species used the sequence information from the human and monkey genes and proceeded rapidly. The mouse *EPO* gene was cloned using monkey and human EPO DNA segments as hybridization probes [42, 43]. Subsequently, *EPO* genes from other species were cloned by hybridization or by polymerase chain reaction using probes or primers based on the known EPO sequences, including the genes from the rat [44], pig [45, 46], sheep [45, 47], and cow [48], among other animals.

Expression of the *EPO* gene

Clones of the gene for EPO were inserted into CHO cells, which synthesized the 193 amino acid precursor protein, removed the signal peptide and carboxy-terminal arginine, added *N*- and *O*-linked carbohydrate to glycosylation sites, and released the mature protein into the culture medium. Immunologic, biologic, and biochemical assays showed that the recombinant hormone had the *in vivo* biologic activity and was immunologically equivalent to human EPO, as revealed within the limits of the available assays [39]. EPO expressed by CHO cells has a molecular weight of 30.4 Kd and contains 40% carbohydrate [49].

Discussion

The isolation and expression of the gene for human EPO was a major achievement that capped almost 155 years of exploration into the nature of anemia and the production of red blood cells. EPO is present in minute quantities in the blood, and difficulty in isolating and purifying the hormone in amounts that would allow investigation of its properties posed a significant obstacle to the development of EPO as a therapeutic agent. After Miyake, Kung, and Goldwasser developed a technique to purify urinary EPO, a strategy for the cloning and expression of the human gene could be devised. The innovative approach of Lin et al. allowed the successful isolation, cloning, sequencing, and development of the recombinant protein. Large-scale production began and clinical trials started. The recombinant protein, epoetin alfa, produced dose-dependent increases in erythropoiesis that paralleled the expected response to endogenous EPO. The commercial production of epoetin alfa has been translated into benefits for millions of patients (Tab. 1). Epoetin alfa ameliorates the debilitating symptoms of anemia and allows these patients to have more normal lives. Other chapters in this volume will elaborate on the clinical use, production, formulation, and other important and evolving aspects in the study of rHuEPO.

Table 1. Some benefits of epoetin alfa therapy

• Increased exercise tolerance
• Improved central nervous system function
• Reduced heart enlargement
• Reduced extreme fatigue
• Increased ability to perform daily functions of life
• Reduced risk of alloimmunization in transplant recipients
• Improved coagulation

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Commercial production of recombinant erythropoietins

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Introduction

For the production of recombinant products from genetically engineered cells, a number of cell hosts may be used, but they generally belong to one of five categories: plant, bacterial, yeast, insect, or mammalian. Depending on the type of product that is desired, the features of that product, the intended use of the product, and the preferred method of manufacture, an appropriate host cell can be selected. With the appropriate genetic engineering, the gene of interest such as erythropoietin (EPO) can be produced by a host cell. In the case of EPO production, the sequence of amino acids, as well as the amount of glycosylation, must be correct to achieve the desired efficacy *in vivo*. This chapter describes one method of EPO production using cells genetically engineered to secrete recombinant human erythropoietin (rHuEPO). In this method of production, mammalian cells, which are capable of producing glycosylation forms with the desired efficacy in humans, are typically selected as hosts. The host mammalian cells secrete the rHuEPO product into the medium environment in which they are cultured, making the remainder of production a matter of separating the rHuEPO product from the cells and other components in the cell culture broth. This chapter summarizes the generation of rHuEPO-producing cell lines, the production of rHuEPO, the separation of rHuEPO from components of the cell culture broth, and the packaging of the final rHuEPO drug product.

Cell line development

The development of a cell line generates a consistent source of cells that is capable of satisfying commercial demand and that adheres to regulatory guidelines for genetically engineered cells. Development begins with the creation of genetically engineered cells that produce the desired product, and ends with a

cell bank, a homogeneous population of genetically engineered cells frozen at the same population doubling level.

Cell line selection and screening

After the initial genetic modification of a host cell population to produce the product of interest, an appropriate production cell line must be isolated and selected. Generally, populations of cells may be screened for desirable properties, such as productivity and the ability to secrete product of the desired quality. Products, such as rHuEPO, may be posttranslationally modified by the host cells (e.g., glycosylation), and these modifications can impact *in vivo* efficacy. Different populations of genetically modified cells may produce product with varying degrees of posttranslational modifications. Therefore, product quality can be an important endpoint when screening for an appropriate production cell line. Selecting the final production cell line may involve several stages of isolating (i.e., cloning) and screening candidate cell lines. To ensure a homogeneous production cell line, the final cell line will have typically been single-cell cloned.

Single-cell cloning

The industry standard for generating a homogenous population is single-cell cloning. Single-cell cloning is accomplished by placing one cell in a microculture environment, and then expanding that cell through increasingly larger cultures to generate a greater number of cells (e.g., 1×10^9 cells) (Fig. 1).

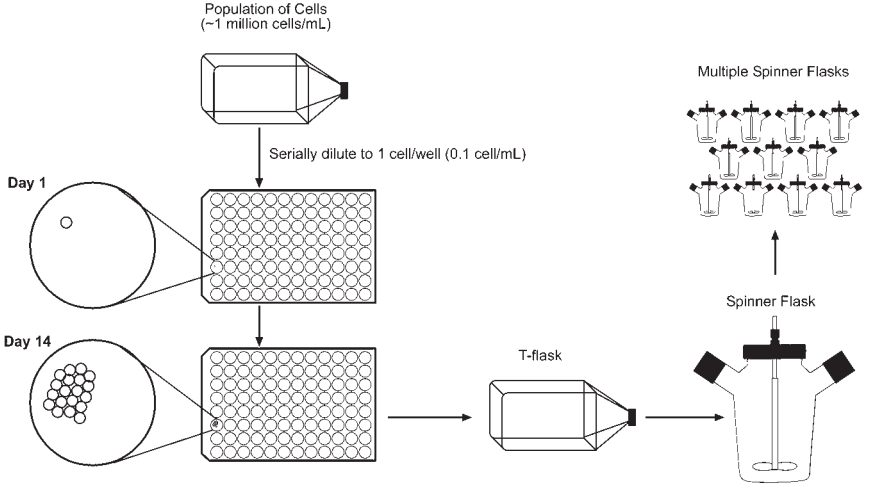

Figure 1. Single-cell cloning technique

Doubling of a single cell should produce two identical cells. Doubling again results in four cells that are assumed to be identical. Within a limited number of generations, expansion of the cells in this manner results in a homogenous culture of cells with identical genomes. When sufficient cells are amassed, cell banks may be created.

Cell bank

Once a cell line has been identified as the manufacturing line, a cell bank must be generated. The cell bank provides the starting material for all lots of product manufactured. For this reason, it is carefully scrutinized from both a business and regulatory perspective.

The master cell bank is the base starting material for the life cycle of the product. Cell banks are created in a two-tier system (Fig. 2). Each vial of master cell bank material can be used to create a working cell bank. Likewise, a single vial provides the starting material for a commercial cell culture lot.

Two-tier system: creation of master and working cell banks

A two-tiered approach is commonly used in industry to generate a supply of cell bank vials for the lifetime of a product. The master cell bank is generated first and typically consists of 100–400 vials. The master cell bank is not used by the manufacturing facility directly. Instead, the second-tier cell bank, the working cell bank, is used by the manufacturing facility. The working cell bank is generated by expanding one vial of material from the master cell bank. The working cell bank typically contains 300–1,000 vials.

Safety assessment

Once cell banks are generated, they must be characterized before they can be used to manufacture a drug for clinical use, as per regulatory guidelines [1–3]. Characterization is critical to ensure that the correct protein is being produced by the cells and that the cell line is not contaminated by adventitious agents.

The cell bank is the primary source for the recombinant protein produced, so verification that the correct molecule is being produced must be done at the inception of the bank. Confirmation of the DNA and/or RNA coding sequence ensures that the cells encode the required genetic sequence for the protein. Verification of the genetic sequence within the cell must be followed with verification of the amino acid sequence of the purified protein. Additionally, the cell bank must be examined to confirm that it is not producing any altered forms of the protein. For example, during gene integration in mammalian cells (after transfection or amplification), the cell's genome is rearranged, and

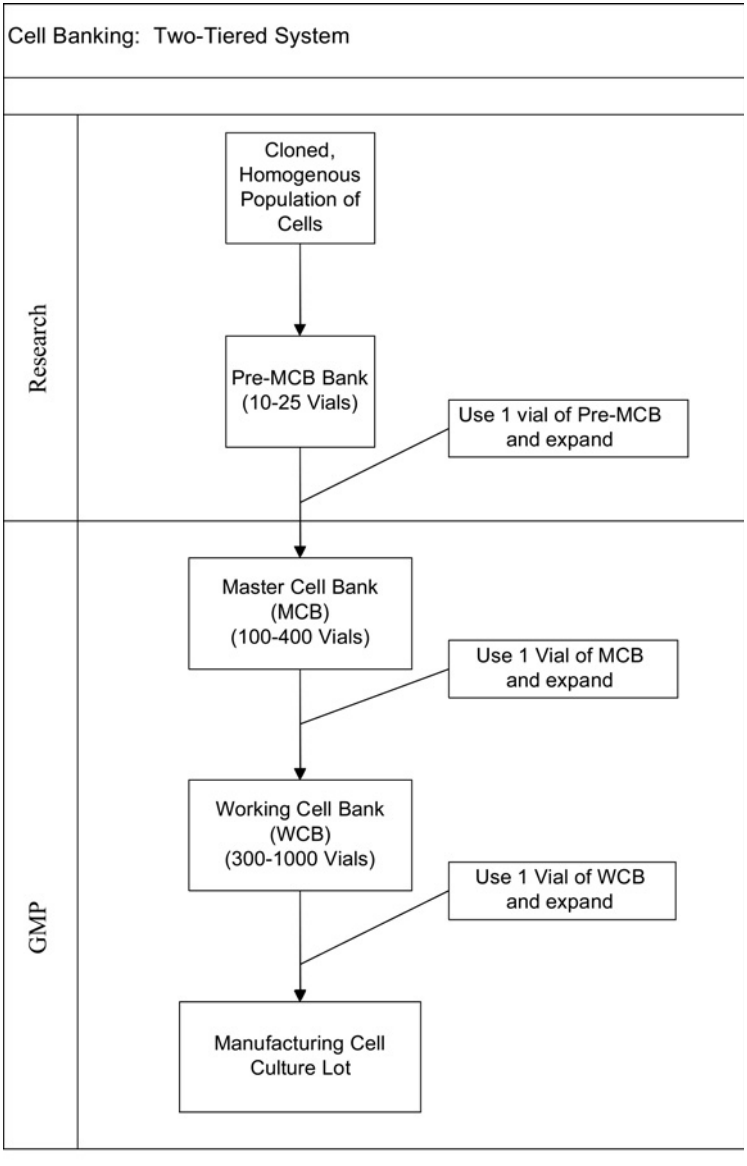

Figure 2. Cell banking is a two-tiered system. GMP = Good Manufacturing Processes; MCB = master cell bank; WCB = working cell bank

rearrangement possibly may involve the coding sequence of the protein of interest. One concern is that the rearrangement could occur at a site that results in a molecule containing a portion of the correct protein and a portion of another protein. In this event, the rearranged molecule could retain some of the correct protein's characteristics and be carried through a purification process;

however, the net result may be either the incorrect protein or a mixture of correct and incorrect proteins. Therefore, the cell bank is examined in great detail to ensure the absence of rearrangements that could lead to unwanted proteins being carried through manufacturing.

Ensuring that adventitious agents are not present is crucial. When a cell bank comprises mammalian cells, which theoretically can act as hosts to viruses, the cell bank is examined for mycoplasma, bacteria, fungi, and viruses. Numerous *in vitro* and *in vivo* assays are used to assess viral contamination. In the end, all of the aforementioned tests for adventitious agents must be negative. If they are not, the cell bank cannot used to make material intended for clinical use.

Cell bank stability assessment

The master cell bank is designed to last the lifetime of the product, so monitoring it and the working cell bank for storage stability is important. Cell banks are generally stored at –130 ºC or below to ensure cell stability. The cell banks are monitored by testing cells for viability upon thawing. If the cell bank's viability is stable, then no other testing is necessary. If viability is not stable, the bank is re-examined, and another master cell bank may need to be generated.

Cell culture process

One method of EPO manufacture uses cell lines genetically engineered to produce rHuEPO. In this chapter, an example of rHuEPO production in a cell culture process is presented, where cells from a mammalian cell bank are grown and product is secreted into the cell culture medium. The ability of the cell culture to produce product is affected by the nutrient environment and other physical parameters, such as temperature, pH, osmolality, and concentrations of dissolved gases. The cell culture process is designed to support the production of rHuEPO of a consistent quality, tailored to enable the product specifications and efficacy targeted by the manufacturer.

Raw materials

The raw materials for the cell culture process consist of the cell bank and the nutrients used to sustain and expand these cells. Nutrients in the cell culture medium generally are amino acids, additional carbon sources (e.g., glucose), vitamins, trace elements, growth factors, hormones, and salts. A review of media composition is given in [4]. Historically, cell culture medium also has contained serum from bovine sources, which may vary in composition depending on its source or the processing. Guidance for the industry for use of raw materials for the manufacture of biologicals is provided by CBER/CDER [1].

Process options

Many process options are available to produce a recombinant protein. One of the simplest cell culture options is batch mode, where the cells and the nutrient-containing media are added to a production vessel, and no further additions are made. Physical parameters (e.g., temperature, pH, and dissolved oxygen concentration) may be controlled within set ranges. The cells grow to the extent that can be supported by the initial nutrient concentrations; cells will stop growing and begin dying when nutrients become insufficient and/or if the amounts of waste products (the by-products of nutrient consumption by the cells) increase.

Fed-batch processes differ from batch processes by providing for the addition of nutrients into the culture as required. This method allows a much greater number of cells to be supported than batch processes. More cells typically translate to greater productivity due to the constitutive production feature engineered into most commercial cell lines. With constitutive production, as long as a cell is present and viable, it will continue to make product. Thus, the viable cell number correlates closely with the amount of recombinant protein produced.

In addition to supplementing a culture with nutrients as required, spent medium in the culture may be removed to reduce the amount of waste products. The process of feeding fresh nutrients and concurrently removing spent media is called perfusion. The spent medium, called the harvest, contains the protein product; the device used to separate the spent medium from the cells is called a cell retention device.

Operationally, batch and fed-batch processes are relatively easy to execute and perform reliably; perfusion processes are relatively more complex to exe-

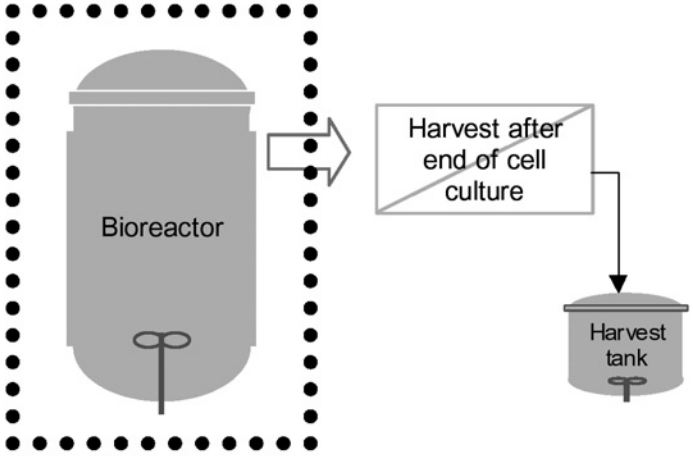

Figure 3. Schematic of a bioreactor operating in batch mode. The sterile envelope, depicted by the dotted line, encompasses only the bioreactor. The harvest operation, which includes cell separation from the conditioned medium and concentration of the conditioned medium, is not performed under sterile conditions

cute and in general use longer production times. The numbers of pieces of equipment that must be operated in a sterile manner within the sterile envelope are depicted in Figure 3, Figure 4, and Figure 5. The number of pieces and complexity of equipment in the sterile envelope correlates with the complexity in executing the three types of processes. A schema of the equipment (bioreactor, media feed tank, and harvest tank) used for each of these process options and the relative scale of tanks and production vessels is depicted. For batch and fed-batch processes, the production vessel is typically the largest piece of

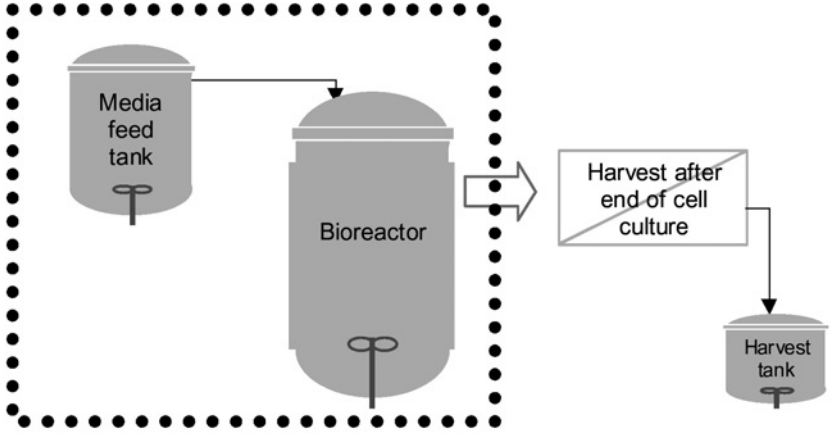

Figure 4. Schematic of a bioreactor operating in fed-batch mode. The sterile envelope, depicted by the dotted line, includes the media tank and the bioreactor. The harvest operation, which includes cell separation from the conditioned medium and concentration of the conditioned medium, is not performed under sterile conditions

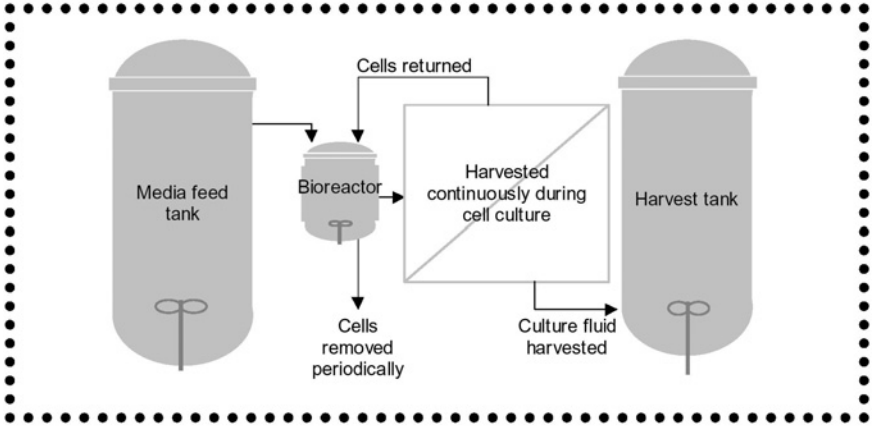

Figure 5. Schematic of a bioreactor operating in perfusion mode. The sterile envelope, depicted by the dotted line, includes the media tank, bioreactor, cell separation device (harvest unit), and harvest tank. The harvest operation here does not include any concentration of the conditioned medium.

equipment used in the cell culture process; for perfusion processes, the feed and harvest tanks are typically the largest equipment used.

The size of the equipment and the time over which each piece of equipment needs to be occupied for a given production run will influence the design of a manufacturing facility and the number of production runs a manufacturing plant can execute. The manufacturing facility is designed to comply with regulatory guidelines for GMP manufacture (i.e., CFR parts 210 and 211 are used in the US; guidelines differ slightly in other countries). Additional information on process options is available [5, 6].

Commercial manufacture

A commercial cell culture manufacturing process consists of a seed train followed by a production step. The purpose of the seed train is to generate a sufficient number of cells with which to initiate production in the production vessel. The required process conditions are imposed on a production vessel to generate optimal quantity and quality of product.

Seed train

A seed train describes the expansion of cells from a frozen cell bank vial to the inoculum of the production reactor. The volumes involved may range from 1 mL to 10,000 L. The vessels involved may include t-flasks, shaker flasks, roller bottles, disposable bag bioreactors and stirred tank reactors (Fig. 6). The reactors may be operated in batch, fed-batch, or perfusion modes. The purpose of each step of the seed train is to generate a sufficient number of cells of known quality to enable inoculation of the subsequent step. The culmination of the seed train occurs at inoculation of the production reactor.

Given a cell bank vial containing 1×10^7 viable cells, the initial seed train time to the production reactor may be estimated. First, the doubling time for the cell line must be established. For mammalian cells, doubling time typically ranges from 16–48 h. Second, the requirements for cell number generated at the end of the seed train must be established. For example, if the process uses a 10,000 L production reactor and the production reactor requires an inoculum density of 1×10^6 viable cells/mL and an initial volume of 7,500 L, then 7.5×10^{12} viable cells are required. The number of cell doublings (n, or population doubling level) from vial thaw to production reactor inoculation can be given by the formula:

$$n = LN(XS / X0) / LN(2)$$
$$n = LN(7.5 \times 10^{12}/1 \times 10^7) / LN(2)$$
$$n = 19.5$$
$$n \sim 20$$

where LN = natural logarithm and XS final cell number = 7.5×10^{12} and X0 = initial cell number = 1×10^7

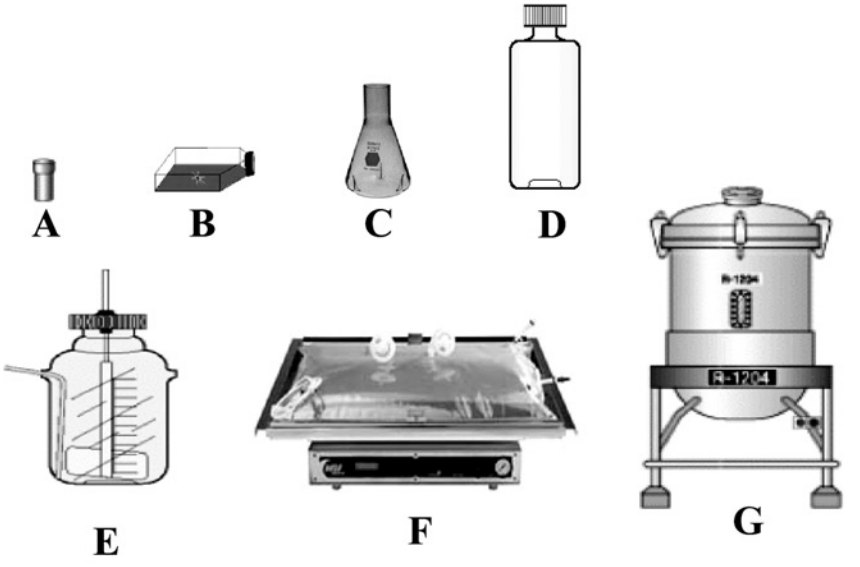

Figure 6. Exemplary vessels used in the preparation of a seed train: (A) freezer vial, (B) T-flask, (C) shaker flask, (D) roller bottle, (E) spinner flask, (F) Cellbag, (G) stirred tank reactor

Using a conservative estimate of 2 days for the doubling time (td), the overall seed train time is $n \times td = 20 \times 2$ days = 40 days, or approximately 6 weeks.

Multiple seed trains using culture taken from an existing seed train may be initiated if cell numbers allow and if the cell line's productivity remains stable. Thus, multiple production reactors stemming from a single cell bank vial may be considered to shorten the overall time to inoculate production reactors. Alternatively each production reactor may be derived from a single vial thaw.

Production

Once the seed train has generated a sufficient number of cells from the cell bank, these cells can produce rHuEPO. Cells can adhere to a solid substrate and secrete rHuEPO into the liquid cell culture medium. Examples of solid substrates include tissue culture plastic such as roller bottles or microcarrier beads that may be made of ceramic or other porous materials. Alternatively, cells can be freely suspended in liquid medium where they secrete rHuEPO. Exemplary cultures in roller bottles and tank vessels are shown in Figure 7 and Figure 8, respectively.

Commercial cell lines are designed and selected for constitutive production; therefore, the amount of rHuEPO produced correlates with the viable cell number (Fig. 9). For adherent culture, the number of cells is limited by surface area. To increase production of rHuEPO, the surface area is increased, typically by increasing the number of roller bottles or microcarrier beads. Since nutrients are supplied in the liquid phase and products are secreted (by the cells) from the

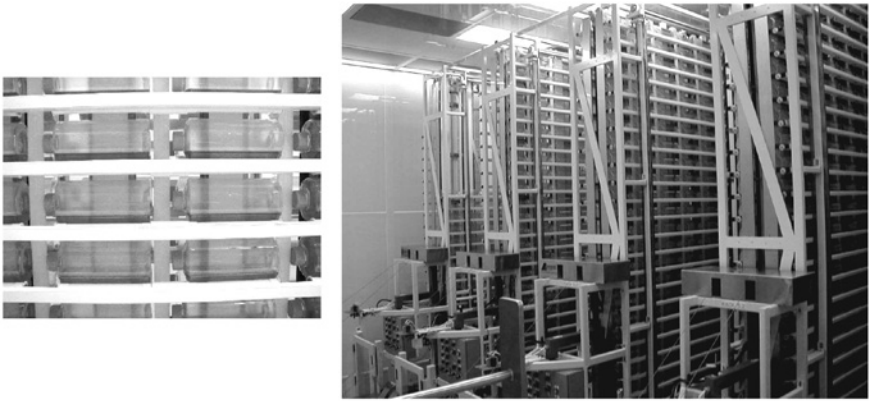

Figure 7. Production of rHuEPO in roller bottles (left panel). The right panel shows large racks containing many of the same type of roller bottle cultures.

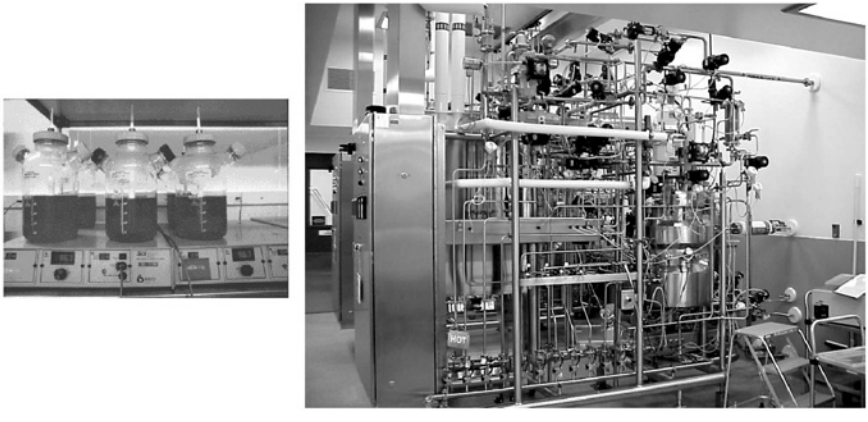

Figure 8. Production of rHuEPO in tank vessels. A medium containing rHuEPO-producing cells is shown in the spinners on the left. The stirred tank reactor shown on the right also contains a culture of rHuEPO-producing cells.

solid phase into the liquid phase, the successful mass transfer of nutrients to the cells, and rHuEPO and metabolic waste products from the cells must be carefully considered in the design of the process. A commercial production lot from adherent cultures with roller bottles typically entails using many small production vessels; the challenge of such a process for commercial scale production is to demonstrate equivalent control (i.e., monitoring) of each of the multiple production vessels. The degree of control required is imposed to achieve comparable growth and productivity performance from each production vessel. After product is produced, it is separated from the cells in the harvest process. Since cells are attached to the solid substrate, harvesting the rHuEPO product in the

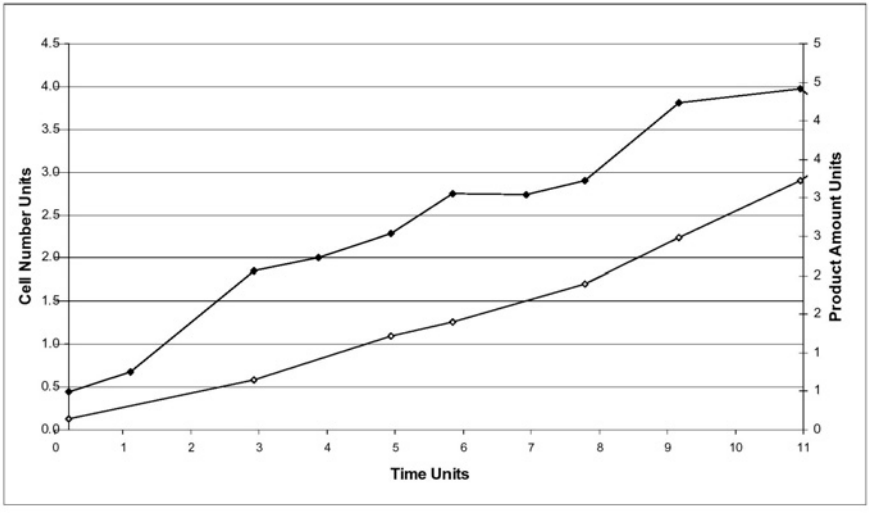

Figure 9. Correlation between number of cells and amount of product produced. Solid diamonds = cell number profile; hollow diamonds = product amount profile.

liquid conditioned medium is operationally straightforward and involves pouring off or decanting the liquid before volume reduction and purification.

For suspension cultures, scale-up is more straightforward. To increase production, the number of cells per unit volume (i.e., cell density) may be maximized, and/or the volume of culture may be maximized. To maximize the cell density, limiting nutrients within the culture environment are identified and supplements are added to the culture. The medium is sufficiently enriched to support the maximal number of cells. To maximize the culture volume, a larger scale of production vessel may be used. Both cells and product are distributed homogeneously in well-stirred reactors so that the growth and production performance within each volume of liquid is consistent. The volume of liquid may be increased as long as homogeneity is achieved (i.e., mixing is adequate). Stirred tank reactors for suspension cultures of mammalian cells typically range between 1,000 L and 20,000 L. To harvest the product, the cells must be separated from the liquid medium. Separation is achieved by microfiltration or centrifugation. The clarified liquid-containing product may then be concentrated for volume reduction and purification.

Within any of the process modes described, a cell line that has been engineered to produce rHuEPO may grow and secrete product encoded by the *EPO* gene. Not all of the secreted product may qualify as the rHuEPO sold by a manufacturer. The EPO molecule has 3 *N*-glycosylation sites and 1 *O*-glycosylation site, leading to the possibility of differently charged molecules with different isoelectric points (i.e., isoforms). Those molecules with the lowest isoelectric points, typically corresponding to those having the greatest amount of sialylation and glycosylation branching, have the greatest *in vivo* efficacy.

Molecules with higher isoelectric points, or less sialylation and less glycosylation, have less *in vivo* efficacy, and can be removed in purification. Additionally, even if the cell produces product of the desired charge profile, this product may be degraded in culture by proteases. Further, the product may form undesired complexes such as aggregates, which must be separated from the desired monomer. The cell may be influenced to produce increasing amounts of the desired charge profile by controlling process conditions, such as temperature, pH, osmolality, and nutrient and waste product concentrations. Similarly, undesired forms of the product may also be minimized.

Recovery and purification of erythropoietin

A number of purity criteria must be met for rHuEPO to be a viable human pharmaceutical. Host cell and other contaminants, such as DNA, host cell proteins, and endotoxin, must be removed to appropriate levels. The removal or inactivation of any theoretical endogenous or adventitious viruses must be shown. Amounts of product-related contaminants, such as aggregates, proteolytically degraded, underglycosylated, or oxidized forms, are minimized. A targeted purity is specified by the manufacturer, so each product lot must meet the criteria described in the manufacturer's product specification. Product specifications may differ among manufacturers.

The clearance rate of rHuEPO in the human body is affected by the number of sialic acid residues on the carbohydrate portion of the molecule. Under-sialylated rHuEPO is cleared in the liver by the asialoglycoprotein receptor [7]. Selecting for molecules with the greatest degree of sialylation gives a product with the desired pharmacokinetic properties, which affects the biological potency of rHuEPO. Different manufacturers may have different selection criteria (and therefore different product quality characteristics) for the EPO molecules that are retained in the recovery and purification processes.

In addition to having a well-understood and reproducible recovery process, a number of regulatory requirements exist for the manufacture of biopharmaceuticals. The product must be produced under Good Manufacturing Practices (GMP), which encompasses a range of topics from plant design, which affects air and water quality, solvent usage, and waste disposal, to day-to-day operations, which include operator training, batch record writing and review, quality assurance, and cleaning validation. Many of these guidelines can be found in Q7A 'Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients' [8].

Concentration and diafiltration

After the clarified cell culture media is harvested, it must be concentrated, and the buffer salts and other high conductivity/low-molecular-weight components

must be removed. Concentration is an important step, since it is difficult to store and handle large pool volumes. The expense of storing large volumes of harvest media in a frozen state can be prohibitive. In addition, the time required to load a large volume of media on to a column decreases plant productivity. Prolonged column load times can also lead to proteolytic degradation of product in the harvest media as it awaits loading onto the column. Removal of buffer salts and other high-conductivity, low-molecular-weight components is important because these may interfere with binding to the purification columns. For example, ion-exchange chromatography depends on ionic interactions between charged groups on the protein and oppositely charged groups on the chromatography media. The high conductivity of the harvest media would prevent protein from binding to the ion-exchange chromatography media.

Concentration and diafiltration are done using ultrafiltration membranes. This operation allows for the passage of low-molecular-weight solutes and water through membrane pores and the retention of larger-molecular-weight solutes, such as rHuEPO and other proteins. The driving force for the passage of these solutes and water through the pores is the pressure difference across the membrane. The membranes can be made of cellulose, polyether sulfone, or other polymers. Membranes are available that have different 'nominal molecular-weight cut-offs', ranging in molecular weight from 5,000 to 500,000. Proteins and other high-molecular-weight materials that do not pass through the membranes are referred to as the retentate; the material that passes through the membranes is called permeate. The rate at which the water and solutes go through the membrane is called the permeate flux rate and is usually measured in litres per minute.

During the concentration phase (Fig. 10a), both water and solutes are forced through the pores, leading to a volume reduction in the harvest medium. The

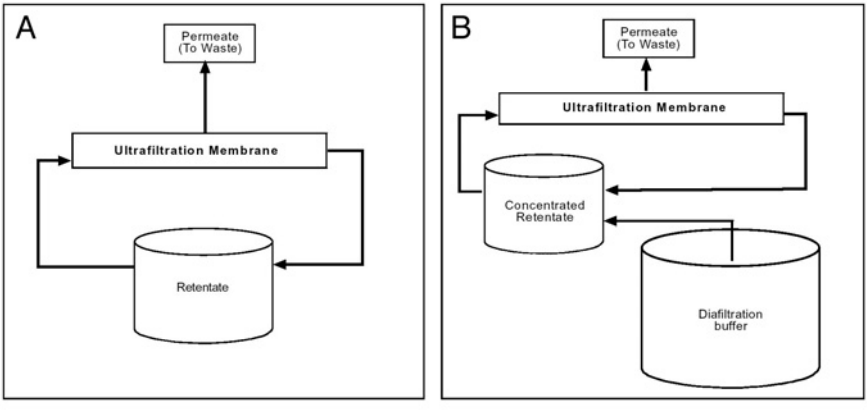

Figure 10. Panel A – Concentration mode. Clarified cell culture medium volume is reduced in retentate to make concentrated retentate. Panel B – Diafiltration (DFM) mode. Removal of high-conductivity components in concentrated retentate to make DFM.

range of this volume reduction may be 5- to 100-fold depending on what is required for a given protein. During the diafiltration phase, the concentrated harvest continues to be fed through the membrane; at the same time, however, a lower conductivity buffer (diafiltration buffer) is pumped into the harvest media retentate at approximately the same rate as the permeate flux rate (Fig. 10b). This process causes a buffer exchange of the original high conductivity buffer for the lower one. The greater the number of retentate volumes pumped though the membrane during the diafiltration step, the more complete the buffer exchange. This product pool is referred to as diafiltered media, which either can be immediately processed through the subsequent purification steps or stored frozen and processed later.

Chromatography: isoform selection

An anion-exchange media can be used to separate molecules on the basis of molecular charge. This chromatography may be used to separate rHuEPO from host cell proteins, nucleic acids, and endotoxin.

Chromatography: removal of contaminant proteins

A reversed-phase chromatography step can be used to remove host cell proteins and additional nucleic acids. The interactions of the column matrix with proteins are mostly hydrophobic in nature. Once bound to the column, a protein can be eluted with another hydrophobic, low-dielectric organic solvent. The protein portion of rHuEPO is quite hydrophobic because of the large proportion of hydrophobic residues and its solubility characteristics after removal of the polysaccharide portion of the molecule. Gel filtration chromatography, also known as size-exclusion chromatography or molecular sieving, may also be used to separate a protein mixture based on molecular weight. The media consists of agarose or acrylic beads of defined pore sizes. Proteins too large to penetrate the pores are excluded from the interior volume of the beads and have a shorter elution path through the column (hence they elute sooner). Smaller proteins either partially or completely penetrate the pores, resulting in a longer elution path and longer elution times.

Viral clearance

If rHuEPO is made in mammalian cells, the ability to remove theoretical endogenous or adventitious viruses from the product must be shown. Because virus titers are well below the level of detection, bench-scale studies are done in which different types of model viruses are deliberately added to process streams to demonstrate the ability of the process to remove viruses. Usually

several types of viruses are used, including enveloped and nonenveloped viruses.

Dosage form (drug product) manufacturing

In general, dosage form manufacturing of rHuEPO, as with all recombinant protein products, is governed by national governmental regulatory agencies to ensure that biologic production facilities adhere to sound quality control and current GMP. Facilities that produce rHuEPO are routinely audited and inspected to ensure compliance and patient safety. In the United States, biologic production for parenteral applications is governed by the Food and Drug Administration (FDA CBER/CDER); regulations are covered under section 21 of the Code of Federal Regulations Parts 210 and 211. These regulations differ slightly from country to country. All manufacturing plants and processes are validated and filed with the FDA (or regional authority) before marketing approval is given. All operations are conducted in an aseptic, temperature-controlled, highly monitored environment, with final filling operations done under conditions that typically allow only 100 particle counts/cubic foot of air in the filling operation suite. For dosage form/drug product manufacturing, maintaining validated processes, quality control, and aseptic conditions are critical to patient safety and product integrity.

In addition to regulatory compliance, a primary concern for recombinant proteins, and in particular rHuEPO, is the maintenance of product integrity by minimizing physical or chemical degradation. Product integrity is maintained from the bulk stage through the final dosage form by adjusting process parameters and the composition of the final formulation. To mitigate process impacts, rHuEPO is formulated in solutions that often contain human serum albumin or polysorbate that acts as a protector against surface adsorption, surface denaturization, shear forces, chemical degradants, and other deleterious factors. Because of the relatively low concentrations of rHuEPO used clinically, additives are important to protect the product during processing. Formulations are optimized for stability and parenteral delivery. Ingredients such as citrates, chlorides, and phosphates at relatively neutral pH are often used. Nonoptimized formulations can result in degradants that arise before the end of shelf life. The formation of degradants can lead to loss of activity or by-products that may potentially be antigenic.

rHuEPO is marketed in a variety of formulations and concentrations, providing the practitioner with a wide selection of dosages for achieving optimal hematocrit values for patients. The final dosage form available in clinics is typically an aqueous formulation in a vial. Prefilled syringes, lyophilization, frozen liquid, or other delivery forms also can be used. This variety of formats can complicate production because of the need to formulate at different strengths, different fill volumes, and different delivery vehicles.

Process

Typical processing of rHuEPO consists of several unit operations: buffer preparation, formulation of the purified bulk, filtration of the formulated product, filling into a delivery vehicle, lyophylization (i.e., freeze drying if applicable), unit inspection, and packaging. Figure 11 depicts a generic process flow from bulk though packaging operations. A formulation-and-fill process may comprise several vessels, a fill line, and inspection packaging lines. One vessel may be used for buffer preparation, another for active addition to the buffer, and a third for holding the final formulated product before filling. The set up can vary depending on batch size and the formulation being produced.

General processing conditions must be closely examined to ensure product compatibility. Typically, the following items must be examined: materials in the processing vessels and transfer lines; duration of product exposure to various temperature and sterility conditions; and shear forces associated with mixing, filtration, and filling. During the formulation-and-filling operation, rHuEPO primarily comes in contact with stainless steel. Exposure to silicone tubing, various filtration membranes, ceramics, and glass can occur, however. Generally, process conditions are tested, and the product is placed on a stability testing protocol to ensure that no deleterious effects result from processing.

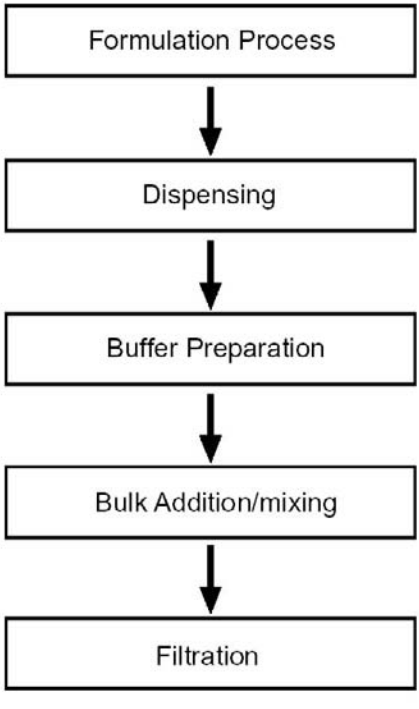

Figure 11. Generic process flow from bulk to packaging operations

The initial step of the dosage form manufacturing is bulk dispensing and addition of materials associated with the final formulated product. Product amounts are adjusted according to batch size and concentration (potency). Additional excipient raw materials are added before production. Before use, each material is examined to ensure that safety, quality standards, and product-formula requirements are met. Buffer preparation is completed by the addition of the excipients to purified Water For Injection in a processing vessel. The buffer can be filtered to clarify and to remove particulates. After buffer preparation, the bulk rHuEPO is added. Additional components that protect or stabilize the rHuEPO can also be added. The formulations or stockkeeping units that are used in multidose settings will contain an antibacterial agent to minimize microbial growth after initial breech of the stopper septum. The bulk rHuEPO product together with the excipients added to make up the formulation is referred to as the formulated bulk.

The formulated bulk is sterile filtered into a holding vessel and stored until filled. Dosage-form components such as vials or syringes are filled using automated equipment in a clean-room environment. The filled vials and syringes are immediately sealed with a stopper, and capped (in the case of vials). Product contact components such as stoppers, vials, and syringes are washed and depyrogenated at high temperature or sterilized in the presence of steam. Some components can be purchased from vendors pre-sterilized and ready to use.

Each filled vial or syringe should be inspected for particulate matter in solution or cosmetic defects, such as marks or scratches. Additionally, each unit is inspected to ensure that proper lot number and expiration date have been applied to the label.

Release

Before the release of any rHuEPO lot, the final material is thoroughly tested to ensure sterility, concentration, lack of endotoxin and bioburden, and product integrity according to the manufacturer's quality control requirements. Additionally, batch records from the manufacturing run are reviewed to ensure that no deviation was made during the production run. Finally, as each lot is released, the product is shipped to wholesalers according to validated procedures.

Summary

An example of commercial production of rHuEPO is discussed in this chapter, where the manufacture starts with a cell line that is engineered to produce product in commercial quantities of required quality. The cell line is banked, and the cell bank is used as the starting material for the manufacturing process. In this example, rHuEPO is secreted from genetically engineered mammalian

cells in a cell culture process and then recovered and purified as rHuEPO bulk in a purification process, to achieve the desired product characteristics specified by the manufacturer. Product from different manufacturers may have different quality characteristics. The rHuEPO bulk drug substance is formulated to achieve the required dosage forms for the final drug product. The manufacturing process is performed in a cGMP facility and monitored for consistent performance. Regulatory and other safety requirements are followed to reproducibly produce a safe and efficacious product.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Biosimilar epoetins

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Introduction

Recombinant DNA technology and hybridoma techniques enabled the development of therapeutic proteins such as the interferons (IFN), erythropoietin (EPO), insulin, growth hormones, cytokines, and monoclonal antibodies [1]. These proteins have revolutionised the treatment of many diseases, including, cancer, hepatitis, and multiple sclerosis. The patents of the first generation of biopharmaceuticals have expired or are about to expire, which enables marketing of noninnovator versions of these products, named 'biosimilars' in the European Union (EU), and 'follow-on protein products' in the USA. The EU has established a legal and regulatory framework for acquiring a marketing authorisation for biosimilars to market [2–9], while in the United States the regulatory pathway is still being developed [10].

Biosimilars are defined as biologic products similar, but not identical, to reference products that are submitted for separate marketing approval after patent expiration of the reference products [3, 11–13]. Biosimilars are not considered to be generic versions. Conventional generics of classical chemical drugs are allowed on the market if pharmaceutical equivalence (i.e., identical active substances) and bioequivalence (i.e., comparable pharmacokinetics) compared with the innovator drug have been established. Thus, clinical efficacy and safety studies are not needed. The term 'biogeneric' should be avoided because this implies the active substance of a biosimilar can be shown to be identical to the active substance of the reference product. This generic paradigm cannot be applied to therapeutic proteins that, in general, are a collection of isoforms and not a single molecule as usually the situation with conventional small-molecule drugs.

The market approval is more complicated for biosimilar compared with classical generic drugs. To establish therapeutic equivalence of biosimilars with reference products, clinical trials are essential [12, 14, 15]. Safety of biosimilars is also an important issue. The main difference concerning safety between biopharmaceuticals and conventional drugs is the potential to induce an immune response (immunogenicity) [16, 17]. The European Medicines Evaluation Agency (EMEA) outlined the requirements for market approval of

biosimilars [18]. The EMEA also published public assessment reports for the approved biosimilar products that discuss product characteristics and the clinical data supporting approval. The EMEA has approved biosimilar versions of recombinant somatropin [19, 20] and recombinant human EPO (rHuEPO) [21–25]. One follow-on somatropin product has been approved in the United States [10].

Immunogenicity of proteins

Nearly all therapeutic proteins are immunogenic, and may induce antibodies by two different mechanisms. In the case of products of plant or microbial origin, a classical reaction as to any foreign substance is induced. Human homologues, such as IFNs and rHuEPO, induce antibodies by breaking immune tolerance existing normally to self-antigens. The mechanisms by which tolerance is induced or broken are not completely understood. An important factor to break tolerance is to present the self antigens in a repetitive way. A periodicity of these antigens as present in aggregates of proteins is apparently very efficient in activating ignorant or anenergetic B cells that are responsible for tolerance.

The clinical manifestations of an immune response are highly dependent on the mechanism involved. The classical activation of the immune system by foreign proteins and the breaking of B-cell tolerance by human proteins differ in time of onset and response level. Also the way these types of response are activated differ and, therefore, also the characteristics of the products.

Immune tolerance exists to products that are copies of endogenous proteins such as IFNs, colony stimulating factors, and rHuEPO. Breaking B-cell tolerance starts with the activation of autoreactive B-cells with aggregates as the most important risk factor.

These aggregates may appear during storage making stability and solubility important in predicting immunogenicity. Factors important for the formation of aggregates are oxidation and reduced solubility by the lack of glycosylation, e.g., if glycoprotein is expressed in a prokaryotic host.

Different methods can identify the presence of aggregates and include size exclusion chromatography, sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE), and Western blotting. These methods, however, may miss immunogenic aggregates. Other methods, such as analytical ultracentrifugation, field-flow fractionation, and light scattering techniques, have shown to be able to detect aggregates that go undetected by standard assays.

The only biologic test to study the capacity of a protein product to break B-cell tolerance are mice that have been made transgenic for the specific protein. These mice are immune tolerant and there is a good correlation of an immune response between these mice and patients. Although these models have helped to identify the factors important for breaking B-cell tolerance and also have been useful in improving the formulation of products, not enough

experience allows them to be used as absolute predictors of immunogenicity of human proteins. The only way to test the immunogenicity of any human product, including biosimilars, is through clinical trials.

When designing clinical trials for testing the immunogenicity of human proteins, many factors must be taken into account. Breaking tolerance by human protein takes, in general, more than 6 months of chronic treatment. The route of administration influences the likelihood of an antibody response independent of the mechanism of induction. The probability of an immune response is the highest with subcutaneous administration, less probable after intramuscular administration, and intravenous administration is the least immunogenic route.

The biologic activities of the product also influences its immunogenicity. An immune-stimulating therapeutic protein is more likely to induce antibodies than a immune-suppressive protein. Impurities may influence immunogenicity. Oxidised proteins were shown to induce antibodies that crossreacted with the unmodified product, and host cell-derived endotoxins have been reported to act as adjuvants. The probability of an immune response, therefore, increases with the level of impurities.

Sex, age, and ethnic background have been reported to influence the incidence of antibody response to specific therapeutic proteins. Type of disease has been identified as the main risk factor of patients. Patients with cancer are less likely to produce antibodies to therapeutic protein than other patients for reasons that are not clear.

Concomitant therapy should be considered. Immune suppressive therapy reduces the probability to develop an immune response to proteins. In addition, immune suppressive drugs, such as methotrexate, are used in conjunction with monoclonal antibodies and other protein drugs to reduce the immune reactions. Thus, immunogenicity data obtained in one population cannot always be extrapolated to another population.

Consequences of antibodies

There may be no clinical consequence for developing an immune response to a biopharmaceutical. The patient may develop binding antibodies that do not significantly affect the activity of the biopharmaceutical or the endogenous protein. On the other hand, anti-product antibodies can bind to and influence the pharmacokinetics of a biopharmaceutical.

A severe consequence of immunogenicity is the loss of efficacy of the product, which can be a severe problem if there is no alternative treatment and the product has a clinically important effect. An example is the case with antibodies to factor VIII that inhibit its activity in patients with haemophilia. Side effects, such as anaphylaxis-like reactions and serum sickness, are mainly caused by the formation of immune complexes seen when the protein is administered in high doses. Major clinical impact can occur if the endogenous

protein with essential biologic activity is also neutralised. For example, neutralising endogenous EPO can result in a rare condition known as antibody-mediated pure red cell aplasia (PRCA).

Between 1998 and 2003 a large increase in the incidence of antibody-mediated PRCA occurring in patients with chronic kidney disease and anaemia who had been treated with Eprex, a formulation of epoetin alfa marketed by Johnson & Johnson [17, 25–27]. The PRCA cases were associated with a breakdown of immune tolerance to treatment with rHuEPO, particularly with subcutaneous administration, resulting in neutralising antibody formation against both recombinant and endogenous EPO [28]. Previously, PRCA caused by production of neutralising anti-EPO antibodies occurred very rarely with treatment. The apparent increase in immunogenicity coincided with a relatively minor formulation change for Eprex – replacement of human serum albumin as a stabiliser with glycine and polysorbate 80. The withdrawal of this formulation of epoetin alfa for subcutaneous administration led to a subsequent decrease in the incidence of PRCA. The reason why Eprex induced PRCA is still not fully understood. A number of possible causes have been proposed [28–30], including micelle formation from polysorbate 80 and epoetin alfa [29, 31] or leachates from rubber stoppers breaking B-cell tolerance through an adjuvant effect [30, 32, 33]. Although many factors are reported to influence the immunogenicity of therapeutic proteins, aggregates play a role in most cases. The Eprex preparation showed an increase in the amounts of aggregates during storage, although the amount was reported never to have exceeded specifications. Product specifications, however, are not defined on the basis of biologic effects, and aggregates may still be relevant for PRCA induction.

The Eprex case highlights concerns regarding the unpredictability and seriousness of immunogenicity of biopharmaceuticals and the potential clinical consequences of their extensive use. Only a small change in the manufacturing process appears to have altered the product's characteristics with a drastic impact on clinical outcome.

The biosimilars assessed by the EMEA

The EMEA provides information on the approval process for human medicines (the European Public Assessment Report [EPAR]), including a scientific discussion on the clinical data submitted for approval. Generally, the EPAR for biosimilars have stated that the biosimilar received approval because it was shown to have a comparable quality, safety, and efficacy profile to the reference product [34–37]. Despite the comparability of these biosimilars to the reference products, clinicians should be aware of some of the issues that emerged during the development and approval of these products that highlight the challenges of biosimilars.

Two biosimilar somatropins, Omnitrope and Valtropin, have been approved by the EMEA. Omnitrope is a biosimilar version of the reference product,

Genotropin. Like Genotropin, Omnitrope is a recombinant form of human somatropin that is manufactured with recombinant DNA technology synthesised in *Escherichia coli*. During the development of Omnitrope, an immunogenicity issue emerged with an early version of the product. As many as 60% of patients enrolled in a study developed anti-growth hormone antibodies, which did not appear to affect growth rate. The cause of immunogenicity was linked to excess host cell protein contamination, which was resolved by the manufacturer with additional purification steps [41].

The first biosimilar epoetin alfa received marketing authorisation in August 2007, which was followed by the publication of the EPAR of Binocrit (Sandoz GmbH), Epoetin alfa Hexal (Hexal Biotech Forschungs GmbH), and Abseamed (Medice Arzneimittel Pütter GMBH & Co). Although the biosimilars are marketed by three companies, they are the product of the same manufacturing plant and the applications for marketing authorisations were identical.

The second biosimilar products of epoetin alfa were approved for marketing in October 2007, and the EPAR of Silapo (Stada) and Retacrit (Hospira) were published in January 2008.

'Biosimilar' is a regulatory term and not a scientific term. Only the evaluations by the regulatory agencies will ultimately define how similar a 'similar' must be. The introduction of biosimilars will reveal how the unavoidable differences between the products translate in biologic and clinical differences. EPAR, however, only provide restricted information, especially information concerning the physical-chemical characteristics of the biosimilar.

As expected, the biggest differences in the structure are in the glycosylation profiles. Compared with the reference epoetin alpha (Eprex), Binocrit contains more high mannose, while Silapo has a reduced amount of *O*-glycan, sialic acid, *N*-glycolyl neuraminic acid, and *O*-acetyl neuraminic acid, which the EMEA considers to be 'undesired variants'.

In the case of Binocrit, this difference has no influence on the *in vitro* behaviour. In the case of Stada, the *in vitro* data were incomplete, showed significant differences between batches, and did not always included direct comparison with the reference product. It is apparent that Stada has a slightly reduced specific activity that translates into reduced pharmacodynamic and clinical activity when dosing patients on the basis of mass (μg).

The reduced specific activity may be related to the possible differences between the product and the standard provided by the European Pharmacopoeia Commission. The suggested different dose-response *in vitro* and *in vivo* of the Stada makes the use of this standard a problem. The use of the murine model to test the relative potency is intrinsically not very precise and allows a relative high variation.

Differences in specific activity are not unusual between comparable products. The two IFN-β products produced in Chinese hamster ovary (CHO) cells (Avonex and Rebif) show a considerable difference in specific activity but are clinically comparable.

For the biosimilar epoetin products, the Committee for Medicinal Products for Human Use (CHMP) has issued a product-specific guideline that describes that two phase III clinical trials are necessary. In the case of Binocrit, however, the European regulators have accepted the data from a single trial, because the subcutaneous route of the comparator Eprex was contraindicated at the time of the studies.

Stada was evaluated in two pivotal trials, a correction phase study and a maintenance study. Both studies with the Stada met their primary endpoint with respect to mean haemoglobin concentrations. In the correction phase study, however, the prespecified equivalence margins of the treatment difference in the dosage were not met. In both studies, Stada showed a reduced clinical activity, which is probably based on the difference in specific activity that was considered clinically not relevant by the CHMP.

Both the Hexal and Stada have exploratory clinical studies assessing efficacy and safety of the biosimilar in the treatment of chemotherapy-associated anaemia. Overall, no significant difference was seen between the treatment groups for the incidence or type of adverse events. Both biosimilar epoetins had a safety profile similar to that of the innovator comparator Eprex.

The possible immunogenicity of the biosimilars and the possible development of PRCA has been an important issue in the evaluation. The Eprex-associated PRCA was the result of a very specific change in the formulation. It is unlikely, therefore, that other immunogenicity problems with an erythropoiesis-stimulating agent (ESA) will result in PRCA.

An antibody response induced by another epoetin may have a different character and may lead to different clinical consequences. With both biosimilars, a radioimmune precipitation assay (RIA) used to monitor the immunogenicity. This assay has been used as the gold standard in the diagnosis of antibody induced PRCA. How well this assay is capable to determine early immune responses in a low-risk population, however, is far from clear. With this assay, a transient binding antibody response was seen before treatment in a number of patients with renal disease who were treated intravenously in the pivotal trials with the innovator and/or biosimilar products. The questions remain whether the specificity of RIA is sufficient to screen for immunogenicity in low-risk populations.

In the risk management programs of both biosimilars, a cohort study is described to assess the incidence of PRCA after marketing authorisation. The number of patients to be involved or the length of the study is not disclosed, but considering the rarity of PRCA, these studies will need to enrol tens of thousands of patients to assess the rate of the event.

The CHMP has clearly taken no position in the discussion about the naming of the biosimilars. The innovative producers of the original products have argued for each biosimilar to have a specific International Nonproperity Name (INN). The CHMP has accepted both the INN name to be identical to the original epoetin alfa in the case of Hexal. In the case of Stada, however, it has accepted epoetin zeta as the INN name.

The approval of these biosimilar products does not substantiate interchangeability with reference products [42]. Furthermore, the EMEA has not approved all biosimilar applications. Alpheon, a biosimilar version of Roferon-A (IFN alfa-2a), was rejected by the EMEA. The manufacturer of Alpheon had submitted nonclinical data (protein structure, composition, and purity) on the biosimilar and conducted a randomised controlled trial study in patients with hepatitis C to demonstrate comparable efficacy and safety between the biosimilar and reference product. The reasons for the rejection by the EMEA included quality and clinical differences between Alpheon and the reference product, inadequate data on the stability of the active substance, inadequate validation of the process for the finished process, and insufficient validation of immunogenicity testing [43].

Conclusions

Biosimilar products are very complex molecules and, therefore, cannot be treated the same as conventional generic drugs. Although a variety of assays are available, they may not be adequate to reliably predict the safety and efficacy of a biosimilar product. The regulatory approval of biosimilars requires much more than the demonstration of pharmaceutical equivalence and pharmacokinetic bioequivalence associated with conventional generics.

The immunogenicity of recombinant therapeutic proteins has become a significant safety concern. Ultimately, only clinical studies and post-authorisation pharmacovigilance to monitor potential immunogenicity will provide definitive evidence for product comparability to the innovator product with respect to safety and efficacy [13, 15].

Outstanding issues will need to be resolved, including substitution, naming, and labelling [44]. It is unclear whether biosimilars and innovator biopharmaceuticals should be considered interchangeable. The labels of the approved biosimilars are nearly identical or are very similar to those of the reference product. A more transparent label that included relevant clinical data for the biosimilar, i.e., the data included in the EPAR, would help clinicians make informed treatment decisions.

Epoetin products from manufacturers outside Europe and the USA have been available in areas of the world without patent protection. These products were shown to differ widely in composition [45]. Although this is no proof that these products are clinically inferior to the innovator product, some of the products failed to meet their own specifications. Some of the manufacturers do not have adequate control over their production process. The content of the vials often did not meet the labelled activity. This mislabelling may lead to undesired clinical effects. Many biopharmaceuticals, including epoetin, are heterogeneous with respect to isoform distribution. As the contribution of different isoforms to the overall activity and toxicity is unknown, consistency between production runs is essential. With that in mind, the most alarming

aspect of this comparison was the variation observed between different lots of the same product (Fig. 1). This inconsistency between batches exposes major flaws in the control of the production process, or worse yet, may even hint to different sources of what is claimed to be an identical product.

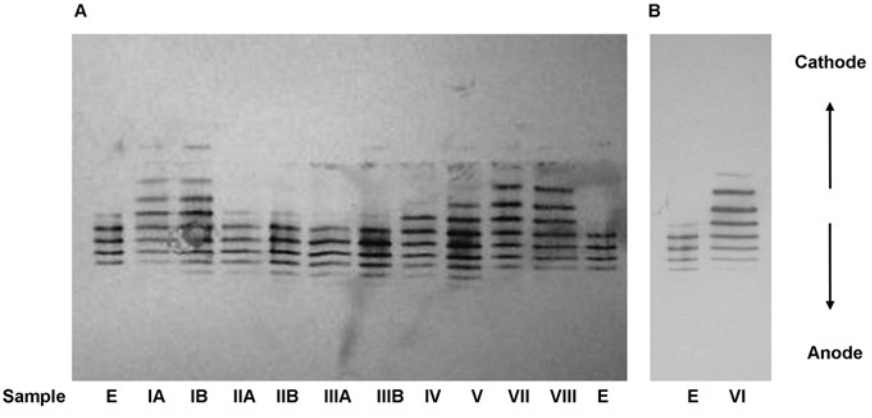

Figure 1. Isoelectric Focusing/Western Blot. Isoform distribution of each sample is shown. For comparison, the Eprex (E) control is shown in the first and last lanes of Figure A and in the first lane of Figure B. I-VIII are epoetin alfa formulations from different manufacturers. A and B means different batches from the same manufacturer.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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New molecules and formulations

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Introduction

Recombinant human erythropoietin (rHuEPO; epoetin alfa), is a glycosylated hormone commonly used for the treatment of anemia associated with chronic kidney disease [1–4]. It is also indicated for the treatment of certain anemias associated with cancer, HIV infection, and for use in surgical situations to reduce allogeneic blood transfusion requirements. A number of studies have demonstrated that rHuEPO is well tolerated and effective at ameliorating anemia, restoring energy levels, and improving patient quality of life in these indications [5–7]. It has also been shown to reduce transfusions, a procedure that can carry inherent risks including transmission of infectious agents and iron overload. In addition, the blood supply is limited and transfusion-induced immune reactions can complicate organ transplantation, for example in patients with kidney transplants.

A more effective anemia treatment option was needed and the discovery and development of rHuEPO via cloning of the human *EPO* gene began in the early 1980s (*see* chapter by M.A. Foote). While rHuEPO has a defined amino acid sequence, differences in its production result in changes in post-translational modifications including glycosylation, conformation, and impurities. Currently it is not possible for another manufacturer to duplicate exactly the product profile of the innovator [8]. Thus, the term ‘generic’ is not used to describe rHuEPO molecules made by different manufacturers. Instead, the descriptors ‘follow-on biologics’ (FOB), ‘generic biosimilars’, or ‘generic biopharmaceuticals’ are used [9].

The first commercial rHuEPO product introduced in the United State and European Union was epoetin alfa (Epogen, Amgen Inc; Procrit, Ortho Biotech Eprex, Ortho Biotech). It was followed by the introduction of a second product, epoetin beta (e.g., NeoRecormon, Roche). Epoetins alfa and beta have minor structural differences but similar physiological effects [10, 11]. Both epoetins are produced by Chinese hamster ovary (CHO) cells. Other FOBs have also been developed. Their approval and acceptance in the EU has been slow, primarily because of incomplete information regarding their efficacy and safety profiles (e.g., likelihood that the agent will promote pure red-cell aplasia [PRCA]).

As patents for epoetins alfa and beta expire, follow-on epoetins, or biosimilar epoetins, have been approved by the European Medicines Agency (EMEA), including three epoetins alfa: Binocrit (Sandoz), HEXAL (Hexal Biotech Forschungs), and Abseamed (Medice Arzneimittel Puetter). An epoetin produced in baby hamster kidney cells, epoetin omega, differs somewhat from epoetin alfa and epoetin beta in its glycosylation profile. While it was available in Eastern European countries and parts of Latin America, it is no longer marketed. Epoetin delta (Dynepo, Shire), which is produced from an engineered human fibrosarcoma cell line HT1080, also has been marketed [12].

In addition to epoetin alfa, epoetin beta, and FOBs, other classes of molecules that may stimulate erythropoiesis have been explored. Together with the epoetins, these types of agents have been given the name 'erythropoiesis-stimulating agents' or ESAs. Most of these agents are peptidic in nature; however small molecule drugs also have been explored. ESAs can also be categorized according to their mechanism of action and where in the erythropoiesis process they may intervene (Fig. 1).

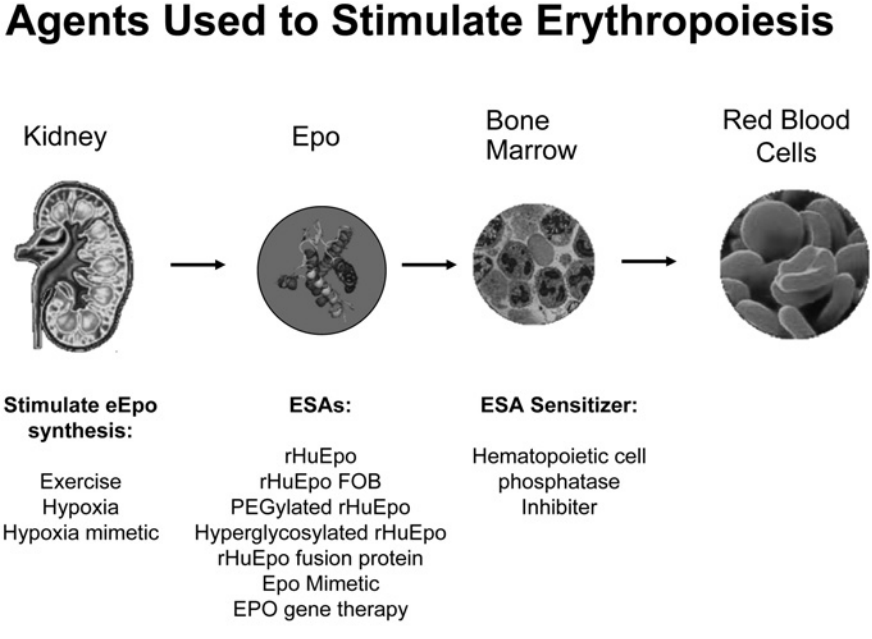

Figure 1. ESAs and points of intervention ESAs can induce EPO synthesis by hypoxia or through use of hypoxia mimetics. Endogenous EPO, rHuEPO, or mimetics of EPO can bind and activate EPOR stimulating erythropoiesis. EPOR activation results in signal transduction involving phosphorylation of EPOR and phosphorylation of JAK2 followed by downstream events. Signal transduction is halted by dephosphorylation of EPOR by hematopoietic cell phosphatase (HCP) or induction of suppressors of cytokine signaling (SOCS). Constitutive phosphorylation of EPOR, constitutive activation of JAK (or JAK2 agonists), loss of HCP activity or inhibition of SOCS activity can induce erythrocytosis.

Considerations of new formulations and drug entities

The purpose of any therapeutic intervention is to treat the patient with an appropriate risk-benefit profile. rHuEPO has been particularly successful in this regard. The molecule is effective at stimulating erythropoiesis with a well characterized safety profile. This risk-benefit profile has created a high standard against which any new erythropoiesis-stimulating molecule will be measured. Thus, the preferred properties of any new drug or formulation should retain or increase efficacy and avoid new or unwanted side effects or toxicities.

One particular concern that has arisen with some rHuEPO molecules is anti-EPO antibodies [13, 14]. Antibody formation to drug entities is of concern not only because the drug may lose efficacy, but also because such antibodies might cross-react with endogenously-produced EPO resulting in pure red cell aplasia (PRCA), a very serious and severe form of anemia [14]. Potential causes for antibody formation not only include the structure of the molecule itself, but also the breakdown products or aggregates generated during manufacturing or storage. Accordingly, new drugs should be designed and manufacturing procedures put in place to minimize this risk.

The production and clearance rates of endogenous EPO protein are naturally optimized for maintenance of hemostasis in the body. This natural regulation, however, is difficult to recreate using a therapeutic. For example, rHuEPO is indicated for three times per week administration in humans. Needlesticks and the inconvenience to the patient and healthcare provider burdens both groups. The clinical setting and other practical concerns may also influence the choice of ESA: areas that lack of refrigeration may desire ESAs that can be stored at room temperature. Infrequent subcutaneous administration of rHuEPO or orally available molecular entities may be useful for home administration. Patients who have frequent visits to the clinic, such as is common for hemodialysis, who already have intravenous access, may prefer frequent administration of intravenous formulations so their hematocrit and drug dose can be monitored frequently. These and other factors are considered in design and development of new molecular entities and formulations.

Approaches to enhancing or modifying a drug's properties have included new formulations and delivery systems of the existing drugs. Pumps or slow-release formulations can be used to maintain the circulating concentration of the drug for extended periods of time (sustained delivery). Modification of the drug by fusion with polymers or other proteins or peptides can be used to extend the half-life (sustained duration of action). Attempts to modify the protein itself by *in vitro* mutagenesis also have been explored. Glycoengineering has been successfully applied to rHuEPO. With this process, new carbohydrate attachment points were introduced into the protein by *in vitro* mutagenesis of the *EPO* gene and followed by expression in mammalian cells. Some of the glycoengineered forms had an increased number of attached carbohydrates, increased *in vivo* activity and serum half-life. The new molecule may be administered with extended dosing intervals with no loss of efficacy.

Some limitations of rHuEPO due to its peptidic composition can potentially be bypassed with new molecular entities such as small molecules or antibodies (EPO mimetics). These compounds may have advantageous properties not present in rHuEPO, such as an oral delivery (small molecule) or structural conformations that hold little in common with EPO, thus exploiting different routes of absorption or elimination. Finally, stimulation of erythropoiesis by mechanisms different from those of rHuEPO, such as those steps upstream or downstream from the receptor activation step have been explored. This chapter discusses new ESAs, their formulations and delivery systems being considered or developed and their limitations, if any.

Erythropoiesis-stimulating agents

EPO molecules with altered activity

One approach to increase activity of EPO is to alter the interaction with the EPO receptor (EPOR). EPO activates erythroid precursor cells by binding and activating EPOR on the surface of erythroid progenitor cells [15]. Receptor activation occurs as a result of a conformational change in the EPOR triggered by EPO binding [16, 17]. The conformational change is induced when the two binding sites on a single EPO molecule crosslink 2 EPOR [18–21]. The two binding sites on rHuEPO have different affinities: high (approximately 1 nM) and low (approximately 10 μM) [22, 23]. Initially, one receptor binds to the high-affinity binding site and is followed by binding of a second receptor to the low-affinity binding site.

Molecules that have increased affinity at either of the two sites can have increased *in vitro* potency. Yet despite this property, no ESAs suitable for clinical development have been reported that have increased biologic activity *in vivo* as a function of increased receptor affinity. The reasons are several-fold. First is the theoretical concern that antibodies, were they to develop against such a modified ESA, could negatively affect patient outcome. Although there are numerous mechanisms to prevent the generation of antibodies against endogenous proteins, administration of a slightly modified form of an endogenous protein may trigger antibody generation. In general, these antibodies recognize the regions of the protein that have been modified. If an ESA was modified in the region that contacts the EPOR (the EPO:EPOR interface), it is likely that antibodies developed against this region would disrupt the EPO:EPOR interaction, thereby inhibiting ESA-stimulated erythropoiesis. If in addition to binding the modified ESA, the antibodies also neutralized endogenous EPO, PRCA would likely result.

A second reason ESAs with increased EPOR affinity haven't been developed is that increased affinity does not always translate into increased *in vivo* potency [24]. Recombinant HuEPO binding to EPOR can trigger rapid internalization of the EPO:EPOR complex with subsequent degradation [25, 26].

Because erythropoiesis requires continuous stimulation of multiple EPOR through multiple binding events, serum half-life is a more important determinant of *in vivo* potency than is receptor binding activity. The EpoR-signaling pathway is down modulated shortly after activation [26, 27]. As a consequence, molecules cleared quickly have low *in vivo* activity [24], which has been observed with carbohydrate-modified EPO analogs that demonstrate increased EPOR binding *in vitro* and decreased *in vivo* efficacy [28–30]. Increased dose can partially compensate for the increased clearance; however, these compounds may need be administered more frequently.

In contrast to molecules with increased affinity for EPOR, some ESAs approved for clinical use have reduced receptor affinity, e.g., darbepoetin alfa [24, 31] (Tab. 1) and pegylated epoetin beta [32]. Usually reduced EPOR binding activity results in both decreased *in vitro* and *in vivo* activity; however, some molecules that demonstrate reduced receptor-binding activity can have increased *in vivo* potency because the increased serum half-lives of these ESAs can more than compensate for reduced receptor binding activity [24, 31]. This activity is highlighted by results shown in Table 1 where rHuEPO analogs with mutations in the receptor-binding sites (either the high-affinity site, site 1; or the low-affinity site, site 2) had reduced EPOR-binding activity that correlated with both reduced *in vitro* and *in vivo* activity. However, darbepoetin alfa had increased *in vivo* activity despite its reduced receptor binding activity because of its increased serum half-life.

Table 1. Activity of rHuEpo analogs

Analog[a]	Description[b]	Receptor binding[c] % of rHuEpo	*In vitro* activity[d] % of rHuEpo	*In vivo* activity[e] % of rHuEpo
rHuEPO	Control	100	100	100
darbepoetin alfa	Increased carbohydrate content	20	11	171
NM311(R14/Q)	Site 2 mutation, Same carbohydrate content	10	2.0	5.9
NM335 (N147/K)	Site 1 mutation, Same carbohydrate content	0.8	0.5	1.7

Activity was normalized to that of rHuEPO, arbitrarily set at 100.

[a] Mutations were introduced into the coding region of an rHuEPO cDNA by *in vitro* mutagenesis. rHuEPO and analogs were expressed in CHO cells and purified as described previously [35]. Aranesp has two additional *N*-linked carbohydrate chains compared with rHuEPO.

[b] Site 1 mutation: high-affinity-binding site, site 2 mutation: low-affinity-binding site. Carbohydrate content of NM311 and NM335 similar to that of rHuEPO.

[c] Receptor binding activity determined using competitive binding to OCIM1 cells [20].

[d] *In vitro* activity measured using EPO dependent 32D cells according to ref 20.

[e] *In vivo* activity determined using a mouse exhypoxic 59Fe uptake assay [24].

While ESAs are typically chosen because they have high-receptor binding activity, there are conditions where certain analogs with low EPOR-binding activity may have clinical utility, such as in patients with polycythemia. Compounds that bind through the high-affinity site but do not dimerize and activate EPOR because of reduced binding at the low-affinity site can function as antagonists [23]. These molecules may bind to EPOR, are inactive themselves, but inhibit binding of endogenous EPO. Such molecules may be monovalent Fab (fragments of antibodies, small molecules with only one binding site) or rHuEPO analogs where the low-affinity binding site is removed [33].

Other changes in rHuEPO that alter physical properties have been considered. Changes in rHuEPO amino-acid sequence can result in increased stability. These changes can include removal of amino acids that are unstable (e.g., Trp) [34]; or are subject to oxidation (e.g., Met), deamidation (e.g., Asn), or changes that confer increased conformational stability, such as those that stabilize alpha helices or connecting loops. Such molecules may be more amenable to long-term storage or suitable for formulations where more stable EPO molecules may be useful, such as in slow-release formulations or automated delivery systems. Removal of proteolytic cleavage sites by *in vitro* mutagenesis can enhance *in vivo* stability. It may be possible to remove antigenic sites, thereby reducing immunogenicity.

Molecules with increased serum half-life

A longer duration of action can allow for reduced frequency of administration and increased *in vivo* potency. One approach that has been successfully applied to rHuEPO is glycoengineering [35]. Glycoengineered molecules bind and activate the EPOR in the same manner as rHuEPO, resulting in similar biologic responses while at the same time reducing clearance and enhancing *in vivo* activity [31]. Other strategies to increase duration of action of EPO included chemical modifications, such as the addition of a polyethylene glycol molecule (PEGylation) or gene fusions between EPO and other proteins. In these cases, the goal is to reduce clearance rate by increasing hydrodynamic size.

Glycoengineering

Recombinant human EPO is a glycoprotein hormone consisting of approximately 40% carbohydrate [36]. The carbohydrate component consists of three *N*-linked carbohydrates attached to Asp at amino-acid positions 24, 38, and 83; and an *O*-linked carbohydrate attached to Ser at amino acid position 126 [36] (Fig. 2). Unlike the invariant protein sequence, the carbohydrate is variable in structure, resulting in glycoforms with modest differences in sizes, structures, and sugar content [37, 38]. A typical *N*-linked carbohydrate made by mammalian cells is branched with 2–4 arms. The end of each arm is typically capped

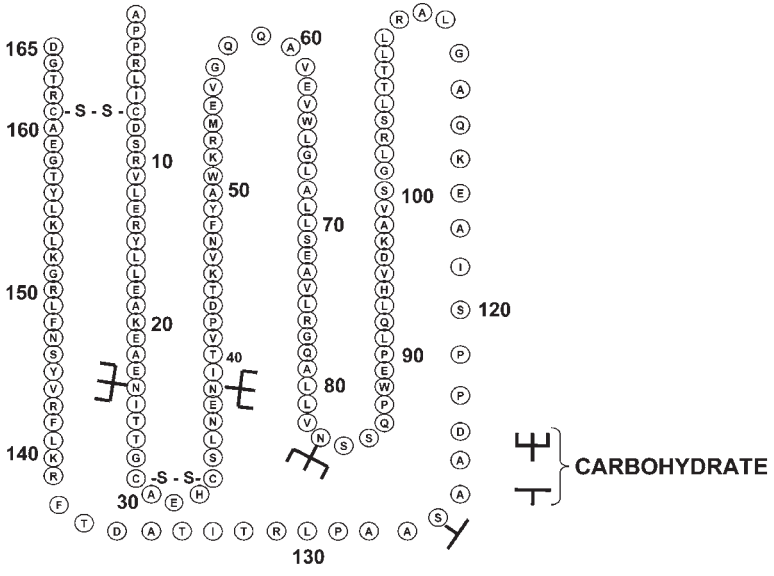

Figure 2. Amino Acid Sequence of Human EPO. Recombinant human erythropoietin (rHuEPO) is 165 amino acids in length. Disulfide bonds (-S–S-) join Cys 7 to Cys 161 and Cys 29 to Cys 33. The 3 *N*-linked glycosylation attachment points are at Asn 24, Asn 38, and Asn 83 and the *O*-linked carbohydrate is attached to Ser 126. Forked structures depict the attached carbohydrates.

by a sialic acid; sialic acid content exhibits microheterogeneity in the different glycoforms. The sialic acid is of importance because it is the only negatively charged sugar on the carbohydrate. Variations in the amount of sialic acid can affect the electrostatic properties of the molecules to which it is attached.

The carbohydrate is essential for *in vivo* but not for *in vitro* biologic activity [24, 28, 29]. The sialic acid component of carbohydrate, in particular, plays a critical role in the *in vivo* biologic activity of rHuEPO. Removal of sialic acid from the carbohydrate of EPO results in increased *in vitro* activity but almost complete loss of *in vivo* activity [24, 29]. Studies on glycoforms of rHuEPO containing different sialic acid contents demonstrated a direct relationship between increased sialic acid content and increased *in vivo* activity [39]. The increased *in vivo* activity was due to an increased serum half-life of the molecule and not increased affinity for the EPOR. The theoretical maximum number of sialic acids on rHuEPO is 14 (up to four sialic acids for each of the three *N*-linked carbohydrates and up to two sialic acids for the *O*-linked carbohydrate) [38]. It was hypothesized that *in vivo* activity may be increased beyond that observed with rHuEPO by adding new sialic acid containing *N*-linked carbohydrates. Each new *N*-linked chain could add up to four additional sialic acids.

To add more *N*-linked carbohydrate, *N*-linked glycosylation sites were introduced into the amino acid sequence of EPO. *N*-linked carbohydrate is

attached to an Asn present in the consensus sequence Asn-Xxx-Ser/Thr (where Xxx can be any amino acid except proline) [40]. This sequence is necessary but not sufficient for *N*-linked carbohydrate addition [41]. During synthesis of a glycoprotein, appropriate consensus sequences are recognized by oligotransferases in the cell, resulting in attachment of carbohydrate. This carbohydrate is subsequently modified by the action of additional intracellular enzymes. The glycosylated protein is then secreted from the cell into the circulation [42].

For the purpose of potentially developing a new drug with properties superior to then available products, it became apparent that simply adding an *N*-linked consensus sequence to rHuEPO would not be sufficient. The changes needed to be introduced in such a way that the resultant molecule was efficiently glycosylated and retained activity, conformation, and stability. To increase the likelihood of success, the amino-acid changes were introduced into regions of the molecule distal to the receptor-binding site to ensure that the molecule would efficiently bind to and activate the EPOR. This effort was aided by structure-function studies that defined the active sites of rHuEPO and determination of amino acids important for maintenance of structure [20, 43, 44].

EPO glycosylation analogs with introduced *N*-linked glycosylation consensus sequences were constructed and tested [35]. Several candidates containing additional carbohydrate had acceptable activity and conformation characteristics. The consensus sequences successfully introduced into two of these candidates (Asn30Thr32Val87; and Asn88Thr90) were combined to generate a new molecule with two additional *N*-linked carbohydrates. This molecule (darbepoetin alfa, Aranesp) had near-normal *in vitro* activity, was glycosylated efficiently, and had a similar conformation and stability to rHuEPO. The carbohydrate content was increased from 40% to 51%, the size from approximately 30,400 Da to approximately 37,100 Da, and the maximum number of sialic acids was increased from 14 to 22.

Studies in mice with darbepoetin alfa revealed that more rHuEPO was required to obtain a response similar to that of darbepoetin alfa [31, 35, 39]. In nonclinical studies, animals were administered rHuEPO or darbepoetin alfa at various dose intervals. The relative *in vivo* activity difference of rHuEPO and darbepoetin alfa increased as the dosing interval increased [45]. Three-fold more rHuEPO than darbepoetin alfa was required to elicit a similar response when the drugs were administered three times per week. This difference increased to 13-fold when the molecules were administered at weekly intervals. The increased *in vivo* activity and the differing potencies with changes in dose interval could be explained by an observed three-fold increase in serum half-life of darbepoetin alfa over rHuEPO [24, 31]. The observation that the serum half-life increased in proportion to the number of added carbohydrate chains indicated that the carbohydrate directly affected clearance.

Testing of darbepoetin alfa in humans mirrored the results found in animals. Compared to epoetin alfa, the serum half-life of darbepoetin administered intravenously was increased approximately three-fold [46]. In clinical trials, patients were converted from rHuEPO administered two to three times per

week to darbepoetin alfa administered once per week or from weekly rHuEPO to once-every-other-week darbepoetin alfa. The hemoglobin concentrations were successfully maintained with the less frequent dosing schedule [47, 48]. Additional clinical results demonstrated that hemoglobin concentrations can be successfully maintained when darbepoetin alfa is administered at once every 3 to 4 week dosing intervals [49–51].

One theoretical concern with any alteration in a protein's amino-acid sequence or structure is immunogenicity. Several characteristics of darbepoetin alfa and its methods of manufacture minimized the potential for antibody formation. The particular amino-acid substitutions in darbepoetin alfa had a minimal effect on structure and stability. The carbohydrate and sialic acid content of the material selected during the purification process was maximized for several reasons: first, the higher carbohydrate content enhances the *in vivo* activity; secondly, carbohydrate can increase solubility and stability of proteins thereby inhibiting formation of aggregates and other byproducts that may trigger antibody formation [52–54]. Finally, *N*-linked carbohydrate is large relative to the peptide backbone giving the carbohydrate a 'shielding' effect potentially inhibiting antibody formation. Antibody formation was monitored during clinical trials with darbepoetin alfa, and there was no enhanced formation of neutralizing antibodies compared to that of epoetin alfa made in the United States [14].

It was found that carbohydrate content could be increased even further than that contained on darbepoetin alfa by introducing additional *N*-linked glycosylation sites. AMG205, a rHuEPO glycosylation analog with four additional carbohydrates compared with epoetin alfa, had low 9G8a immunoreactivity, indicating a similar conformation to epoetin alfa [35]. 9G8a is a monoclonal antibody whose binding was increased to rHuEPO analogs with altered conformation or stability and assays with this antibody were used to assess conformation of rHuEPO analogs.

AMG205 *in vivo* potency and serum half-life were increased compared with darbepoetin alfa. In rats, AMG205 had nearly twice the half-life of darbepoetin alfa (intravenous, 31 *versus* 17.2 h; subcutaneous, 28.2 *versus* 13.2 h), respectively [55]. However, AMG205 had an *O*-linked glycosylation deficit. Another candidate, AMG114, exhibited increased efficiency of *O*-linked carbohydrate addition. It also had low 9G8a immunoreactivity. The serum half-life in rats was similar to that of AMG205 (16.7 h). In dogs, the serum half life of AMG114 administered intravenously was 68.9 h compared with 20.4 h for darbepoetin alfa.

Clinical testing in subjects with chemotherapy-induced anemia administered AMG114 revealed an approximately two-fold increase in serum half-life compared with darbepoetin alfa (131 *versus* 73.7 h) and a dose-dependent increase in hemoglobin concentrations when administered at every-3-weeks dosing intervals [56]. These results demonstrate that serum half-life could be increased in humans even further than that observed with darbepoetin alfa and that such molecules could maintain hemoglobin concentrations in humans.

The carbohydrates on darbepoetin alfa, AMG114 (and AMG205) are positioned distant from the recepotor binding sites (Fig. 3). The new chains are opposite to the two chains found on darbepoetin alfa. The areas of the molecule changed are distal to the region of rHuEPO that is involved in the interaction with the EPOR explaining the retention of *in vitro* activity of both darbepoetin alfa and AMG114.

AMG114 has not been developed for clinical use primarily because existing molecules including epoetin alfa and darbepoetin alfa can meet clinical needs.

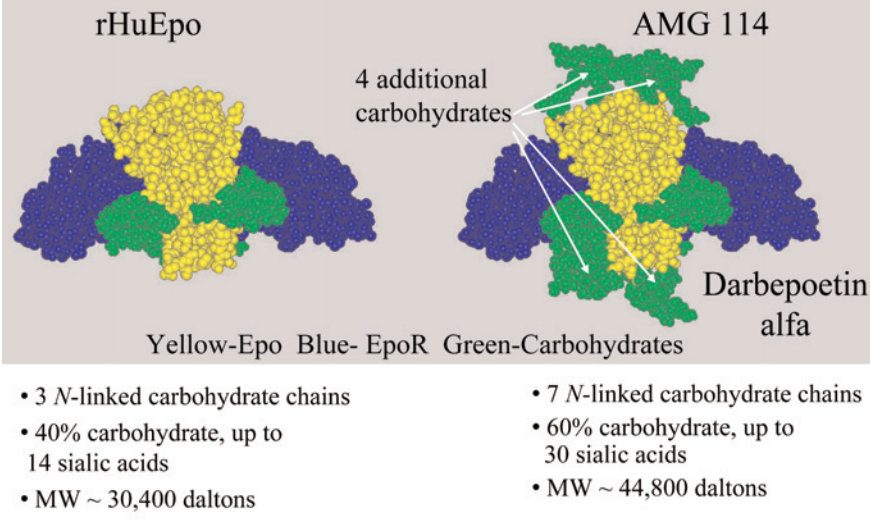

Figure 3. Darbepoetin alfa has two and AMG 114 has four additional carbohydrates distal to the receptor binding regions. A three-dimensional model of rHuEpo (yellow) was based on the crystal structure of EPO bound to the extracellular domains of 2 EPOR (blue)[18]. *N*-linked carbohydrates addition sites are green. The AMG114 glycosylation sites are at the end opposite the from the naturally occurring and added glycosylation sites in darbepoetin alfa. All the new *N*-linked carbohydrates in AMG114, including those in darbepoetin alfa, are distal from the EPOR- binding sites.

In addition, there was concern that molecules with very long half-lives might be more difficult to use clinically because of a potential increase in swings in hemoglobin concentrations.

PEGylation

Mixing activated polyethylene polymers with proteins under appropriate chemical reaction conditions produces PEGylated proteins. PEGylation involves chemical attachment of the polymer, polyethylene glycol (PEG), to reactive regions of proteins or carbohydrates. The attachment of PEG is thought to improve solubility and possibly reduce immunogenicity due to

shielding by the conjugate. PEGylated molecules can have an increased hydrodynamic size because they create a 'water shell' around the molecule. In addition, the increased hydrodynamic size can result in reduced clearance and thus increased *in vivo* activity. Clearance studies with polyethylene glycol polymers showed a direct relationship between size of PEG and its rate of clearance. PEGylation has been used successfully to increase serum half life of many classes of proteins [57]. The attachment of PEG to rHuEPO would increase its serum half-life in proportion to the increased hydrodynamic size of the PEGylated ESA [58]. Polyethylene glycol (PEG) is thought to be relatively inert and nonimmunogenic by itself so it is a suitable starting material for protein conjugate therapeutics.

One issue with drugs made by solution or solid-phase chemistry can be poor specificity of conjugation in the chemical reaction or generation of undesirable byproducts. Many PEGylation chemistries have been tried in an attempt to reduce undesirable byproducts, improve the specificity and efficacy of polyethylene glycol attachments, and minimize immunogenicity risk of the protein conjugate while maximizing the *in vitro* and *in vivo* activity of the resultant molecule [59]. The current chemistries typically target the reactive amino groups on Lys or the amino terminal amine. rHuEPO has 8 Lys, some of which are part of the active site [18, 20]. Therefore, some PEGylated EPO molecules contained PEG conjugated to Lys may have low activity because the PEG interferes with receptor binding and activation [32]. Other PEGylated EPO molecules may have low activity because the attached polymer introduces structural alterations that interfere with receptor binding.

Amino acids can be introduced to increase the specificity of PEG attachment. For example, PEG moieties can be specifically conjugated to the Cys sulfhydryl group. Therefore Cys substitutions at specific regions in the protein may allow targeted PEGylation [60–62]. Another strategy is to make PEGylated EPO synthetically: during synthesis, a PEG-conjugated amino acid could be introduced instead of the unconjugated amino acid. This approach allows specific targeting of particular amino acid positions for PEG attachment, such as the glycosylation sites, and reduces heterogeneity and the potential for loss of *in vitro* activity [63, 64]. It is not clear that these molecules will retain the same stability, *in vivo* activity, and lack of immunogenicity as their glycosylated counterparts, however.

Fusion proteins

Several EPO gene fusion proteins have been reported, including EPO/interleukin (IL-3) [65] and EPO/granulocyte-macrophage colony-stimulating factor (GM-CSF) [66]. EPO dimer has also been generated as a potential therapeutic [67–69]. EPO dimer may have increased *in vitro* activity due to altered avidity to the receptor. However, the clinical relevance of such molecules is unclear. The two protein partners are typically joined by a linker peptide that

includes Gly, Ala, and Ser. These three amino acids are thought to result in linkers that are flexible and relatively inert, allowing independent folding of the two proteins into their appropriate three-dimensional structures.

The promise of such molecules is that they might impart to the fusion protein biologic properties of both molecules. One can imagine that co-administration of a growth factor such as IL-3 that stimulates the early stages of erythropoiesis with rHuEPO can increase efficacy of rHuEPO. Because the fusion protein is larger and may have increased serum half-life, it may impart increased potency for both partners. The fusion protein also ensures that both molecular entities are simultaneously present. Simultaneous administration by fusing two drugs can simplify administration, especially when the two proteins have different pharmacokinetic parameters. The ability to independently adjust dosing of the fusion partners is lost, however. Full *in vitro* activity of both proteins in fusion proteins does not always occur [65]. Furthermore, the difficulty in retaining a nonimmunogenic structure has been a challenge [70] and no ESAs with protein fusions have been approved for use in humans.

EPO mimetics

rHuEPO is currently administered by either subcutaneous or intravenous injection. Because of its large size and peptidic nature, delivery by other routes such as oral, transdermal, or by inhalation can be quite challenging. One possible solution is a small molecule, an EPO mimetic, capable of stimulating the EPOR. EPO mimetics are compounds that mimic the activity of EPO but bear no structural homology. EPO mimetics can have new biologic or biophysical properties not present in EPO. Designed appropriately, such a compound has the potential to be delivered by routes that may be more convenient than those currently in use for rHuEPO, e.g. oral.

Significant challenges are associated with the identification and development of a useful small molecule EPO mimetic. First, the mimetic needs to be small to be delivered orally, yet it needs to be large enough to have sufficient affinity for the EPOR and to ensure it persists long enough in the serum to be efficacious. Finally, the compound should not have unwanted side effects due to either toxicities associated with off-target effects of the compound or from its breakdown products. In spite of these challenges, work has proceeded and progress has been made in attempt to identify compounds that may be amenable to oral delivery.

Several strategies have been used to identify EPO mimetics. The first is to screen peptide and small molecule libraries for those that can stimulate erythropoiesis using *in vitro* bioassays as screens. According to this strategy, the target of the drug may not be known, and compounds active in the assay may stimulate erythropoiesis downstream from the EPOR. Another strategy is to identify molecules that directly bind to and agonize the EPOR in a manner similar to that of rHuEPO. The latter strategy may be performed in two steps:

the first step is to identify compounds that bind the EPOR and the second step is to covalently link the compounds into bivalent dimers that can agonize the receptor by EPOR dimerization.

The latter strategy takes advantage of the observation that an EPOR mutant containing an Arg129 to Cys129 mutation was constitutively active [21, 71]. A disulfide bond formed between the Cys129 residues on the receptors resulted in dimerization and receptor activation demonstrating one instance where EPO was not essential for receptor activation. X-ray crystallography results demonstrated that the EPOR forms a 2:1 complex with EPO [18]. Each receptor uses the same region on its surface to bind to two surfaces on EPO, resulting in receptor dimerization. Further confirmation of the dimerization mechanism was the discovery of agonist monoclonal IgG antibodies that could dimerize and activate EPOR [19]. The anti-EPOR antibodies activated EPOR because they were bivalent, had two binding sites, and could simultaneously bind and cross-link 2 EPOR (Fig. 4). Monovalent Fab fragments could bind but did not agonize the receptor, confirming a requirement for bivalent binding.

Peptide phage libraries were screened and one peptide was identified that could bind and agonize EPOR [72]. This peptide, AF11154, had no homology

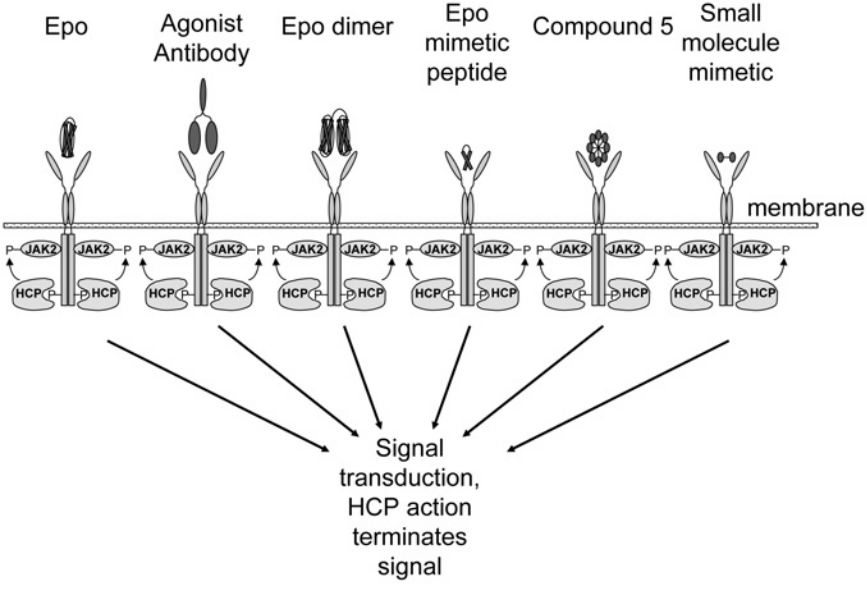

Figure 4. Mechanisms of EPOR Activation. EPOR are activated and when it is dimerized by binding of the two asymmetric receptor-binding sites by rHuEPO. EPO binding results in phosphorylation of EPOR, JAK2 recruitment, and phosphorylation of JAK2. The activation of JAK2 results in downstream signaling events. HCP can bind the activated (phosphorylated) receptor resulting in dephosphorylation of JAK2, thereby terminating signal transduction. The EPO mimetic compounds; agonist antibody [19], EPO dimer [67, 69], EPO mimetic peptide [72], compound 5 [81], and small molecule mimetics [79, 81] can all dimerize and activate the EPOR in a manner similar to that of rHuEPO.

to EPO. It self associated into dimers to form a bivalent molecule that could dimerize EPOR in a 2:2 mimetic:EPOR complex [73]. Additional sequence modification of this peptide resulted in EMP1, a 20-amino acid peptide with an approximate 50-fold increase in affinity over the starting peptide (Tab. 2). The affinity was increased more by covalent linkage of the 2 peptides [74]. The activity of this peptide was still significantly lower (500-fold) than that of rHuEPO when tested *in vitro*. In addition, the *in vivo* activity was very low (25,000-fold less than rHuEPO). However, this work demonstrated that a molecule smaller than rHuEPO could successfully dimerize and activate the receptor. An attempt to discover other EPO agonist peptides by another group was also successful [75]. However, the *in vitro* potency was not increased (Tab. 2).

One explanation for the low *in vivo* activity of mimetic peptides is likely their rapid clearance. One group addressed this problem by creating a fusion protein between EMP1 and a larger protein, plasminogen activator inhibitor (PAI1) [76], resulting in an increase in molecular weight from 4,800 Da to 66,000 Da (Tab. 2). The *in vivo* activity was significantly increased (2500-fold); its *in vivo* activity, however, was still significantly less than that of rHuEPO (100-fold) and the ability to be delivered orally was compromised by the size increase.

In another approach, additional screening of peptide mimetics of EPO produced a molecule with increased stability and affinity for EPOR compared with EMP-1. PEGylation of this molecule reduced its clearance and it is currently in clinical development (Hematide; Affymax Inc Palo Alto, CA) [77, 78].

The peptide mimetics described are significantly larger (approximately 4,200 Da) than the preferred size of an orally bioavailable compound (<600 Da). To overcome this issue, the peptides were used in an attempt to design lead compounds of smaller size. Some nonpeptide small molecule agonists have been isolated based on the EMP1 structure [79]; however, their *in vitro* activities were low (Tab. 2). An independent approach was to directly screen for small molecule EPO mimetics that could dimerize EPOR. Small molecule libraries containing compounds with two-fold symmetry were screened to find dimerizing compounds that agonize the receptor [80, 81]. This strategy did not result in discovery of agonist compounds, however. A small molecule (compound 1, approximately 500 Da) that bound but did not agonize EPOR was discovered. Compound 1 was made active *in vitro* by oligomerizing it with a multivalent crosslinker resulting in a molecule (compound 5) containing eight compound-1 molecules joined together. Compound 5 binding to EPOR was increased somewhat (10-fold) over that of compound 1; however, the size (6,400 Da) was greater than that required to be orally bioavailable. In addition, its *in vitro* activity was low relative to rHuEPO and the compound had toxicity. The feasibility of discovery of small molecules that could agonize EPOR was demonstrated, however to date there are no reports of success in identification of molecules suitable for clinical development.

Table 2. Activity of erythropoietin and mimetics

	MW KDa	*In vitro* activity EC50	Binding IC50	*In vivo* activity Units/mg	Rel difference in activity molar	Notes	Ref
rHuEPO	18,200	5–10 pM	100 pM	>100,000	1	peptide monomer	19, 20, 24, 36
EMP1	4200	400 nM	200 nM	2	25,000	peptide dimer	72,73
EMP dimer	4800	approx 20 nM	approx 2 nM	200	250	peptide dimer	73
EMP-PAI1	63,000	NA	NA	5000	100	peptide dimer	76
ERP	3085	100 nM	approx 1 nM	NA	NA	peptide dimer	75
ERB	2170	approx 3 nM	45 nM	NA	NA	peptide monomer	82
EPO Mab 71	150,000	200 pM	1 nM	NA	NA	peptide dimer	19
Compound 1	500	0	60 μM	NA	NA	nonpeptide monomer	80, 81
Compound 5	6400	1–5 nM	4 μM	NA	NA	nonpeptide dimer	81
A1B10C1	2100	1–10 nM	NA	NA	NA	nonpeptide dimer	79

NA = not available

Stimulation of erythropoiesis through other mechanisms

Difficulties with development of small molecules that activate EPOR by dimerization can be bypassed by targeting a different mechanism or a different step in the erythropoiesis pathway. One report describes a peptide that activates EPOR by binding to a domain on EPOR similar to MHC peptides (Fig. 4). This 23-amino acid peptide (ERP) is reported to have both *in vitro* and *in vivo* EPO activity [82]. The molecule appears to activate by binding EPOR at a region distal to its point of dimerization (transmembrane domain), suggesting that it is activated by binding to a different site than that caused by rHuEPO. The mechanism may be similar to that of the virus envelope protein, gp55 that also activates EPOR by an interaction with the EPOR transmembrane region [83, 84].

Another approach to mimetic discovery is to modulate steps downstream from receptor activation such as inhibition of hematopoietic cell phosphatase (HCP) [85, 86]. HCP is an enzyme that dephosphorylates JAK2, a kinase that is part of the EPOR signal transduction cascade [15]. JAK2 is normally activated (phosphorylated) as a consequence of EPOR activation. HCP binds to activated (phosphorylated) EPOR and then dephosphorylates JAK2, terminating signal transduction. Truncated EPOR lack the HCP binding site, and thus HCP, cannot dephosporylate JAK2, resulting in hypersensitivity of the receptor to EPO. Naturally occurring truncations have been described in humans whereby the affected individuals have increased hemoglobin concentrations but very low EPO concentrations due to a hypersensitive EPOR [87–89]. These observations suggest that small molecule antagonists of HCP may result in increased EPOR activity that increases erythropoiesis in the absence of added EPO.

One concern of HCP inhibitors relates to observations associated with HCP mutations in mice. These mice (motheaten) have a defective *HCP* gene [90] and have multiple hematopoietic abnormalities, including increases in macrophages, lymphocytes, and erythrocytes. HCP is a negative regulator for several different cytokine receptors besides EPOR [85, 86, 91, 92]. Although HCP inhibitors may be effective at increasing erythroid cell number, increases in cell number of other hematopoietic cells may limit the usefulness of these compounds.

Hypoxia mimetics

A more direct approach in stimulation erythropoiesis would be to identify molecules that stimulate production of endogenous EPO. Recently the mechanism by which hypoxia regulates *EPO* gene transcription has been partially elucidated and those pathways may yield novel drugs [93]. One of the several transcription factors that promotes *EPO* gene transcription is hypoxia inducible factors (HIF). The active form includes HIFα and HIFβ subunits of which sev-

eral forms have been described. The HIFα genes are transcribed constitutively but their levels are dependent on the pO2 of oxygen which affects the rate of metabolism and thus the level of active HIF complex. In normoxia, HIFα is rapidly degraded due to oxygen dependent hydroxylation of the alfa subunit of HIFα by a prolyl hydroxylase (HIF-PH). Hydroxylated HIFα associates with Von Hippel Lindau protein which promotes ligation of the protein with ubiquitin followed by rapid degradation by the proteosome. In the absence of oxygen (or iron), HIF-PH activity is reduced and *EPO* gene transcription increases due to accumulation of HIF.

Genetic alterations in one of the three known HIF-PH encoding genes have been reported to result in polycythemia as does certain mutations in VHL [94–97]. Early observations that cobaltous ions can stimulate erythropoiesis [98, 99] are now explained according to their ability to inhibit HIF-PH. Indeed the original erythropoietic biologic unit (cobalt unit) was defined as the erythropoietic response associated with administration of 1 ml of 5 mM cobalt chloride to a starved rat [100]. Thus, alteration of this pathway can stimulate erythropoiesis and attempts to identify clinical candidates are underway.

Initial attempts to stimulate erythropoiesis by administering cobalt clinically were successful at stimulating erythropoiesis in humans [101–103]; however, toxicities associated with cobalt limited its usefulness [104]. Another molecule that inhibits HIF-PH is desferrioximine (DFO) [99, 105]. It is thought to inhibit by reducing the availability of iron, which is also critical for HIF-PH activity [105]. Its poor pharmacokinetic properties and poor efficacy has prevented clinical use [106].

Despite issues with cobalt and DFO, results with these molecules supported additional work to identify molecules with improved safety/efficacy profiles. Other small molecule antagonists of HIF-PH have been described and these can stimulate erythropoiesis in animals [93, 107]. One molecule FG2216 (Fibrogen, San Francisco) has advanced to Phase I/II clinical trials and dose-dependent increases in hemoglobin have been demonstrated in patients with chronic renal insufficiency. Clinical development was halted when a patient died because of drug-related liver failure, but clinical trials have resumed. Attempts to identify other molecules with better safety/efficacy profiles continue.

Gene therapy

Controlled delivery of *EPO* genes to humans is another promising approach for EPO therapy. Early work in this field depended on direct injection of plasmid DNA containing constitutively active *EPO* genes into the muscles of mice [108], resulting in a measurable increase in hematocrit. Several concerns became apparent from these studies, including inefficient and variable delivery of the *EPO* gene and subsequent variations in EPO concentration. In addition EPO expression decreased over time. Improvements in expression systems and gene delivery methods have been reported [109–112]. Current *EPO* gene ther-

apy protocols require repeated administration of *EPO* genes. In addition, there is concern that the therapy may be irreversible or the vector may integrate into the genome at positions that alter gene expression, resulting in tumorigenicity. Constitutive high level expression of *EPO* genes could also result in life-threatening erythrocytosis [113]. Monkey *EPO* genes introduced into Cynomolgus macaque monkeys with their anterior tibialis muscle cells engineered to express monkey EPO initially showed excessive erythrocytosis [114]. Some animals developed antibody-mediated PRCA through an unknown mechanism, creating concerns about safe delivery of gene-delivered EPO.

The efficiency and irreversibility concerns of *EPO* gene therapy have been addressed by developing implantable capsules containing EPO-expressing cells [115]. The capsules can be removed, halting EPO delivery. A further improvement is to control EPO expression with a small molecule such as tetracycline, enabling increased EPO expression in response to oral administration of the gene activator [116] such as tetracycline [109, 110, 117], mifepristone [118], or rapamycin [119]. Additional advances are the development of vectors where EPO expression is controlled by oxygen tension [116], or to target the kidney for gene transfer [120]. Despite these advances, safe and controlled EPO delivery using gene therapy methods suitable for human use remains a distant but tantalizing opportunity.

Clearance of hyperglycosylated and PEGylated derivatives

Despite several decades of work, the mechanism by which ESAs are cleared is not completely understood. In addition, while clearance of ESAs is altered by hyperglycosylation and PEGylation, the pathways impacted by these modifications are also not completely understood. Clearance of ESAs was first thought to be mediated primarily through liver hepatocytes that express an asialoglycoprotein receptor (ASGR) [30, 121, 122] or through the kidney [10, 123–125]. Clearance through the liver appears to be limited to asialo EPO, not fully glycosylated EPO protein, and evidence of clearance through the liver of ESAs is not supported by other studies [122, 126, 127]. Initial data suggesting a major elimination pathway for rHuEPO through the kidneys were not supported by subsequent studies showing that rHuEPO clearance is independent of kidney function [126, 128, 129]. In healthy men, only a small amount of intact radiolabeled epoetin beta (<5% of the dose) was found to be excreted in the urine, suggesting that rHuEPO is degraded elsewhere in the body [130]. Consequently, the role of the liver and kidney in rHuEPO clearance is thought to be minimal.

Binding of EPO to EPOR can lead to cellular internalization and intracellular metabolism [25, 26, 131–133]. The bone marrow where EPOR expressing cells reside is hypothesized to play a role in clearance through EPOR-mediated binding, endocytosis and intracellular degradation. Only indirect evidence,

mostly derived from chemotherapy experiments [134–137], support a role for clearance by this pathway *in vivo*.

To explore the possible role of an EPOR-mediated clearance pathway, an engineered rHuEPO analog (NM385) that was devoid of detectable receptor binding but retained similar structure and carbohydrate content to rHuEPO, was constructed and its clearance properties were examined [138]. After a single intravenous dose of 30 μg/kg in rats or mice, NM385 had a slightly longer terminal half-life but similar clearance compared with rHuEPO, suggesting that ESAs may be cleared to some degree through this pathway. The impact of complete elimination of receptor-binding activity on clearance of NM385 was substantially less than that observed with hyperglycosylated or PEGylated ESAs, suggesting that derivatives with PEG or additional carbohydrate have reduced clearance due to their impact on other pathway(s). In further support of this proposal, PEGylated rHuEPO and PEGylated NM385 both had similar clearance properties, suggesting that EPOR-mediated clearance is minimally impacted by hyperglycosylation or PEGylation. Taken together, these observations indicate that while EPOR-mediated pathways may play some role in clearance of ESAs, nonEPOR-mediated pathways account for the majority of elimination from the body.

Hyperglycosylated and PEGylated ESAs have other biophysical characteristics such as increased hydrodynamic size. Thus reduced clearance of hyperglycosylated or PEGylated derivatives might be explained by stearic factors. One pathway that may be impacted by size is degradation or metabolism in the interstitium, such as by cells involved in the reticuloendothelial scavenging pathway or lymphatic system [139]. ESAs with larger hydrodynamic size may have reduced transport from the blood to the interstitial fluid where degradation takes place. Consistent with this hypothesis, the lymphatic system is believed to play an important role in the reduced bioavailability after subcutaneous administration of proteins [140]. Only small peptides or free ^{125}I and not intact material are detected in tissues after intravenous administration of ^{125}I-darbepoetin alfa, suggesting that degradation may occur in tissue [138]. Additional work is required to test this hypothesis.

New formulations and devices

Endogenous EPO level is exquisitely controlled in the body by rapid changes in expression. In contrast, protein therapeutics may be stored in a nonphysiologic environment for extended periods of time, which in some instances may be years. Safe storage in any formulation requires that conditions and formulations be designed to minimize formation and accumulation of unnatural breakdown products or alterations in EPO structure. Inappropriate formulations that do not maintain the integrity of the product can risk exposing the patient to an abnormal form of the protein.

Formulations containing rHuEPO have been successfully developed and used safely and effectively for more than a decade. Despite the success of current formulations, change is sometimes required to keep up with regulatory or safety concerns or to allow for new technologies, such as new devices or delivery systems. Such manufacturing and formulation changes included removal of excipients, such as human serum albumin or bovine-derived products.

Prolonged stimulation of erythropoiesis is one desirable property that may be addressed by new delivery systems, including devices that allow controlled release of rHuEPO over long time periods. This approach necessitates that the molecule remain stable in the device for prolonged periods of time. Another approach is to introduce rHuEPO into a biodegradable matrix that degrades slowly over time (slow release) [141, 142]. This strategy requires development of methods to immobilize rHuEPO in a matrix, such as microparticles, that breakdown at predictable rates and release the product in a controlled manner.

The use of an appropriate slow-release process has not been successful for several reasons. The main reason is a requirement that the protein remain intact and unchanged during both the processing of the material and during the prolonged exposure in the body. Protein integrity is a particularly difficult requirement in biodegradable matrices because the protein is in a concentrated hydrated state at physiologic temperatures for extended periods of time. Small amounts of contaminating rHuEPO aggregates, misfolded rHuEPO, or breakdown products may compromise not only efficacy but also safety (e.g., may introduce immunogenicity). Another concern is that rapid breakdown of the matrix may result in excessive delivery resulting in overdose.

Conclusions

More than two decades have passed from the heady days when the *EPO* gene was cloned and rHuEPO was first administered to a patient. Recombinant HuEPO has proven to be a breakthrough as EPO replacement therapy, setting the bar high for any improvements that may follow. Nonetheless, there remains a desire for better erythropoietic molecules, new formulations, or more useful delivery systems. The discovery, development and regulatory approval of darbepoetin alfa (Aranesp) shows that it is possible to discover and develop newer molecules that can be clinically approved to provide different dosing options and improve patient convenience, e.g., by providing flexible options for dosing frequency. Further progress is anticipated as new devices that can simplify the administration of these drugs are developed. Additional changes in rHuEPO or in rHuEPO formulations are anticipated that may need to be developed to allow more effective use of these delivery devices. The future will be exciting as small-molecule orally active EPO mimetics are discovered; though matching the safety profile of rHuEPO presents a substantial hurdle to any small molecule program. Permanent correction of anemia may occur through gene therapy, thereby allowing additional treat-

ment opportunities. These developments may require extensive research and testing; however, many believe that these developments are not a question of if, but when.

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Erythropoietin receptor

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Structural basis for the signal transduction of erythropoietin

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Introduction

The structures of recombinant erythropoietin (r-HuEPO), the extracellular ligand-binding portion of erythropoietin receptor (EPOR) and several novel peptides have been extensively studied using a variety of methods including X-ray crystallography and nuclear magnetic resonance (NMR) [1, 2]. The receptor for EPO has been classified as a member of the class 1 cytokine receptor super family [3, 4]. EPOR is a transmembrane receptor that is activated when EPO binds to the extracellular portion of the receptor causing dimerization [5]. The dimerization of the extracellular portion of the receptor induces tyrosine kinase proteins associated with the cytoplasmic side of the receptor to initiate the signal transduction events that eventually cause the committed erythroid–colony-forming units (CFU–E) progenitor cells to eventually mature into red blood cells [6] (Fig. 1).

Studies on molecules that cause 2 EPOR to form a dimer have demonstrated that EPOR can be bound and activated by structurally unrelated molecules. This oligomerization of EPOR can be caused by an engineered extracellular disulfide bond in EPOR that can stimulate constitutive signal transduction [7]. Antibodies with epitopes that react with the extracellular domain of EPOR also stimulate the EPOR [8] as well as several different peptides that demonstrate both agonist as well as antagonist activity [9–12]. In addition to r-HuEPO, a novel hyperglycosylated analog of r-HuEPO (Aranesp, darbepoeitin alfa) with improved pharmacologic properties, has demonstrated activation of EPOR. The common modality for all the molecules that cause signal transduction is that they all result in the formation of an r-HuEPO receptor homodimer. It was initially speculated that EPO functioned mechanistically as a cross-linker that brings the intracellular portion of EPOR into close proximity producing the signal transduction events that eventually allow the cell to mature into a red blood cell. However, recent data suggest that EPOR receptor may initially form a dimer on the cell surface [13], and that EPO provides the proper orientation of EPOR outside the cell, thereby allowing the intracellular kinases to be in the precise geometry for biologic activity.

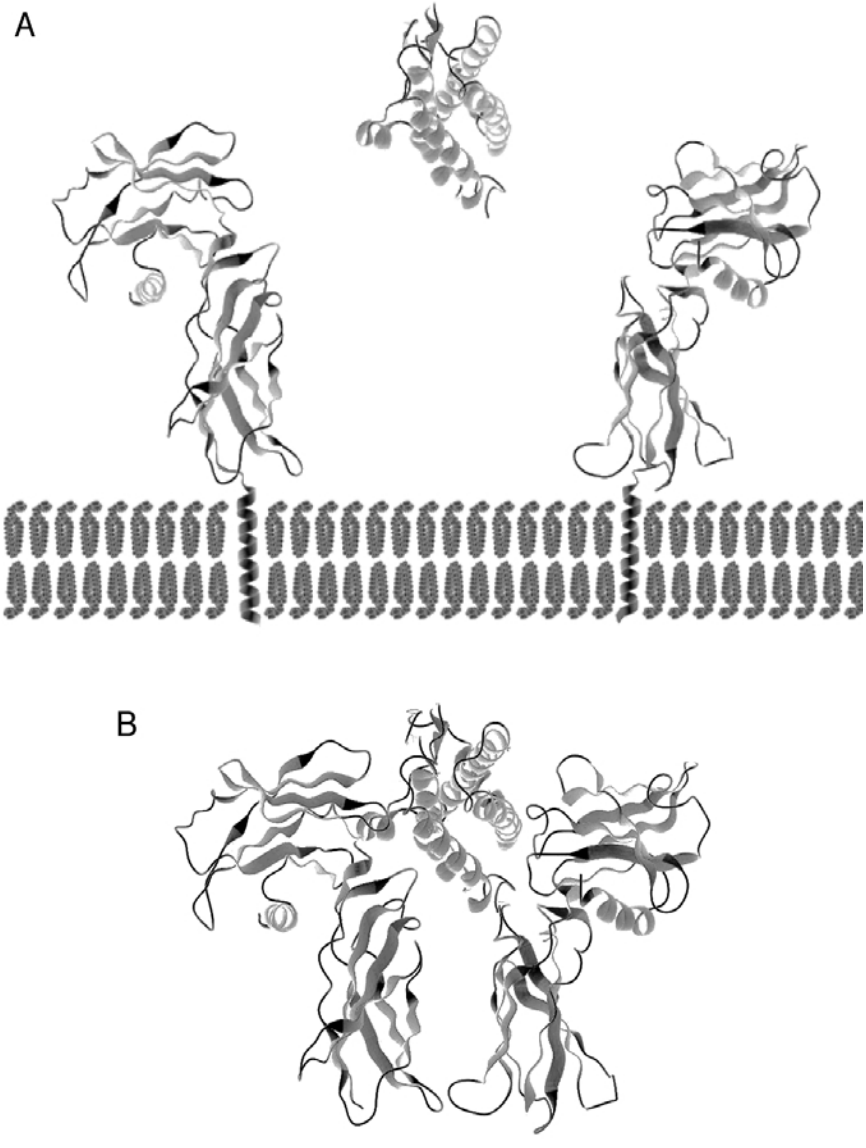

Figure 1. (a) Model depicting the structures of the two EPO receptors imbedded into the cell membrane. EPO, which is produced in the kidney and released into the bloodstream, functions by binding to two receptors, causing dimerization and receptor activation. The complex of EPO and two EPO receptors allows the precise geometry required by the intracellular portion of the EPOR (not shown in this diagram) for biological activity. The relative proximity of the two intracellular regions caused by the mechanical association of EPO with the EPO receptors allow the association and phosporylation of kinases which is the initiating event which eventually allows the cell to mature into a red blood cell. (b) The X-ray crystallographic structure of EPO: EPOR. The structure resolved by Syed et al. (1EER.pdb) shows the interaction between the 'sides' of the EPO alfa helical bundle and two EPO receptors.

With the introduction of pegylated epoetin in Europe, which is epoetin beta pegylated with a 30 kDa methoxy-polyethyleneglycol polymer connected to the amino terminus of rHuEPO and the σ-amino groups of Lys45 or Lys52 [14, 15], a third derivatized protein is being introduced into the clinic. The concept of pegylating a protein to improve its pharmacokinetic/pharmacodynamic properties of therapeutic proteins is well documented in the development of pegfilgrastrim (Neulasta) [16], which is a pegylated form of recombinant human granulocyte colony-stimulating factor (rHuG-CSF) and Pegasys (pegylated interferon alfa-2a) [17]. The binding of the pegylated epoetin (CERA, continuous erythropoietin receptor activator) is greatly reduced to approximately 50- to 100-fold lower affinity for EPOR binding sites. However, with respect to Lys45, it is anticipated that pegylation at this site would greatly reduce receptor binding affinity, as this residue has been mapped out to be within the high-affinity-binding site for binding to EPOR [2].

EPO shares with other cytokines the general property of binding to its receptor with high affinity (100–200 pM), relatively short half life (10 h), and low blood circulation levels (10 pg/mL). In addition, it takes only a small fraction of the possible EPOR (10%) to be occupied for the EPO signal transduction pathway to be stimulated. What becomes strikingly evident by the continued introduction of additional polymers, either synthetic as is the case of the pegylated epoetin beta or through glycosylation with respect to darbepoetin alfa, is that in the clinical setting the absolute binding affinity of the these molecules to EPOR is less important than the additional half life these molecules have with respect to the naturally occurring EPO.

Erythropoietin structure

The fundamental issue in resolving the X-ray crystallographic structure of the r-HuEPO ligand and receptor was obtaining a construct of r-HuEPO that was suitable for crystallization.

Proteins which are highly glycosylated such as r-HuEPO are often not good candidates for crystallization. When proteins are expressed in bacteria, the protein is not glycosylated and often forms aggregates presumably because the residues which were covered by the carbohydrate structures are now exposed [18]. Multiple bacterially expressed constructs were put through the crystal screening process until a suitable crystallization candidate could be determined. Among the variety of constructs that were produced included N24K, N38K, N83K, P121N, and P122S. The Pro mutations were suggested based on the possible cis-trans conformation heterogeneity that had been reported in other unrelated protein structures [19].

The first significant structural data came when the structure of the receptor and an EPO peptide mimetic was first revealed [10]. Within only a few years after this breakthrough structure, the structures of the r-HuEPO/EPOR2 complex [1] and the NMR structure of the unbound ligand [2] were published. The

NMR and crystal structures were very similar with the exception of the region around the Pro mutations at residue 121 and 122. An artifact of these mutations caused a small alpha helix to be formed in the crystal structure which is not found in the NMR structure. These data provided a further insight into the structure of the ligand and its structural relationship to the receptor.

r-HuEPO has a structural topology that is similar to other members of the cytokine hormone family. This family of proteins shares a four helical bundle motif in which the first two helices go in the opposite direction relative to the second set of helices. This 'up-up-down-down' motif requires two long loops between the first and second helix, a small loop between the second and third helix, and another long loop between the third and forth helix [20]. The class of proteins can be subdivided into proteins with long alpha helices and short crossing angles between the helices that are found in G-CSF and human growth hormone (HGH). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is representative of a cytokine structure where the helices are significantly shorter and the crossing angles between helices are relative large. Often the cytokines with the shorter helices have a small beta strand connecting the long loops between helix A and B and C and D.

After the structure of EPO was elucidated, it was found that the EPO structure was a hybrid between the two classes of four helical bundles. The characteristic topology of r-HuEPO, and cytokines in general, is a core structure composed of a four helical bundle (Fig. 2). There is one pair of antiparallel long helices, A (residues 8 to 26) and D (residues 137 to 161), and another pair of shorter helices, B (residues 56 to 83) and C (residues 90 to 112). A long loop connects the first and second helix (AB) and a short loop connects the second and third helix (BC) and another long loop connects the third and fourth helix (CD) [21]. The core bundle has a left-handed twist and the crossing angles of the helices (–148° to 167°) [2] also provide a further classification of the cytokine family. Before the elucidation of the structure of r-HuEPO, most cytokines could be classified as either having long helices and short crossing angles (e.g., G-CSF) or short helices with long crossing angles (e.g., GM-CSF) [20]. Often the short helical cytokines have two antiparallel beta strands that structurally link the long loops AB and CD. The r-HuEPO structure illustrates a hybrid of the two classifications. r-HuEPO has longer helices and shorter crossing angles than the other shorter helical cytokines, but inconsistent with the longer helical classification, r-HuEPO has an antiparallel beta sheet: β1 (residues 39 to 41) and β2 (residues 133 to 135).

The first of two disulfide bonds structurally link together the amino terminus and the carboxy terminus at residues Cys7 to Cys161. The proximity of this disulfide bond relative to the amino terminus and carboxy terminus is presumed to hold the four helical bundle together at one end while a beta strand and second disulfide bond stabilize the quaternary structure of the protein at the opposing end. The second disulfide, Cys29 to Cys33, links the end of helix A with the AB loop. The hydrophobic core residues on helix D pack against

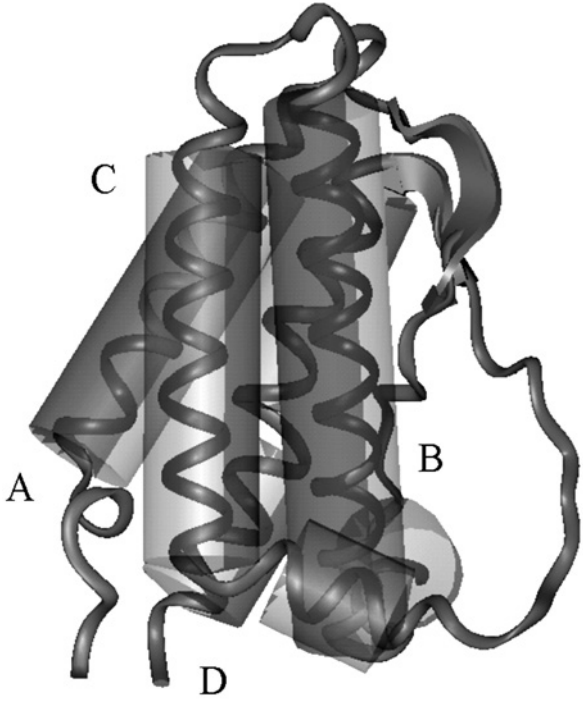

Figure 2. A secondary structure depiction of EPO. EPO has a structural topology which is similar to other members of the cytokine hormone family. The characteristic topology of EPO, and cytokines in general, is a core structure composed of a four helical bundle. There is one pair of antiparallel long helices, A (residues 8 to 26) and D (residues 137 to 161), and another pair of shorter helices, B (residues 56 to 83) and C (residues 90 to 112).

the hydrophobic residues of helices A, B and C that reflect the pattern of the approximate 3.5 residues per turn in a standard alpha helix.

In the X-ray structure of r-HuEPO bound to the EPOR, there are two additional short helices, the B' helix (residues 47 to 52) orthogonal to B and the minihelix C' (residues 114 to 121) following C with a 90° tilt beginning at Gly113. The second minihelix is not found in the NMR structure and may be an artifact of the mutations made around this area to develop a protein suitable for crystallization.

Structure of EPOR

The extracellular domain of EPORr has two β sandwich domains, designated D1 (*N*-terminal) and D2 (*C*-terminal), each of which shares an overall topology with the fibronectin type III fold. The D1/D2 domains are almost perpendicular to one another and form an inverted 'L' with the ligand-binding site located between the monomers. As is found in other members of the cytokine

family of receptors, EPOR has a WSXWS, box that is a sequence of amino acids containing tryptophans and serines, which are essential for the biological activity of the receptor [22].

In addition to the predominantly β sheet structure, there is an α-helix found at the amino terminus which may have important structural implications (Fig. 3). Adopting the position inside the 'elbow' of the D1 and D2 domains, the α-helix makes extensive contacts with both D1 and D2 domains of the receptor, with a buried surface area of approximately 200 $Å^2$. The α-helix is amphipathic, having both hydrophobic and hydrophilic regions, and presents several residues towards a hydrophobic patch on the D1 domain of the receptor. Phe11 and Leu18 of the α-helix form the basis of hydrophobic interactions with Phe39, Leu27 and Phe29 in the D1 domain. *N*-terminal to the WSXWS box in domain D2, Phe208 also makes contacts with the hydrophobic pocket. Two electrostatic interactions occur between the α-helix and other regions of

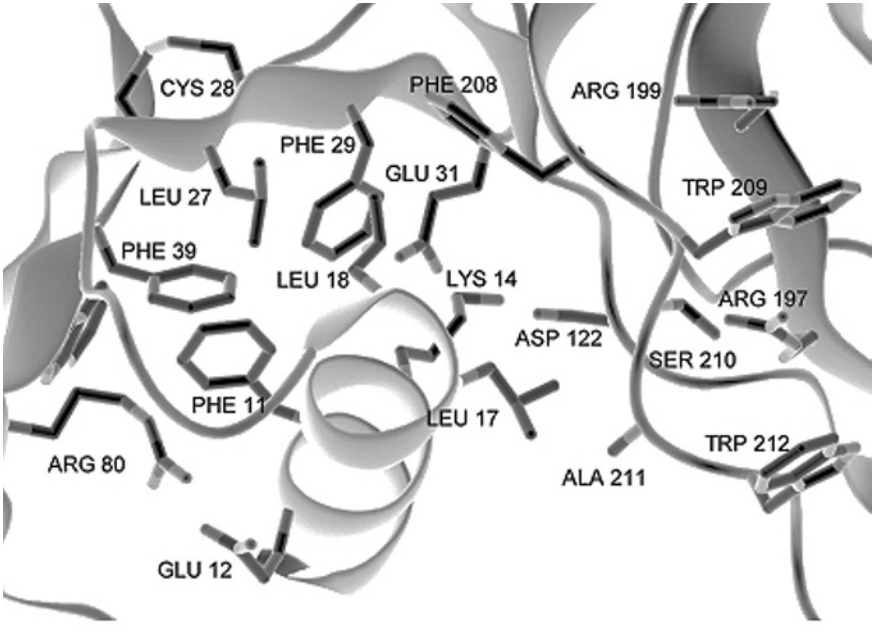

Figure 3. Amino Acid Contacts of the amino terminal alpha helix of the EPO receptor. Ribbon representation of the soluble EPOR receptor (EPOR). Selected residues at the helical interface are a depicted using a stick model. Residues 209–212 represent the putative cytokine family WSXWS box. The EPOR N-terminal α-helix is amphipathic and presents several hydrophobic residues towards a hydrophobic patch on the D1 domain of the receptor. PHE11 and LEU18 of the α-helix form the basis of hydrophobic interactions with PHE39, LEU27 and PHE29 in the D1 domain. N-terminal to the WSXWS box on domain D2, PHE208 also makes contacts with this hydrophobic pocket. Two electrostatic interactions occur between the α-helix and the rest of the protein. One salt bridge is formed between GLU12 and ARG80 and the other salt bridge links all three domains via the electrostatic interactions of LYS14 with GLU31 (on domain D1) and ASP122 (on domain D2).

EPOR. Several salt bridges function to link the α-helix with D1 and D2 including the electrostatic interactions between Lys14 with Glu31 (on domain D1) and Asp122 (on domain D2).

Alteration of the WSXWS sequence disrupts r-HuEPO binding and receptor signaling. The WSXWS motif has been shown to be critical for the folding and transport of the receptor to the cell surface, and an A211E mutation further improved the efficiency of the processes [22]. In the structure of an r-HuEPO/EPOR complex, Glu211 occupies the X position. The side-chain of Glu211 in the WSXWS motif is closest (<4.5 Å) to Leu17 of the *N*-terminal receptor helix. Additionally, the side chains of the tryptophans in the WSXWS motif, residues Trp209 and Trp212, sandwich the hydrophobic side chain of Arg197 in the receptor fold, while Ser210 and Ser213 are within hydrogen-bonding distance of Ala198 and Val196, respectively (not shown in figure). The observation that the WSXWS motif interacts with the *N*-terminal helix and the β-sheet residues Val196, Arg197, and Ala198 in the r-HuEPO/EPOR complex, suggests that the *N*-terminal helix may be important in stabilizing the folded EPOR through an interaction with WSXWS. The interaction between WSXWS and the α-helix may therefore be essential in holding the two domains, D1 and D2, in the precise geometry allowing dimerization and signal transduction by EPOR.

The r-HuEPO/EPOR complex is a 2:1 stoichiometry and the receptor molecules are held together through two distinct surface areas on r-HuEPO that are located on opposing faces of the molecule. These r-HuEPO/EPOR interfaces have been identified as high affinity (kDa approximately 1 nM) and low affinity (kDa approximately 1 μM) binding sites and are referred to as site 1 and site 2, respectively [23]. Site 1 is comprises r-HuEPO residues from helices A, B', D, and part of the AB loop, whereas site two comprises amino acids in the A and C helices exclusively. Presumably site 1 binds more tightly than site 2 because of the larger number of residues participating in the interaction with EPOR of the first site relative to the second. Site 1 is characterized by a hydrophobic-binding region in the middle of the binding site with flanking amino acids that allow electrostatic interactions with EPOR. The EPOR loops L1, L5 and L6 interact with the hydrophobic r-HuEPO residues with Phe93 of EPOR as the dominant hydrophobic contact. Phe93 is a pivotal residue firmly held in place by hydrogen bonding to r-Hu-EPO residues Thr44 and Asn147. Mutagenesis has shown that Phe93 on the receptor is a critical r-HuEPO-binding determinant, and mutation of this residue eliminates any detectable binding [24]. The site-1 binding site of r-HuEPO also has contributions from the A-B linker polypeptide residues (Thr44–Phe48) of r-HuEPO. The hydrophobic binding surface of site 1 itself is surrounded by hydrophilic interactions with Asn147 of r-HuEPO that functions as a major contributor to the electrostatic interactions. Asn147 binds to EPOR using three hydrogen bonds, one of which is between its Nδ atom and the carbonyl oxygen of Phe93. On either side of the Asn147 in r-HuEPO are arginine residues, Arg143 and Arg150, that form salt bridges with acids Glu60 and Glu117 of r-HuEPO, respectively.

r-HuEPO site 2 interactions with EPOR are less extensive than site 1, which explains its lower affinity. Most of the site-2 interactions are between residues of the C helix of r-HuEPO and the L3 loop of EPOR. The hydrophobic surface is created on EPOR by residues Phe93, Phe205, and Met150, although because of the positioning of the D1 domain relative to the site 2 interactions with r-HuEPO, there is a relatively flat EPOR surface that r-HuEPO residues can interact with. The closest nonpolar contact in site 2 includes r-HuEPO Leu108 and r-HuEPOR Phe93 Cγ3.9 Å). The rest of the C helix residues are positioned at a greater distance from EPOR hydrophobic surface than at the site 1 interface. Phe93 is again the major contributor to the central hydrophobic binding pocket (Leu5, Val11, Tyr15, Ser104, Thr107, and Leu108 of r-HuEPO) are within 4.5 Å of EPOR Phe93. Met150 of EPOR, however, is more buried than in site 1, making van der Waals contacts with Arg10, Val11, and Arg14 of r-HuEPO.

In general, the side-chain interactions at both interfaces are predominantly between positively charged lysines and arginines of r-HuEPO and negatively charged aspartate and glutamate side-chains of EPOR. Site 1 contains almost twice as many side-chain–side-chain interactions as site 2. However, the number of hydrophilic contacts involving main-chain atoms from either r-HuEPO or EPOR is equal for both sites. In addition, site 1 contains two hydrogen bonds that involve main-chain atoms from both r-HuEPO and EPOR. Hydrophilic interactions from three EPOR residues are common to both interfaces, two of which involve hydrogen bonding from a main-chain atom. Although most of residues that interact across both interfaces are polar, the expected contribution to binding affinity is greatest from the hydrophobic contacts.

The sequence identity of EPO between different mammals is reportedly about 60% [25]. Inspection of the two bindings sites with comparison to other species suggest why cross species reactivity is so high as 100% of the binding site residues on EPO are conserved within the mammalian species studied. The sequence (NFLRGK) of the high-affinity binding site is evolutionarily conserved also among vertebrates and is found in EPO sequence of the green puffer fish (*Tetraodon nigroviridis*).

Structure of an r-HuEPO peptide mimetic bound to EPOR

An elegant series of experiments resolved the structure of a soluble EPOR complexed with an agonistic peptide, which reportedly had no sequence identity to the naturally occurring ligand [10] (Fig. 4). Included in this body of work was the bacterial expression, purification, and refolding of a soluble form of EPOR (EBP) and the isolation of the self-dimerizing 20 amino acid peptide EMP1 (GGTYSCHFGPLTWVCKPQGG). The peptide has β-sheet structure and a disulfide bond constraining the peptide. The stoichiometry of the EBP/EMP1 complex is one peptide homodimer bound to two r-HuEPO receptor monomers. EMP1 was originally identified using peptide phage display technology. The 20 amino acid peptide mimicked the biologic activity of

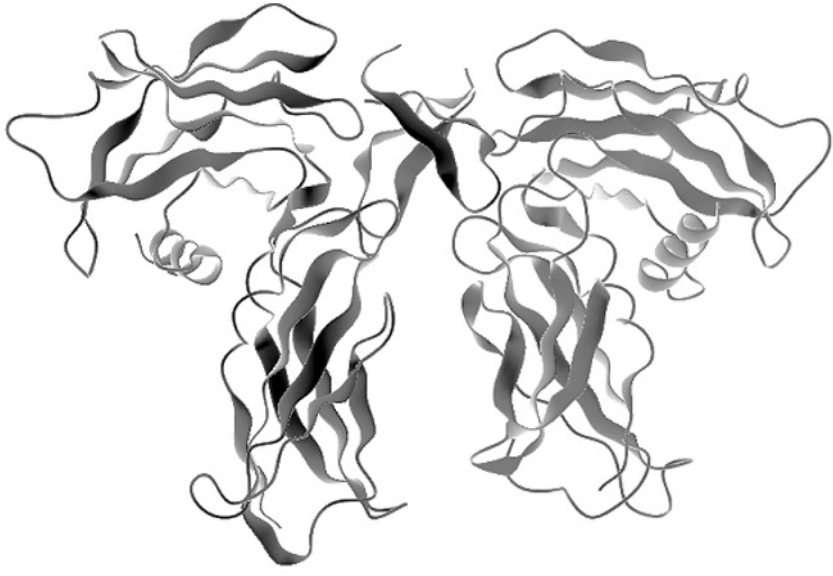

Figure 4. Ribbon depiction of the complex between the extracellular domain of Erythropoietin (r-Hu-EPO) and an agonist peptide. The X-ray crystallographic structure of the r-Hu-EPO receptor complexed with an agonist peptide was resolved by Livnah et al. The two-fold symmetry of the peptide allows the dimerization of 2 EPOR. The functional mimicry of EPO by a 20-residue peptide with a totally unrelated sequence was of considerable value in understanding the structure of the receptor and its signal transduction mechanisms.

EPO and functioned as an agonist. The binding affinity of the peptide compared with r-HuEPO binds about a thousand fold less at 200 nM, compared with 200 pM.

The interface between the peptide and EPOR is mainly hydrophobic in nature with very few invaginations and charged residues that would be characteristic of a naturally occurring binding pocket. The three residues on EPOR contributing the most binding energy to EMP1 are Phe93, Met150, and Phe205. These amino acids make contact with the Phe10 and Tyr4 of the peptide. The dimeric and symmetrical nature of the peptide is reflected in the nature of the binding interaction with EPOR. Both interaction sites on the EMP1 dimer are identical, therefore, the two receptors adopt orientations and relative affinities of the two binding sites that are different than that found in EPOR dimerized by r-HuEPO. If the two complexes (r-HuEPO/EPOR and EMP1/EPOR) are viewed along a plane that is perpendicular to the membrane, there is almost a 60° difference between the angles of the D1 domain of EPOR with respect to the r-HuEPO and peptide complexes. In addition there is a significant difference of about 45° in the angle of the hinge region between D1 and D2 with respect to the binding of the r-HuEPO and EMP1.

Conclusion

A number of different molecules can effect the dimerization and signal transduction of EPOR. Recent data suggest that the receptor may actually be self-associated on the cell surface in the absence of the natural ligand, suggesting that it may not merely be EPOR dimerization but an even more subtle conformational change that occurs only after the natural ligand, r-HuEPO binds.

The quality and scope of the structural biology research in understanding the interactions of EPOR with several different molecules has provided a detailed understanding of the role of individual amino acids within the receptor. These structures provide a rationale in the design of the next generation of erythropoiesis-stimulating molecules and may, in the future, provide the basis for a true small molecule mimetic that could effectively allow the activation of EPOR required for a signal transduction comparable in effect to the natural ligand.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Intracellular signaling by the erythropoietin receptor

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Introduction

The erythropoietin receptor (EPOR) is crucial for promoting the survival, proliferation, and differentiation of mammalian erythroid progenitors [1]. Differentiation from colony-forming units–erythroid (CFU–E) to late basophilic erythroblasts is highly EPO dependent, whereas differentiation beyond this stage is no longer dependent on EPO, but requires adhesion of the cells to a fibronectin matrix, through $\alpha 4\beta 1$ integrin [2].

EPOR and other cytokine receptors lack enzymatic activities and rely on the Janus kinase (JAK) family kinases for signal transduction that are bound through a conserved Box1 and Box2 domain in the receptor membrane-proximal portion. EPO binding triggers a conformational change in EPOR dimer, leading to trans-phosphorylation and activation of JAK. The cytoplasmic tail of EPOR contains multiple Tyr that, when phosphorylated by JAK, provide docking sites for SH2-containing cytosolic signaling molecules, including Stat5, phosphoinositide-3 kinase (PI3K), and the Shc and Grb2 proteins that activate the Ras/mitogen-activated kinase (MAPK) pathways. Importantly, phosphorylated receptors and JAK also recruit multiple signaling attenuators, including phosphatases (SHP) such as the protein tyrosine phosphatase SHP-1, several suppressor of cytokine signaling (SOCS) proteins, and members of the newly discovered Lnk adaptor family proteins.

The major function of EPOR is to prevent apoptosis of committed erythroid progenitors, to initiate a predetermined program of three to five-terminal cell divisions, and to activate a program of erythroid gene expression. EPOR activates the same signal transduction pathways that are activated by other cytokine receptors and many receptor tyrosine kinases. Thus, the response of

a cell to a signal generated by EPOR is dependent on its properties and developmental history – presumably erythroid progenitors contain a unique constellation of transcription factors, such as GATA1 and FOG, and other proteins that allow them to interact with proteins, such as Stat5, that are activated in response to receptor signaling [3–6]. Such combinations of regulated and 'cell-specific' factors could regulate the highly specific pattern of erythroid gene expression [7].

We focus on the major signal transduction pathways activated by EPOR that are important for erythropoiesis (Fig. 1, see p. 159).

EPOR-activated STAT5 signaling

Signal transducer and activator of transcription (Stat) proteins were first discovered as mediators of interferon signaling [8] and are key mediators of signaling downstream of multiple cytokine receptors. Stat proteins are latent in the cytoplasm, but once phosphorylated through receptor activation, they relocalize to the nucleus where they act as transcriptional activators. Thus, they provide a rapid, single-step mechanism for cytokine-mediated gene induction.

Of the seven known mammalian Stat proteins, Stat3 and the two highly homologous Stat5a and Stat5b isoforms (here grouped simply as 'Stat5') have pleiotropic functions in multiple tissues. Within the hematopoietic system, Stat5 mediates survival and proliferative functions in hematopoietic stem cells as well as in lymphoid and myeloid progenitors. Stat5 is the principal Stat to be activated by EPOR. Although there have been reports of Stat1 and Stat3 activation by EPOR in some cell lines [9] their physiologic significance is uncertain.

STAT5 functional domains

The Stat proteins range in size between 750 and 900 amino acids and share conserved functional motifs, revealed by sequence comparisons and mutagenic and crystallographic studies. These motifs comprise the amino-terminal, the coiled coil, the DNA binding, the conserved linker, an SH2, a Tyr activation, and the *C*-terminal transactivation domains.

The SH2 domain of Stat5 binds phosphorylated Tyr on the activated EPOR that presumably increases the effective concentration of Stat5 in the vicinity of Jak2, facilitating its Tyr phosphorylation, presumably by Jak2. Of the eight EPOR cytoplasmic domain Tyr, Y343 and Y401 are sufficient for maximal Stat5 activation by EPOR, whereas Y429 and Y431 partially activate Stat5 [10]. The sequence surrounding these Tyr suggests that the Stat5 SH2 domain recognizes a YXXL motif on EPOR, a motif conserved in frog and fish EPOR [11].

EPOR-bound Stat5 becomes phosphorylated on Tyr 694, which then mediates Stat5 homodimerization through reciprocal phosphotyrosine-SH2 domain interactions. A mutant EPOR lacking all cytoplasmic phosphotyrosines can still mediate Stat5 tyrosine phosphorylation, albeit at reduced levels [10], consistent with the finding that an EPOR lacking all cytoplasmic Tyr can still generate survival signals in a Stat5-dependent manner [12]. Similarly, a knock-in mouse expressing a truncated EPOR lacking cytoplasmic tyrosines, EPOR-HM, is viable; we found that Stat5 becomes tyrosine-phosphorylated after EPO addition to primary erythroblasts of the EPOR-HM mouse, though with slower kinetics than wild-type erythroblasts (E. Porpiglia and M. Socolovsky, unpublished observation). One explanation of these observations is that Stat5 can bind directly to EPOR-activated Jak2, without mediation of EPOR cytoplasmic Tyr [13]. Potential mechanisms include an interaction between the *C*-terminal domain of Stat5 and the Jak2 pseudokinase (JH2) domain [14] or an interaction of the Stat5 SH2 domain with tyrosines in the Jak2 activation loop [15].

The Stat DNA-binding domain recognizes a family of palindromic 'GAS' enhancers of the general sequence TTTCCNGGAAA. The optimal sequence for both Stat5a and Stat5b is TTC(C/T)N(G/a)GAA [16]. However, Stat5 optimally binds to two tandem GAS enhancers, separated by six base pairs. These tandem sites bind optimally to Stat5 tetramers, while their binding of Stat5 dimers is weak or absent. Several such putative Stat5 tetramer-binding sites have been identified in known erythroid Stat5 target genes, including *bcl-*x_L and *pim1* [16], suggesting that Stat5 tetramers may induce a repertoire of genes that differs from that of Stat5 dimers.

The *N*-terminal domain is implicated in protein-protein interactions and is responsible for the formation of Stat5 tetramers. Tetramer formation depends on a conserved *N*-terminal Trp residue as well as on a Lys residue in this domain. Stat5 mutants that lack this Trp residue have reduced transcriptional activation properties, presumably due to their inability to form tetramers and cooperatively bind DNA GAS sites [13]. The *N*-terminal domain, together with the adjacent coiled coil domain, participate in a radical spatial reorientation of the Stat1 [18] and Stat5 dimers [17], that stabilizes the dephosphorylated, inactive form of these Stats in an antiparallel homodimer. Tyr phosphorylation results in a flipping of this structure into a parallel homodimer that is stabilized by reciprocal SH2-phosphotyrosine interactions [13, 18, 19].

The transactivation domain (TAD) at the *C*-terminus was originally identified in Stat1, where the splice variant Stat1β, lacking this domain, also lacks independent transcriptional activity. Similarly, a naturally occurring, protease-mediated *C*-terminal truncation of Stat5, Stat5Nsi, has a dominant negative effect on Stat5 transcription [20]. The Stat5 TAD has two Ser phosphorylation sites that are thought to enhance transcription. One of the Ser is within a PSP motif, recognized by MAP kinases [21] but the responsible kinase is not known. The transcriptional activation function of the TAD is at least in part due to its ability to complex with the coactivator histone acetyltransferases CBP/p300 [22].

Regulation of Stat5 tyrosine phosphorylation

Despite its apparent simplicity, gene induction by Stat proteins is subject to multiple regulators that determine the duration and termination of Stat signaling. These regulators affect JAK kinases that lead to Stat phosphorylation, phosphatases that dephosphorylate Stat proteins, and others that modify cytoplasmic/nuclear Stat transport.

Tyr phosphorylation of Stat proteins results in their redistribution from the cytoplasm to the nucleus, a process that is regulated differently for different Stat proteins. In the case of Stat5 and Stat3, but probably not Stat1, the inactive Stat molecules continuously shuttle in and out of the nucleus, although it is not known if they mediate a transcriptional function in this state. After cytokine stimulation, Stat5 nuclear import rate does not change, but the Tyr-phosphorylated dimer conformation may be resistant to nuclear export [23, 24].

Stat5-mediated function in erythropoiesis

Stat5 has a specific and essential role in EPO-mediated survival signaling in committed erythroid progenitors. We described a principal antiapoptotic pathway, whereby EPOR-activated Stat5 induces the immediate-early transcription of the antiapoptotic gene *bcl-x_L* [25]. The bcl-x_L protein is essential for erythropoiesis, and the *bcl-x_L* knockout mouse dies at embryonic day 13 due to severe anemia [26]. Several transcription factors are thought to regulate EPO-independent expression of *bcl-x_L* within erythroblasts, including GATA1. However, EPO signaling synergizes with GATA1 to substantially increase the amount of bcl-x_L in erythroid precursors. The principal mediator of this effect is Stat5**.** Two Stat5 consensus binding sites are located within the first intron of the *bcl-x_L* gene and that bind Stat5 directly after stimulation by EPO [26, 27]. Consistent with Stat5-mediated bcl-x_L induction, cultured erythroid cells that have been deprived of EPO can be rescued from apoptosis by transduction with a constitutively active *Stat5* gene that maintains expression of *bcl-x_L* [26].

To evaluate the role of Stat5 in erythropoiesis *in vivo*, we examined Stat5ab-deficient mice. The erythroid phenotype of these mice was initially overlooked [28–30]. However, Stat5ab-deficient mice do have an erythropoietic rate deficit manifesting as fetal and neonatal anemia. Anemia persists in approximately half the mice that survive into adulthood. The remainder attain a near-normal hematocrit, but are deficient in their response to acute phenylhydrazine-induced erythropoietic stress [25]. The anemia of Stat5ab-deficient mice is attributed to increased apoptosis of Stat5ab-deficient erythroblasts. Stat5ab-deficient fetal liver cells also undergo higher apoptosis and have a blunted response to EPO *in vitro* [25, 32]. Stat5ab-deficient mice were originally generated by deletion of the first exon each of the *Stat5a* and *Stat5b* genes [28], and are believed to express variable low levels of a transcription-

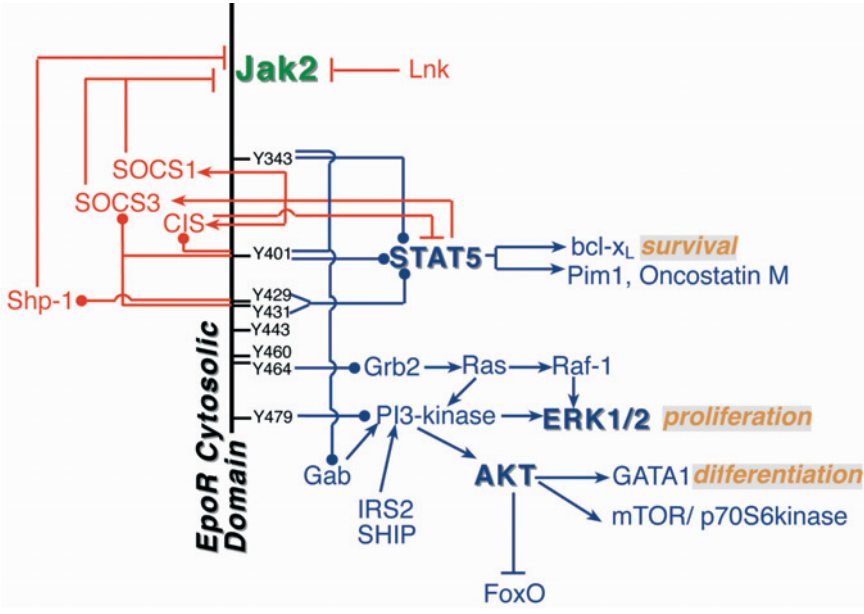

Figure 1. EPOR-activated signaling pathways. Summary of EPOR-activated pathways described in the text. The black vertical line represents the signaling domain of the EPOR. Jak2 (green) binds in the box1/box2 region (not marked) upstream of Tyr343, and its activation is the first step in the signaling cascade. Jak2 activation leads to phosphorylation of up to eight cytoplasmic tyrosines (numbered), which serve as docking sites for signaling molecules such as STAT5 and for negative regulators such as SOCS3 and CIS. Blue lines and arrows indicate positive signaling pathways that lead to survival, proliferation, and differentiation functions. Red lines and arrows indicate negative regulation of EPOR signaling. Lines terminating in a dot extending from specific tyrosines, mark docking sites for the indicated molecules. Arrows indicate activation by upstream kinases or transcriptional activation. Lines terminating in a horizontal line indicate inhibition.

ally active, *N*-terminally truncated Stat5. Stat5-null mice have been generated, where the entire *Stat5a* and *Stat5b* genes are deleted [31]. These mice have a qualitatively similar, but more severe erythroid phenotype, with fetal anemia leading to perinatal death of >99% of the mice.

To investigate the anemia in Stat5ab-deficient mice, we developed a flow-cytometric assay that identifies stage-specific erythroblasts directly in tissue [32]. Erythroblasts form the bulk of erythropoietic tissue and are classified by their morphology into a well-established developmental sequence. The Ter119 marker is specific to all erythroblasts. Ter119 positive cells were subdivided further into subsets of similar maturation by their level of expression of the transferrin receptor (CD71). Early erythroblasts are EPO dependent and express high levels of CD71, which subsequently declines with maturation (Fig. 2a).

Flow-cytometric analysis of Stat5ab-deficient hematopoietic tissue showed a dramatic increase in early ($Ter119^{high}CD71^{high}$) erythroblasts, which were

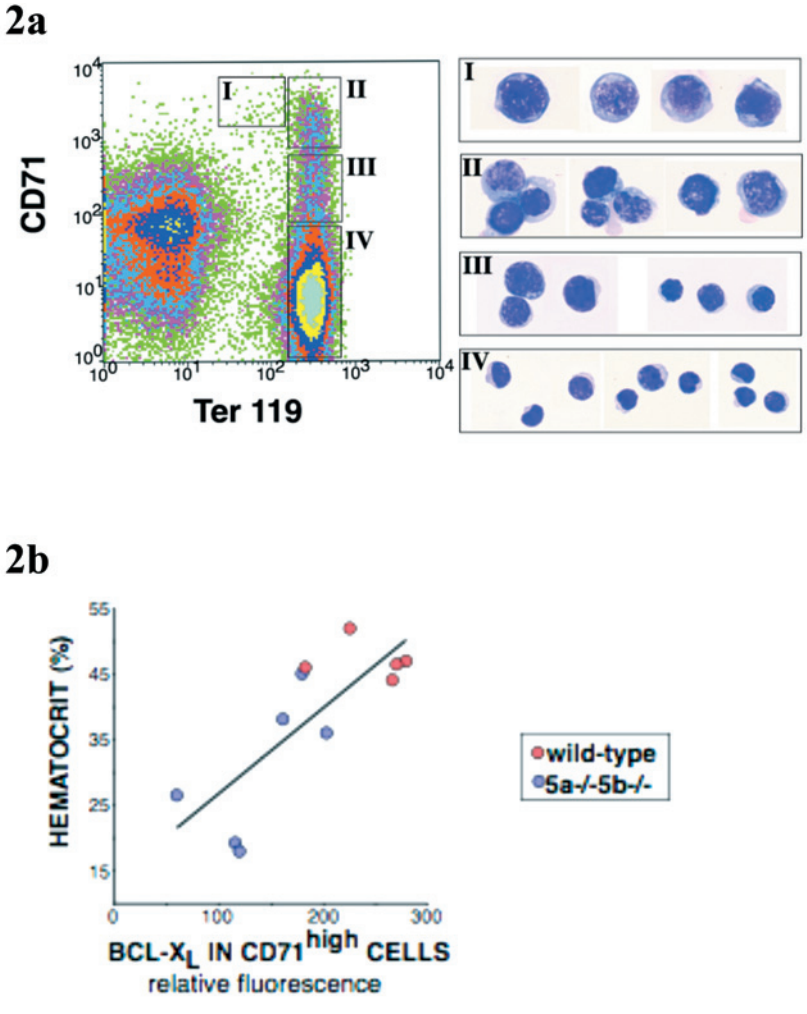

Figure 2. (a) CD71/Ter119 Flow-cytometric assay for stage-specific erythroblasts. Flow-cytometric profile of CD71 and Ter119-labeled spleen cells (right panel) and cytospins of cells sorted from regions I to IV (left panel). Originally published in [32]. (b) A correlation between bcl-x_L expression and hematocrit in Stat5$^{-/-}$ mice. Originally published in [32]. © the American Society of Hematology.

undergoing high rates of apoptosis and failing to differentiate efficiently into red cells. The amount of *bcl-x_L* in these early erythroblasts were reduced in a manner that correlated with the phenotype of the mice: the most severely anemic mice expressed the lowest amounts of *bcl-x_L*, whereas mice with near-normal hematocrit had near-normal *bcl-x_L* (Fig. 2b). These findings underlie the importance of the Stat5-bcl-x_L pathway for the maintenance of basal erythropoietic rate and normal hematocrit [32]. In its absence, increased apoptosis of early erythroblasts (ineffective erythropoiesis) results in anemia.

The role of STAT5 in the erythropoietic stress response

The erythropoietic system has a large reserve capacity, which can increase erythropoietic rate up to 10-fold in response to tissue hypoxia. EPO and EPOR signaling are principal mediators of this stress response. The Stat5ab-deficient mice mount a weak response to, and take longer to recover from, an acute phenylhydrazine-induced erythropoietic stress [32]. Similarly, a knock-in mouse in which the wild-type EPOR is replaced with a truncated EPOR allele that lacks cytoplasmic domain Tyr (the 'HM' mouse), activates Stat5 weakly, and is similarly deficient in its response to acute phenylhydrazine-induced stress. These observations suggest that Stat5 plays a role in the erythropoietic response to stress. In addition to bcl-x_L, *in vitro* studies with HM mouse erythroblasts suggest that high amounts of EPO lead to Stat5-mediated induction of oncostatin-M and Pim-1 [33], but it is not known whether these molecules play a role in the erythropoietic stress response.

The abnormal response of the Stat5ab-deficient and the HM mouse models to stress does not, however, necessarily imply that Stat5 plays a role in the physiologic response to stress of the normal mouse. We found that wild-type mice injected with a single high dose of EPO respond with a transient increase in phosphorylated Stat5 in early erythroblasts in both bone marrow and spleen. We also found a transient increase in the bcl-x_L protein in the same cells, which peaked at 18 h and subsequently declined. By contrast, examination of mouse models of chronic erythropoietic stress does not show an increase in the amounts of either phosphorylated Stat5 or bcl-x_L above those found in erythroblasts in the basal state (E. Porpiglia, Y. Liv and M. Socolovsky, unpublished observation). These findings suggest that the Stat5-bcl-x_L pathway, in addition to its essential role in basal erythropoiesis, contributes to the acute response to erythropoietic stress. The response to chronic stress, however, does not use this pathway. We found that high concentrations of EPO result in suppression of Fas-mediated apoptosis in erythroblasts, both during fetal development and during the response to stress erythropoiesis in the adult mouse. Unlike the Stat5-bcl-xL pathway, EPO-mediated Fas and FasL suppression occurs in both acute and chronic erythropoietic stress [34, 35].

EPOR-stimulated PI3-kinase/AKT signaling

In response to EPO, the PI3K signaling pathway is rapidly activated in EPO-dependent cultured cells and in mouse and human erythroid progenitors. EPOR activation of PI3-kinase (PI3K) signaling is mediated by several distinct mechanisms. Phosphorylated EPOR Y479 binds directly to the p85 subunit of PI3K; this residue (Y479) is essential for EPOR binding to the P85 subunit of PI3K [36–38]. In addition, phosphorylation of Y479 in response to EPO is sufficient for activation of the PI3-K signaling pathway, as has been demonstrated by the ability of the F7Y479 EPOR mutant, in which all cytosolic tyrosines

but Y479 are mutated to Phe, to activate PI3K and extracellular signal-regulated kinase (ERK) but not Stat5 signaling pathway in response to EPO in heterologous cells.

(Phospho) Y479 recruits PI3K to EPOR, resulting in RAS-independent activation of a MAPK cascade (ERK1/2) [36]. A role for protein kinase C (PKC) as an intermediate in this activation has also been suggested [39, 40]. It is not clear whether (phospho) Y479 also supports the activation of other MAPK pathways such as JNK or p38. Each of 2 Tyr, Y464 and Y479, alone can mediate a proliferative signal in EPOR-expressing BaF3 cells. However, only Y479 (but not Y464) is capable of inducing the differentiation of >85% of mature erythroid EPOR null CFU–E ectopically expressing mutant EPOR. (Phospho) Y479 is the only Tyr residue on EPOR that can support both proliferation and differentiation of erythroid cells to the same extent as the wild-type receptor [41], suggesting that pathways additional to ERK also may be activated by PI3K in response to EPO.

EPOR stimulates PI3K signaling pathway through several additional mechanisms. In the primitive hematopoietic UT7 cells and in human erythroid progenitor cells, EPOR is constitutively associated with Insulin Receptor Substrate (IRS)-2 [42]. After EPO stimulation, IRS-2 becomes rapidly Tyr phosphorylated and associates with the P85 subunit of PI3K and with phosphatidylinositol 3,4,5 triphosphate 5-phosphatase (SHIP) [43]. Thus, EPOR-associated IRS-2 provides an alternative mechanism for activation of PI3K in response to EPO. Furthermore, phosphorylated EPOR Tyr343 and Tyr401 recruit Gab proteins leading to their phosphorylation, association with, and activation of PI3K [42, 44]. Src tyrosine kinase may also mediate phosphorylation of EPOR resulting in its association with the P85 subunit of PI3K as has been observed in erythroleukemic K562 cells [45].

The existence of many isoforms and subunits of PI3K has precluded the assessment of the exact requirement for PI3K in the regulation of erythropoiesis. The activation of PI3K is essential for optimal erythropoiesis since the deletion of at least the p85α subunit of PI3K results in significant suppression of primitive and mature erythroid progenitor production [46].

Signaling by activated AKT (PKB) protein kinase

Three Akt (protein kinase B) forms are known in mammals (Akt-1, Akt-2, and Akt-3) and are generated from three genes. PI3K-generated phospholipids bind to the Akt pleckstrin homology (PH) domain. As a consequence, Akt translocates from the cytoplasm to the plasma membrane, where Akt is phosphorylated by regulatory kinases on two residues critical for its activation. These residues are a Thr (Thr308 in Akt1) in the activation loop within the catalytic domain and a Ser (Ser473 in Akt1) within the hydrophobic motif in the *C*-terminal region. PDK1 is the kinase phosphorylating Akt in the activation loop. The kinase phosphorylating the hydrophobic motif, named PDK2,

has been identified as mammalian target of rapamycin complex 2 (mTORC2) [47]. Mammalian TOR (mTOR) is a large protein kinase that exists in two forms, associated with either rictor or raptor [48, 49]. In contrast to the raptor containing TOR (mTORC1), the rictor containing TOR (mTORC2) is not sensitive to rapamycin. The rictor-mTOR complex (mTORC2) directly phosphorylates Akt on Ser 473 and facilitates phosphorylation of Thr 308 by PDK1 [47].

Akt Ser Thr kinase promotes survival by several mechanisms, including phosphorylation and inhibition of several proapoptotic proteins such as caspase 9, Bad, and FoxO transcription factors or enhancement of ubiquitination of p53 through phosphorylation of Mdm2. Akt has many additional functions in the regulation of metabolism, gene expression, and cell fate specification. Akt also modulates cell growth and the protein synthesis machinery through regulation of mTOR/P70S6kinase signaling pathway [50]. The function of mTOR signaling in erythropoiesis is not known.

EPO stimulation of erythroid cells results in rapid phosphorylation and activation of Akt by PI3K in both primary human erythroid progenitors [51] and cultured erythroid cells [52, 53]. Several lines of evidence suggest that in erythroid cells, the PI3K/Akt signaling pathway mediates not only survival but also growth and differentiation. For example, inhibition of PI3K activation prevents differentiation of primary human erythroid progenitor cells in culture [51, 52]. In addition, Akt activity is critical for survival and maturation of primary mouse erythroid precursor cells [54, 55]. In response to EPO stimulation of cultured and primary erythroid cells, Akt phosphorylates the GATA-1 transcription factor at Ser310 and transactivates it [54, 56]. Mutation of Ser310 to Ala suppresses the transcriptional activity of GATA-1 by >30% in erythroid cells [54, 56]. Mice with the GATA-1 S310A mutation do not exhibit an erythroid phenotype, suggesting that molecular consequences associated with loss of phosphoS310 in GATA-1 are compensated *in vivo* [57]. Akt enhancement of GATA-1 activity is not limited to phosphorylating GATA-1S310 and is mediated by both phosphorylation-dependent and independent mechanisms [54]. These findings suggest that Akt regulation of erythropoiesis may be mediated by nonphosphorylation-dependent regulation of GATA-1 and regulation of erythropoiesis independently of GATA-1. One such mechanism may be through regulation of FoxO transcription factors.

Despite these results, mice lacking Akt1, Akt2, Akt3, or all Akt isoforms have not been reported to exhibit any erythroid phenotype [58–60] possibly due to functional redundancy of different Akt proteins. The erythroid phenotype in these mice has not been closely examined. Loss of Pim1/Pim2 leads to a mild hematopoietic phenotype. Mice lacking Akt1 in which genes for Pim1/Pim2 kinases are deleted have a severe erythroid phenotype, suggesting a role for Akt in the *in vivo* regulation of erythropoiesis [61].

Akt regulation of erythropoiesis may be mediated by its ability to phosphorylate and inhibit FoxO transcription factors [62]. FoxO transcription factors are mammalian homologs of DAF-16 in *C. elegans*. FoxO1, FoxO3, and

FoxO4 are ubiquitous, whereas FoxO6 is primarily expressed in the brain. EPO stimulation of cultured and primary erythroid cells leads to rapid PI3K/Akt-mediated phosphorylation of FoxO3 (the mouse homolog of human FOXO3a, FKHRL-1), a member of the FoxO family of Forkhead transcription factors, on three conserved residues (Thr32, Ser253, and Ser315 in FoxO3) [52, 63]. FoxO3 is the most abundant and active FoxO expressed in primary mouse erythroid precursor cells. While FoxO1 is expressed in fetal liver and bone marrow hematopoietic cells, including erythroid progenitors, FoxO1 expression is greatly downregulated during primary erythroid cell differentiation [64]. FoxO4 expression is barely detectable in primary erythroid cells. Phosphorylation of FoxO3 in erythroid cells leads to its cytosolic localization and suppression of its transcriptional activity [65]. FoxO3 regulates the expression of B-cell translocation gene 1 (BTG-1) in *p53* null erythroblasts, which may be important for erythroid cell maturation [66]. Mature CFU–E express the highest concentrations of EPOR; the expression of EPOR is downregulated on postprogenitor erythroid cells suggesting that EPOR signaling is gradually lost during erythroid maturation. Both FoxO3 expression and activity increase as primary erythroid precursors mature, suggesting that loss of EPOR signaling (and therefore Akt signaling) on erythroid precursor cells may be concomitant with enhanced FoxO3 activity [64]. The function of FoxO depends on the cell context. In erythroid cells, FoxO3 is essential for the regulation of lifespan, cell cycling, and maturation, and this regulation is mediated by controlling oxidative stress in these cells [64].

While EPO has been associated with regulation of cell mitosis, the exact downstream effectors are not known. Several cell cycle regulators and transcription factors are modulated by EPOR signaling in mouse and human erythroid progenitor cells including $p27^{KIP}$, Bcl6, $P21^{CIP}$, Cyclin D2, ataxia telangiectasia mutant (ATM), and Cyclin G2. Some of these may be regulated by the PI3K/Akt pathway in primary erythroid cells [64, 67–69]. In particular, $P27^{KIP}$ and Cyclin G2 may be direct transcriptional targets of FoxO3 in primary erythroid cells and presumably are regulated by the PI3K signaling pathway [64]. The function of ATM in erythropoiesis is not known. The activation of the PI3K/Akt pathway has been shown to override the DNA damage-induced cell cycle arrest of hematopoietic cells [70]. Whether this function is mediated by FoxO (and/or FoxO regulation of ATM) or is a direct consequence of Akt function is not known. Clearly further investigation is required to establish the exact role of EPO in the regulation of erythroid cell mitosis.

These studies establish that, by modulating the transcriptional activity of GATA-1 and FoxO transcription factors, Akt signaling participates in the regulation of erythroid cell differentiation and maturation. While it is clear that the PI3K/Akt signaling pathway plays important functions in the regulation of erythroid cell formation, further studies should identify downstream effectors and assess the exact mechanism of Akt function in response to EPO.

EPOR-stimulation of RAS/MAPK pathways

In mammalian cells, Ras activates groups of MAPK, including ERK, JNK, or SAPK (c-Jun *N*-terminal kinase) and p38 (reviewed in [71]). In various EPO-dependent cell lines, different groups of MAPK are activated in response to EPO [72–76]. Activation of the Ras/ERK pathway may occur through the canonical Grb2 binding site at (P)Y464 of EPOR [77] and cell proliferation promoted by this pathway in response to EPO requires Raf-1 [78]. Synergistic activation of ERK1/2 by EPO and stem cell factor (SCF) may be important for proliferation of human erythroid progenitors [79]. Alternatively, the ERK pathway may be activated independent of Ras through the PI3K pathway [36]. JNK, and p38 are implicated in inducing erythroid differentiation of EPO-dependent SKT6 cells [76] and the role of JNK and p38 in inducing apoptosis of erythroid cells remains controversial [76, 80, 81].

To date all the three *ras* genes (*H-, N-,* and *K-ras*) have been knocked-out in mice. Among them, only *K-ras* is essential for normal mouse development since *K-ras*$^{-/-}$ and *N-ras*$^{-/-}$*; K-ras*$^{+/-}$ mice die at mid-gestation stages with anemia [82]. The study of endogenous *K-ras* signaling in erythropoiesis has been greatly advanced by using a system based on highly purified erythroid progenitors from mouse fetal livers [83]. In this system, large amounts of erythroid progenitors and early erythroblasts can be purified through a single-step procedure from mouse fetal livers with approximately 75–85% purity. These cells express high amounts of receptors for EPO and stem cell factor (SCF); thus biochemical studies can be conducted using a pure primary cell population upon EPO or SCF stimulation. Purified erythroid progenitors can be cultured *in vitro* to go through their normal terminal proliferation and differentiation. During the two-day culture period, the cell number increases 15- to 20-fold, and erythroid differentiation can be monitored step-by-step and quantitatively using a flow cytometric analysis based on the expression of erythroid-specific TER119 and non-erythroid specific CD71 (transferrin receptor) surface proteins (Fig. 3).

In this system, overexpression of oncogenic Ras, mediated by retroviral vectors, leads to constitutive activation of its three major downstream signaling pathways, including the Raf/ERK, PI3K/Akt, and RalGEF/Ral pathways [84]. Consequently, it results in a severe block of terminal erythroid differentiation and cytokine-independent growth of erythroid progenitors [83].

In contrast to results obtained when oncogenic Ras was overexpressed from retroviral vectors, endogenous levels of oncogenic *K-ras* fail to constitutively activate the Stat5, Akt, and p44/42 MAPK pathways in primary erythroid progenitors. Rather, this hyperactivates these EPO-dependent signaling pathways [85], which explains previous observations that hematopoietic progenitors expressing oncogenic *K-ras* from its endogenous locus display hypersensitivity to cytokine stimulation in various colony assays [86, 87]. Consequently, expression of endogenous oncogenic *K-ras* leads only to a mild block of terminal erythroid differentiation and a mild hyperproliferation of primary ery-

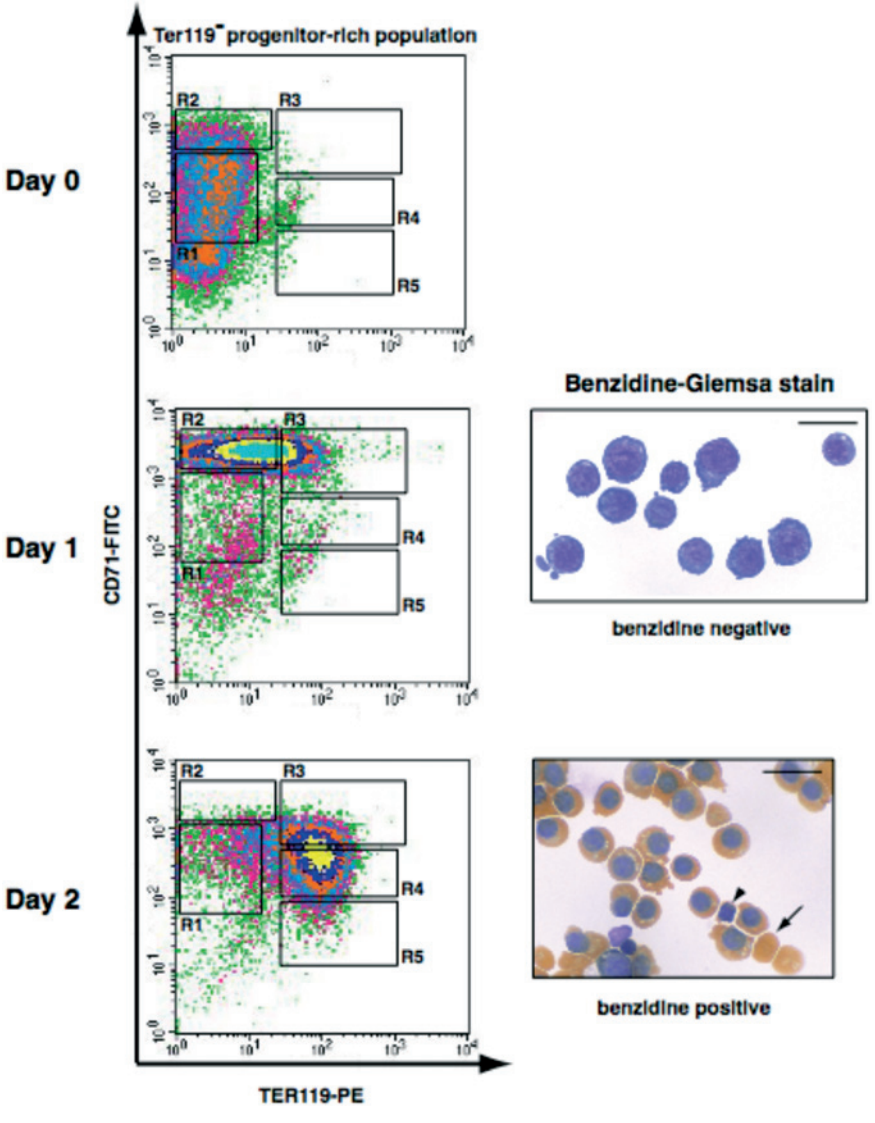

Figure 3. Flow cytometry analysis of fetal liver erythroblasts cultured *in vitro*. TER119-negative progenitor-rich cells were purified on day 0. Purified TER119-negative cells (approximately 70–80% R1 and R2 cells; essentially no R3–R5 cells) were cultured *in vitro* for 16–18 h on fibronectin-coated plates in medium containing serum and EPO. EPO was removed from culture later on. The differentiation profiles of cultured cells were examined by flow cytometry (left panels) and Benzidine-Giemsa stain (right panels). The arrowhead indicates an extruded nucleus and the arrow indicates an enucleated reticulocyte. Scale bar: 20 μm. Originally published in [83]. © the American Society of Hematology.

throid progenitors in the *in vitro* culture system [86]. Importantly, although endogenously expressed oncogenic *K-ras* does result in mild hyperprolifera-

tion of primary erythroid progenitors, it does not support cytokine-independent growth of these cells, in contrast to overexpression of oncogenic Ras [83].

Complimentary to the study of oncogenic *K-ras*, loss-of-*K-ras* function greatly reduces cytokine-dependent Akt activation [88]. Although EPO stimulation activates all the Ras isoforms, it primarily activates *N-* and *K-ras*. Moreover, it is *K-ras* but not *N-ras* that mainly regulates cytokine-dependent Akt activation [88]. The underlying mechanisms remain elusive, but may be due to different expression levels of Ras isoforms as well as their diversified *C*-termini, which greatly modulate their association with downstream effectors [89, 90]. Other studies showed that extensive reduction of Akt activation in *K-ras*-deficient erythroid progenitors leads to delayed erythroid differentiation, which only occurs during cytokine-regulated differentiation stages and is rather mild [88].

Taken together, both of gain and loss of *K-ras* signaling regulate erythroid differentiation through modulation of cytokine-dependent signaling (Fig. 4). Since the erythroid progenitors used in these studies are late progenitors mainly regulated by two cytokines (EPO and SCF), endogenous *K-ras* signaling only has mild effects on their differentiation.

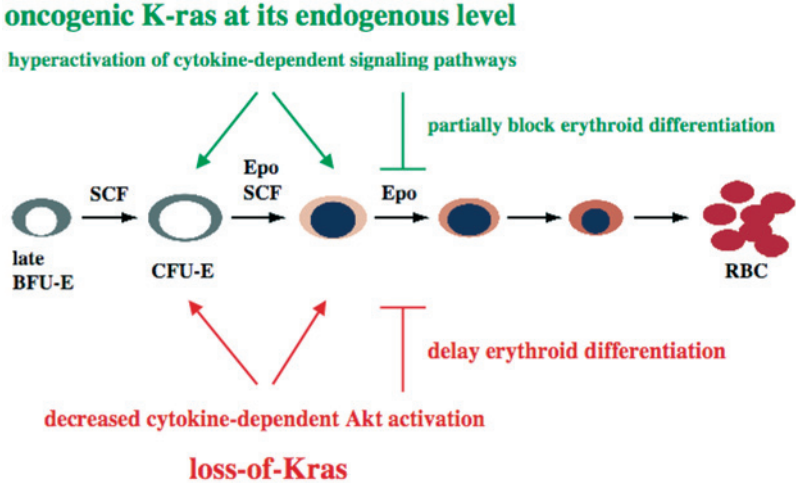

Figure 4. Endogenous *K-ras* signaling regulates erythroid differentiation through moderating cytokine-dependent signaling pathways. Reproduced from [110].

Negative regulators of EPO signaling

Erythroid progenitor cells possess considerable heterogeneity in their sensitivity to EPO, which is believed to provide flexibility to accelerate or decelerate red cell production in response to stress, such as anemia or polycythemia. GATA-1 promoter driven EPOR transgene expression rescues EPOR nullizy-

gous mice from fetal anemia [91]. EPOR transgene expression levels correlate with their sensitivities to EPO in adult bone marrow CFU–E progenitors. Therefore, the magnitude of EPOR signaling pathways within erythroblasts results in different cellular responses to EPO. This intrinsic difference of erythroblasts in their sensitivity to EPO might be tightly regulated through several negative signaling molecules of EPO/EPOR signaling that control the threshold of EPO-dependent survival. Phosphorylated receptors and JAK recruit multiple signaling attenuators that provide checks and balances at multiple steps of cytokine-receptor signaling transduction to ensure tightly controlled cellular response to fit physiologic needs and prevent oncogenic transformation. In particular, the length of the phospho-Stat5 signal is determined by a number of negative regulators, and usually does not persist beyond a few hours.

One class of attenuators is the SH2 domain-containing protein tyrosine phosphatase Shp-1. Shp-1 is relatively limited in its distribution to hematopoietic cells, where it acts as a negative regulator [92]. In the basal state Shp-1 is inactive in the cytoplasm, due to inhibition of its catalytic loop by its *N*-terminal SH2 domain. Binding to phosphotyrosine 429 of EPOR cytosolic domain, through its SH2 domains, activates phosphatase activity. Shp-1 may negatively regulate EPOR signaling by binding the activated EPOR and dephosphorylating Jak2 [93, 94]. Shp-1 may also directly inhibit Stat5 by translocating to the nucleus where it dephosphorylates Stat5b [95]. Another cytoplasmic phosphatase, PTP1B, is a specific Stat5 phosphatase, and other nuclear phosphatases, including TC45, may also act on Stat5 [96, 97].

Another class of negative regulators is SOCS proteins. SOCS proteins are transcriptionally induced by JAK/Stat pathways upon cytokine stimulation, and act within a classical negative feedback loop both to inhibit activation of signaling pathways and to target signaling components for proteasomal degradation [98, 99]. SOCS1 and SOCS3 bind to the catalytic groove of JAK2 to inhibit its catalytic activity. Both SOCS proteins can target JAK2 for ubiquitination and degradation through their SOCS box ubiquitin E3 ligase interaction motif [100, 101]. Additionally, SOCS3 binds through its SH2 domain to Tyr residues of the EPOR: Y401, where STAT5 and SHP-2 also bind, and Y429/Y431, where SHP-1 also binds [102, 103]. This presumably reduces binding and activation of other SH2-containing signal transduction proteins. Importantly, SOCS1-deficient erythroid progenitors exhibit an enhanced sen-

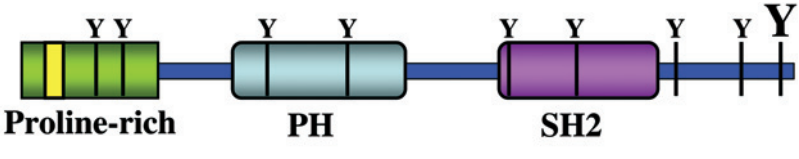

Figure 5. The protein structure of Lnk and its family members, APS and SH2-B. 'Y' indicates tyrosine residues, with the conserved tyrosine near the *C*-terminus shown in a large font. Positions of Y are drawn according to Lnk.

sitivity to EPO in forming erythroid colonies [104], consistent with its role as a principal negative regulator of the EPOR signaling pathway.

Recent work uncovered a new class of signal attenuators for rapidly down-regulating cytokine receptor signaling, the Lnk adaptor proteins [105, 106]. The Lnk family of proteins, which includes SH2-B and APS, are structurally distinct from the above-noted negative regulators in that they do not possess any known enzymatic activity, but contain other protein–protein interacting domains: a PH and a Src homology 2 domain (SH2) and a conserved Tyr near the *C*-terminus [107] (Fig. 5). Lnk-deficient mice have increased numbers of erythroid progenitors in the bone marrow and spleen. Bone marrow CFU–E progenitors are hypersensitive to EPO in generating red cell colonies. Despite having normal, steady-state red blood cell counts, $Lnk^{-/-}$ mice exhibit superior recovery after erythropoietic or myeloablative stress. In addition, Lnk deficiency resulted in enhanced EPO-induced signaling pathways, such as p42/44MAPK and Akt activation, in splenic erythroid progenitors.

Conversely, Lnk overexpression inhibits EPO-induced cell growth in 32D/EPOR cells. In primary culture of fetal liver cells, Lnk overexpression inhibits EPO-dependent erythroblast differentiation and induces apoptosis, without causing cell cycle arrest. Lnk blocks the three major signaling pathways – Stat5, Akt, and MAPK – induced by EPO in primary erythroblasts. In addition, the Lnk SH2 domain is essential for its inhibitory function, whereas the conserved tyrosine near the *C*-terminus and the PH domain of Lnk are not critical. Furthermore, wild-type Lnk, but not the Lnk SH2 mutant, becomes Tyr-phosphorylated after EPO administration and inhibits EPOR phosphorylation and JAK2 activation. Hence, Lnk, through its SH2 domain, negatively modulates EPOR signaling by attenuating JAK2 activation, and negatively regulates EPO-mediated erythropoiesis [106].

Members of the protein inhibitor of activated STAT (PIAS) family have been implicated in the inhibition of STAT and other transcription factors by multiple mechanisms, including the recruitment of transcriptional repressors and sumoylation [108]. Little evidence to date shows that PIAS proteins regulate STAT5.

Unresolved issues

Whereas we know the identity of the major signal transduction pathways downstream of EPOR (Fig. 1), and have a good understanding of several transcription factors regulated by each pathway, many key issues remain unresolved. Many of the same signal transduction pathways are activated by other members of the type I cytokine receptor family, which includes the thrombopoietin receptor, granulocyte colony-stimulating factor, and prolactin receptor, as well as EPOR. They share homology in the membrane proximal region, which includes the Box1 and Box2 domains [109] and all signal by Jak2. Yet the same transcription factors, such as Stat5, regulate transcription of very dif-

ferent sets of genes in these different differentiation pathways. Thus it is important to identify the genes in erythroid progenitors whose promoter sites are occupied by erythroid-important transcriptional regulators such as STAT5 and FoxO3 (mouse homolog of human FOXO3a), at different stages of erythroid differentiation. One must determine other transcription factors, such as the erythroid-important factors GATA-1, and FOG with which these interact and trigger cell- specific gene expression. A related issue concerns the strength and duration of each of the signaling pathways activated downstream of EPOR. As examples, how do the amounts of STAT5 dimers and tetramers or phosphorylated and unphosphorylated Fox03A change with time after EPO stimulation, and as a consequence are different genes activated or repressed over time?

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Mechanism of erythropoietin receptor activation

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Introduction

Erythropoietin receptor (EPOR) is a founding member of the cytokine receptor superfamily [1]. A type I transmembrane protein that binds the ligand erythropoietin (EPO) with high affinity (kDa approximately 400 pM) on the surface of erythroid progenitors, EPOR is devoid of catalytic activity. Given that the extracellular domain of EPOR only contains two cytokine receptor homology modules (D1 and D2), EPOR is considered a simple (or short) receptor, unlike other superfamily members, like the receptors for interleukin (IL)-6 type cytokines or for granulocyte colony-stimulating factor (G-CSF), which possess other extracellular sequences, such as immunoglobulin-like domains [2].

Genetic evidence has established that EPOR is the sole receptor chain for EPO. Knock-out of *EPO* or of *EPOR* genes in mice results in a lethal anemia at day 12.5 of embryonic life [3]. The failure of definitive erythropoiesis in the fetal liver is due to apoptosis of proerythroblasts [3]. EPO and EPOR play essential nonredundant roles from the colony-forming unit–erythroid (CFU–E) stage to formation of red blood cells, but are not absolutely obligatory for generation of blast-forming unit–erythroid (BFU–E) and CFU–E cells [3]. The first protein in the signaling chain triggered by activation of EPOR by EPO is JAK2, a member of the Janus kinase family of tyrosine kinases [4]. JAK2 associates with EPOR and is tyrosine phosphorylated and activated after stimulation with EPO [4]. Knockout mouse embryos devoid of JAK2 also die at day 12.5, due to absence of definitive erythropoiesis [5, 6].

Functional challenges to EPOR from the unique features of the red blood cell compartment

As ‘form follows function’, before examining the mechanisms of activation of the receptor, it is of interest to put forward three essential ideas about EPOR function in red blood cell formation:

- The volume of blood occupied by red blood cells, defined as hematocrit, is approximately 40%. Increases in red blood cell numbers, which are regulated by EPO, cannot be of high amplitude since hematocrit increases above 15% to 20% lead to severe hemodynamic complications and are seen in disease states, such as myeloproliferative neoplasms;
- EPOR has apparently evolved sequences that make its cell surface localization inefficient, which is translated in low cell surface EPOR densities in erythroid progenitors and transfected cells; and
- Signaling by EPOR is very efficient, as occupancy of only a few receptors (5–10% receptor occupancy, detected at 10 pM EPO) suffices to induce a half maximal response, while half maximal binding is detected at much higher EPO concentrations, approximately 0.18 nM Epo [7]. Therefore, variations in EPO concentrations must therefore be translated into incremental and precisely controlled increases in red blood cell numbers.

EPO binding to EPOR

Epo binds to the receptor with a stoichiometry of 1:2 (EPO:EPOR) [7], a stoichiometry similar to that of the growth hormone binding to its receptor [8]. Binding of EPO to EPOR by two opposite sites: one site (called site 1) binds with high affinity (<1 nM) to the extracellular region of EPOR, while an opposite site (called site 2) binds with a much lower affinity (approximately 1 mM) to another extracellular receptor chain [9]. This binding is translated into an overall high affinity (kDa approximately 400 pM) of binding of EPO to intact receptors on living cells.

The numbers of cell surface EPOR molecules per cell are quite low. These range from 1,500 to 2,000 sites/cell surface in proerythroblasts and early basophilic erythroblasts, to 750 in late basophilic erythroblasts and 250 in chromatophilic and orthochromatophilic progenitors to <10 sites/cell surface in late orthochromatophilic erythroblasts and reticulocytes [10, 11]. In stably transfected hematopoietic cells, the maximal numbers are approximately 1,500 to 1,800 surface receptors/cell [12].

Structural features of EPOR extracellular domain

EPOR contains a number of features in the extracellular domain, conserved with other cytokine receptor superfamily members. Two pairs of cysteines in the extracellular domain forming intramolecular disulfide bonds, and a WSXWS motif (Fig. 1A) are absolutely required for folding of the receptor. Mutation of any of the Trp or Ser residues in the WSXWS motif abolishes traffic to the cell surface [13], but the nature of the X residue modulates folding and traffic. A substitution of the Ala residue normally present in EPOR by Glu (present in the growth hormone receptor) increases the number of cell surface

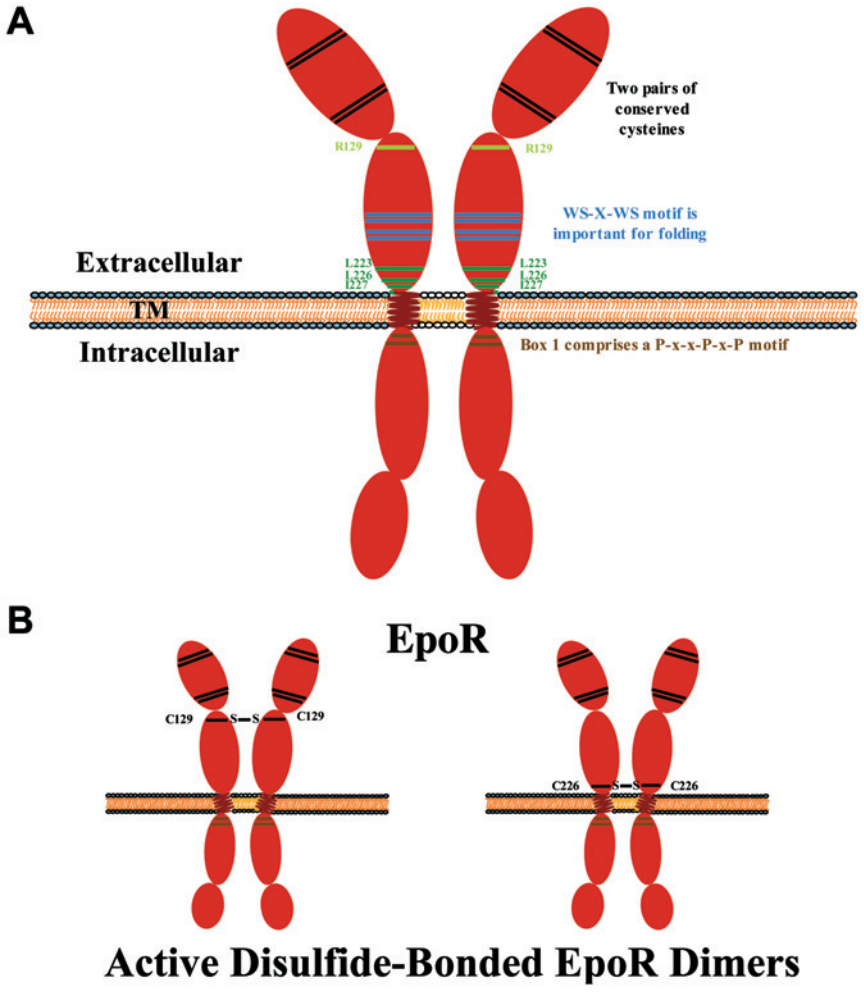

Figure 1. Structure of the erythropoietin receptor (EPOR). (A) EPOR extracellular domain contains two pairs of conserved Cys residues and a WSXWS motif, which are absolutely required for folding and forward traffic to the cell surface. The positions of extracellular residue R129 and juxtamembrane/transmembrane residues L223, L226, and I227 are shown, since mutation of any of these residues to Cys allows close apposition of monomers and formation of disulfide bonded active dimers. The intracellular domain of EPOR contains a Pro rich sequence (PxxPxP) denoted Box 1, which is required for binding the tyrosine kinase JAK2 (not shown, see Fig. 4). (B) Disulfide bond formation between EPOR monomers bearing mutations R129C or L226C results in dimerization in a productive conformation and activation in the absence of EPO. Similarly, when mutated to Cys, certain residues around R129, such as E132 and E133, as well as L223 and I227 at the extracellular-to-transmembrane domain junction yield constitutive active disulfide-bonded EPOR dimers.

receptors by approximately three-fold, most likely by enhancing folding rates in the lumen of the endoplasmic reticulum [12]. Alignment of EPOR sequences from several species suggested a selection against the presence of

residues such as Glu at the X position of the WSXWS motif. Lower amounts of cell surface EPOR might allow finer tuning of red blood cell production by EPO, while higher amounts would be predicted to promote erythrocytosis.

Structure of ligand-bound EPOR extracellular domain

Several X-ray crystal structures have been solved for the extracellular domain of EPOR in isolation. The secondary and tertiary structures agree very well between all these studies, where the extracellular domain was crystallized in complex with a peptide mimetic, with EPO or without ligand [7, 14, 15]. The extracellular domain folds in two domains (D1 and D2) that form an L-shape and that are connected by a four residues helical linker. D1 and D2 are related in structure to fibronectin type III domains, but show differences between them with respect to fold subclass [14]. What differs between the structures of different ligand-EPOR complexes is the relative geometry, i.e., angle in the plane of the membrane surface, between the D1 domains of two monomers [7, 14–17]. An EPO mimetic peptide, which is a weak agonist, induces a 180° angle between D1 domains [14]. Strikingly, a dibromylated derivative of the mimetic peptide, which is an antagonist, induces a 165° angle between D1

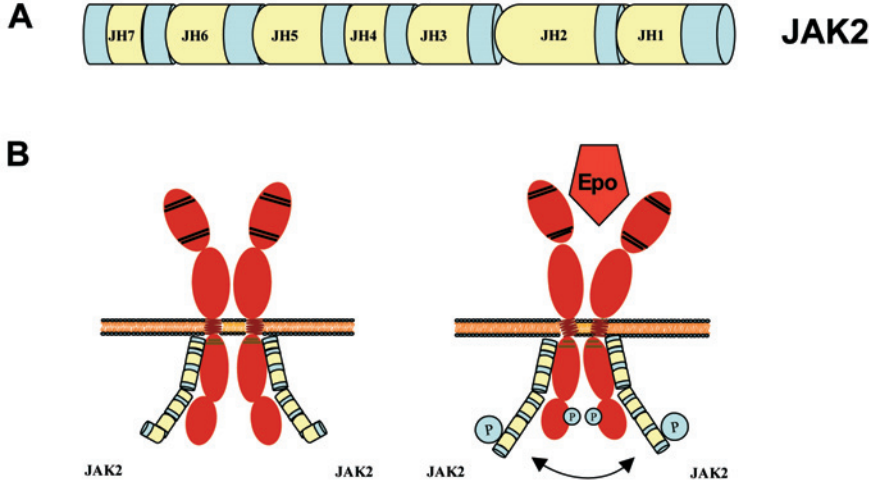

Figure 2. EPOR forms a complex with JAK2 before activation by EPO. (A) JAK2 shares seven JAK-homology (JH) regions with the other three members of the Janus kinase family. JH1, kinase domain, JH2, pseudokinase domain, JH3–JH5 contain an SH2-like sequence, while JH5–JH7 contain a FERM (Band-4.1, ezrin, radixin, moesin)-like domain that is required for attaching to the juxtamembrane cytosolic region of EPOR. (B) JAK2 is appended to the cytosolic domain of EPOR in the resting state, in the absence of EPO. In this conformation, the kinase domain (JH1) is inactive due to the inactive dimeric conformation of the receptor and to the inhibitory interactions between JH1 and JH2 domain. Upon ligand binding to EPOR extracellular domain, the dimer is re-oriented and that leads to transphosphorylation and activation of JAK2, and phosphorylation of cytosolic EPOR Tyr residues.

domains [16]. In contrast, the angle induced by the strong and physiologic agonist EPO is 120° [7]. These geometry differences are provocative, particularly when correlated with the drastically different biologic effects of EMP, of the antagonist derivative of EPO mimetic protein and of EPO [17]. However, caution must be exerted when literally interpreting these conformational differences, since the receptors are normally spanning the plasma membrane through transmembrane domains and are connected to cytosolic domains. Thus, the carboxyl ends of the extracellular domains are normally not free, as they are in these X-ray crystal structures, and the presence of the transmembrane-cytosolic domains may impose a different relative geometry to the two receptor monomers.

Structure of the unliganded EPOR extracellular domain

A surprising result was reported by the X-ray crystal structure of the unliganded EPOR [15]: the extracellular domain was crystallized as a dimer in a completely different conformation, that maintains the carboxyl ends of the extracellular domains (normally connected to the transmembrane domains) far away at a distance of >70 Å. At such a distance, it would be impossible for the JAK2 proteins appended to the intracellular domain to interact. However, whether such a dimeric conformation actually exists for the full-length EPOR is a matter of debate for several reasons: First, mutagenesis and functional studies [18–21], demonstrated that substituting several extracellular, or juxtamembrane-transmembrane residues with Cys leads to active disulfide-bonded dimers of unliganded EPOR (Fig. 1B), which indicates a much closer apposition of juxtamembrane and transmembrane residues for EPOR. Second, in buffers with physiologic salt concentration, the extracellular domain of EPOR is not a dimer, but a monomer, which can be dimerized by EPO addition [22, 23]. Third, the transmembrane domains of EPOR self-associate, and a viral envelope protein can specifically activate EPOR by specifically interacting with EPOR transmembrane domain. Nevertheless, this study [15] indicated that forcing the formation of EPOR extracellular domains dimers in the absence of EPO would lead to a conformation incompatible with signaling.

Epor cytosolic domain interacts with JAK2 in the absence of ligand

The intracellular domain of EPOR contains 239 amino acid residues and is devoid of catalytic activity. Recent evidence from several cytokine receptors indicates that JAK are appended to receptors before ligand binding [24–27] and that JAK are localized to membranes due to their recruitment by cytokine receptors [28]. Thus, the complete functional unit of a cytokine receptor includes the cognate JAK that is appended to that particular receptor (Fig. 2).

Janus kinases

The mammalian JAK family consists of four members, JAK1, JAK2, TYK2, and JAK3 [29]. JAK were initially named JAK for 'Just Another Kinase', as they were identified based on their homology to other tyrosine kinases [30, 31]. JAK2 was the third cloned member of the JAK family of protein tyrosine kinases [32]. Because JAKs proved to be crucial for signaling by all cytokines, which was first discovered for TYK2 and type I interferons [33], and because JAKs possess two kinase domains, one active and another inactive (pseudokinase domain), these enzymes were re-named Janus kinases after the Roman god custodian of the universe, the god of "beginnings and guardian of gates and doors", a god that has two heads and two faces. The metaphore holds up also at the functional level since receptor activation requires oligomerization of at least two JAK molecules, which face each other when receptor complexes are assembled.

JAKs have seven regions of homology, called JAK homology (JH) domains (JH1–7) [34]. From the carboxyl- to the amino-terminus, the JH domains are: the kinase (JH1); the pseudokinase (JH2) domains; JH3, and JH4 domains, which contain an SH2-like sequence; and then JH5–JH7, which contain a FERM (Band-4.1, ezrin, radixin and moesin)-like motif, which is crucial for appending JAK to cognate cytokine receptors (Fig. 2A). The JH1 domain contains all the necessary features of a catalytic tyrosine kinase, while the sequence of the JH2 pseudokinase domain lacks characteristic residues of active tyrosine kinases, making it catalytically inactive [31].

Sequences required for EPOR-JAK2 interaction

JAK2 is appended to the cytosolic domain of EPOR (Fig. 2B) [4]. Biochemical studies showed that certain cytosolic sequences are crucial for binding of JAK2 to the receptor. These included Box 1, a Pro-rich sequence (PxxPxP) located 11 amino acid residues downstream of the transmembrane domain, and Box 2, a charged and hydrophobic amino acid sequence located approximately 50 amino acid residues downstream of the transmembrane domain [35]. Both Box 1 and Box 2 are loosely conserved among type I cytokine receptors. A Trp residue (W282), located between Box 1 and Box 2, was shown early on to be required for physiologic JAK2 interaction and activation [35].

A higher resolution examination of the sequences required for JAK2 interaction with EPOR was possible after the observation that traffic of EPOR to the cell surface is dependent on JAK2 expression [24]. In the absence of JAK2, EPOR remains in an immature glycosylation (endoglycosidase H-sensitive) form in the endoplasmic reticulum. Binding of the FERM domain of JAK2 to EPOR is required for maturation of EPOR to the cell Golgi apparatus and cell surface localization [24]. Studies using chimeric JAK1–JAK2 proteins indicated that the FERM domain sequences and not the kinase or

pseudokinase domains are crucial to impart specificity for receptor attachment [24]. This JAK2-dependency of EPOR cell surface localization provided a sensitive and specific assay for interrogating the sequences required for productive interaction.

Ala scanning mutagenesis established that not only Box 1, Box2 and W282, but also most other residues in the region between Box 1 and Box 2 mediate interaction of EPOR with JAK2 [24]. Interestingly, residues that precede Box 1, or D287 in the region between Box 1 and Box 2, are not required for promoting cell-surface localization of EPOR, but proved to be crucial for activating JAK2 [36].

The cell surface EPOR is a preformed dimer

Several lines of evidence established that the cell surface EPOR is a preformed inactive dimer in the absence of ligand. Such dimerization would be predicted to prevent EPOR from activation in the absence of ligand. In the same time, very low EPO concentrations would readily activate preformed receptor dimers, without the need to recruit a second subunit to a receptor monomer.

Fluorescence co-patching studies

Live cell surface imunofluorescence co-patching between receptors tagged at the amino-terminus demonstrated that EPOR exists on the cell surface as preformed dimers/oligomers in the absence of ligand [37]. In this technique [38], live cells at 4º C (to block internalization) are subjected to patching by a sandwich of antibodies (anti-tag and fluorescently-labeled secondary antibody), leading to formation of patches of cell surface transmembrane proteins, that are either fluorescent green or red, depending on the fluorochrom used for the secondary antibody. Labeling for two tags (e.g., HA and Myc or HA and FLAG) with different antibody sandwiches (primary antibodies must be of different species) will lead to the formation of separate patches, i.e., individual red and green patches, provided that proteins did not interact and did not form complexes before the patching procedure. Yellow patches, resulting form the superposition of red and green patches would be detected if the differently tagged transmembrane proteins were present in mutual complexes before the patching procedure was applied. When HA- and Myc-tagged EPOR proteins were co-expressed in 293-derived BOSC cells, it was noted that most HA-EpoR and Myc-EPOR were in mutual complexes on the cell surface (Fig. 3) [37]. Remarkably, these preassembled receptor complexes do not signal in the absence of ligand, indicating that preformed dimerization is most likely preventing activation.

HA-EPOR does not co-patch with FLAG-tagged thrombopoietin receptor or with FLAG-prolactin receptor (FLAG-PrlR), demonstrating the specificity

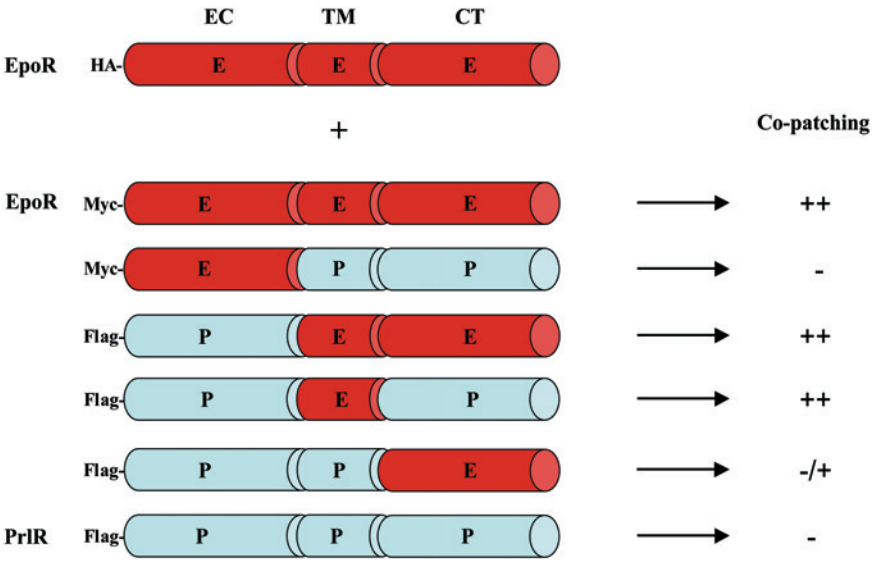

Figure 3. EPOR is a preformed dimer at the cell surface, as revealed by co-patching studies. See *Fluorescence co-patching studies* section in the text for further description.

of this assay. In contrast, chimeric EPOR-prolactin receptors, that contain the transmembrane domain of EPOR (PEP or PEE) do co-patch with EPOR, while those that only contain the extracellular or the cytosolic domain of EPOR do not (Fig. 3) [37]. Thus, the transmembrane sequences, and not the extracellular or the cytosolic sequences of EPOoR, mediate EPOR dimerization at the cell surface in the absence of ligand.

Fluorescence Resonance Energy Transfer (FRET) studies

FRET studies employed fusion proteins where yellow (YFP) and cyan (CFP) fluorescent protein sequences were fused at the amino-terminus of the EpoR. FRET measurements indicated that the majority of receptors are in close proximity (at a distance <100 Å) and oligomerized in the absence of ligand [39]. Like for the GH receptor, addition of ligand did not increase the proportion of dimerized receptors by FRET [39], suggesting that most EpoR chains already are part of dimer complexes. This result is consistent with that obtained by co-patching, where addition of Epo did not significantly enhance dimerization [37]. While EpoR and GH receptor were each shown to form dimers, they did not form heterodimers/heterooligomers, when co-expressed in the same cell, supporting the specificity of the FRET assay [39].

EPOR transmembrane domain strongly homodimerizes

Starting from the observation that heptad leucine motifs mediates self-recognition of artificial transmembrane segments, a data base search identified degenerate versions of these leucine motifs within transmembrane segments of several proteins, including EPOR [40]. In genetic bacterial TOXCAT assays, where activation of fusion proteins containing the ToxR transcription factor is induced by dimerization of test transmembrane sequences, EPOR transmembrane sequence self-oligomerizes to levels higher than those of the glycophorin A transmembrane sequence [40, 41]. These data provide a biochemical basis for the role of the transmembrane sequence in inducing oligomerization of the full-length EPOR. Furthermore, interrupting the α-helicity and dimerization of the EPOR transmembrane sequence, by introduction of a Gly-Pro sequence, impairs EPO-dependent signaling, suggesting a role for transmembrane domain dimerization in supporting ligand-dependent signaling [41].

Activation of EPOR by viral gp55 proteins through specific transmembrane domain interactions

The defective viral envelope protein gp55-P, coded by the polycythemia strain of spleen focus forming virus, binds to the murine EPOR co-expressed in the same cell and activates signaling [42]. Different degrees of EPOR activation can be induced, as a function of the precise transmembrane sequences present in gp55 proteins from different virus strains [43, 44]. For full EPOR activation, the transmembrane domain of the gp55 protein must be a dimer and must contain specific sequences that mediate interaction with the EPOR transmembrane domain. Dimerization of the domain of the strong activator gp55-P (polycythemia-inducing, SFFV strain 'P') was demonstrated in TOXCAT assays [40, 45]. In contrast, the transmembrane domain of the weak activator gp55-A (anemia-inducing, SFFV strain 'A') was shown not to dimerize in TOXCAT assays [45]. Both gp55-P and gp55-A transmembrane domains mediate complex formation with EPOR, as shown by co-patching experiments [45]. Furthermore, sequence-specific interactions between the single transmembrane domains of EPOR and gp55-P were identified to be essential for receptor activation. Computational searches and mutagenesis data indicated that the face of the murine EPOR transmembrane domain containing S238 is predicted to interact specifically with gp55-P, and more specifically with Met390 of its transmembrane domain [44]. Since in the absence of EPO, the EPOR transmembrane domains stabilize an inactive dimeric receptor conformation, our model predicts that the transmembrane sequences of gp55 proteins specifically bind and disrupt the inactive EPOR transmembrane dimer, leading to different degrees of activation. For gp55-P, which is a dimer and contains specific transmembrane sequences like Met390, a conformational change will be

induced in EPOR, leading to receptor activation and formation of red blood cells in the absence of EPO.

Protein fragment complementation assays on EPOR fusion proteins

The use of a protein fragment complementation assay in cultured cells provided evidence that transmembrane unliganded EPOR dimers likely exist in a conformation that prevents activation of JAK2 [46]. These assays were based on fusion proteins between the extracellular and transmembrane domains of EPOR to fragments of dihydrofolate reductase (DHFR). Only when the two fragments of DHFR are in close proximity (induced by dimerization) would fluorescently-labeled methotrexate be able to bind DHFR. For fusion proteins, where DHFR fragments were fused immediately downstream of the transmembrane domain, protein fragment complementation was detected only in the presence of the ligand EPO. This finding suggests that EPOR extracellular-transmembrane domains adopt a rigid structure that either impairs dimerization of intracellular DHFR fragments or locks them in a conformation where complementation cannot occur. When a 30-amino acid flexible linker was inserted between the EPOR transmembrane and the DHFR fragments, spontaneous complementation could be demonstrated in the absence of ligand. These functional data support the notion that dimerization of extracellular and transmembrane domains of EPOR prevents spontaneous activation. Interestingly, when DHFR fragments were fused after the carboxyl-terminus of the entire cytosolic domain, then spontaneous activation was also detected [46], suggesting that most of the intracellular domain might be highly flexible and possible that the full-length receptor is a dimer.

EPOR is activated as a dimer

Long before the X-ray crystal structure of the complex between EPO and the EPOR extracellular domain was available, several key functional studies have indicated that EPOR is activated as a dimer.

The R129C mutation

A single point mutation (R129C) in the extracellular domain (Fig. 1B) enables EPOR to induce EPO-independent cell growth and tumorigenesis [18]. Biochemical studies employing two-dimensional (nonreducing and, subsequently, reducing gel electrophoresis) demonstrated that the introduction of the Cys residue led to formation of disulfide bonds between two receptor monomers [19]. EPOR mutants containing R129S, R129E, and R129P mutations were functionally wild type, demonstrating that Cys, and not the loss of

Arg, was required for constitutive activation [19]. Both monomers and disulfide-linked EPOR R129C dimers were found at the cell surface, and were shown to exhibit normal EPO-binding characteristics. Thus, the EPOR R129C chains do not form disulfide bonds with 100% efficiency. Interestingly, mutations in the critical extracellular WSXWS motif abolished not only cell surface appearance of the receptor, but also the formation of the disulfide bond by the mutated EPOR R129C monomers [19]. These data indicate that disulfide bond formation at C129 require proper receptor folding and does not represent aggregation in the endoplasmic reticulum.

When the interface of the two EPOR monomers was modeled on the basis of the dimeric interface of the growth hormone receptor [8], it became evident that the R129C mutant will place the Cys residue in the interface of the dimer. Several other residues were predicted to be located in the interface. For some of those, E132 and E133, it was shown that their mutation to Cys also yields disulfide-bonded constitutive active receptors [47].

Truncated receptors where the entire cytosolic domain was deleted, were able to block, in a dominant manner, the activation of the wild type EPOR by EPO [47]. The dominant negative effect required excess expression levels of the truncated *versus* wild-type receptors, and was also induced, albeit less efficiently, by a less severely truncated receptor that conserved 60 cytosolic residues. These results established that activation of EPOR by EPO requires receptor dimerization.

Cysteine mutations at the juxtamembrane-transmembrane border of EPOR

Random and Cys scanning mutagenesis studies of the juxtamembrane and transmembrane domains of EPOR identified three EPOR point mutations, L223C, L226C, and I227C that promote constitutive activation (Fig. 1B) [20, 21]. Disulfide bonds are formed by Cys residues at positions 223, 226, and 227, that lock the receptor in an active dimeric conformation [20, 21].

When examining disulfide bond formation by single Cys mutants of EPOR in nonreducing gels or by chemical cross-linking, it became clear that mutation to Cys leads to formation of disulfide bonds for most residues in the interval 220 to 229. The extent of disulfide bond formation did not correlate with activity, in that the efficiency of disulfide bond formation was not higher for the three mutants, L223C, L226C, and I227C, that were constitutively active. The implication of these data is three-fold:

- Simply dimerizing the receptor and bringing in close apposition the transmembrane domains does not suffice for activation;
- Only some and not all dimeric conformations are active. A model where inactive receptors would be dimers that would have the transmembrane domains very far apart (>70 Å) [15] is inconsistent with these data since, in such a model, disulfide bond formation at positions 223, 226, and 227

would never form; and
- The lack of periodicity of disulfide bond formation for the entire region between residues 220–229 suggests that this region is not α-helical, and that some of it might be flexible.

Nuclear magnetic resonance studies suggest that residues 226–230 form a helix-cap structure, which prevents self-activation [20]. Consistent with this, when the residues predicted to form a helix-cap at the very start of the transmembrane domain are replaced by a stretch of Leu residues, which are known to form α-helices, spontaneous activation of the receptor was detected [20]. Therefore, the junction between the extracellular and transmembrane domains is key to preventing receptor self-activation and might emerge as a possible target for small molecule regulators of EPOR signaling.

Remarkably, this point is supported by the random mutagenesis study, where several other mutants were selected to induce activation, most of them being concentrated at the same residues at the junction between the extracellular and transmembrane domains, i.e., NKK, NRE, or NTH mutants of positions 226–228, single and double mutations at 226 and 227 [21]. Only one middle transmembrane domain double mutant LI → WK at 236–237 induced constitutive activation. For this WK mutation, individual mutants L236W and I237K were not active [21], emphasizing the power of randomly mutating groups of residues, which enabled the identification of unique combinations of mutations that lead to activation.

Dimer reorientation in EPO-induced EPOR activation

Rather than simply inducing dimerization of monomers, ligands for receptors like EPOR and growth hormone receptor are now presumed to induce reorientation of preformed inactive dimmers [37, 39]. The existence of these inactive and active dimeric orientations is supported by the observation that the transmembrane and juxtamembrane cytosolic regions of several cytokine receptors adopt a rigid α-helical orientation, and that intracellular signaling depends on the pitch (register) or these α-helical orientations [37, 39, 48].

Alanine scanning and alanine insertion mutagenesis

Ala scanning of the proximal 50 cytosolic juxtamembrane residues of EPOR was performed to identify key residues for JAK2 activation. Two assays were used as read-outs of this scan: the ability of EPOR Ala mutants to respond to EPO in cytokine-dependent Ba/F3 cells, and the ability of JAK2 to promote traffic of the mutant EPOR to the cell surface. Three classes of mutations were isolated:

- Mutations of Box 1, Box 2 and of residues between them, which lost both JAK2-dependent traffic and response to EPO (already discussed);
- mutations that did not affect either traffic or function; and
- mutations that did not abolish traffic, but blocked activation of JAK2 after EPO-dependent stimulation of EPOR. Residues belonging to this last group were denoted 'switch residues' as they are normally required to transmit the signal from the receptor to JAK2. They consist of hydrophobic residues preceding Box 1 (L253, I257, W258) and D287. The first three hydrophobic residues form a hydrophobic motif (hydrophobic-x-x-x- hydrophobic-aromatic/hydrophobic) that is conserved in several cytokine receptors [25, 26] and is required for signaling. The exact mechanism by which the 'switch residues' enable activation of JAK2 is not clear, but might involve interactions between JAK kinase/pseudokinase domains and receptors, while residues between Box 1 and Box 2 are required to bind the FERM domain residues of JAKs.

In conclusion, one key event that must occur upon activation is represented by a change in position of the 'switch' residues, which would in turn change the relative positions of JAK2 proteins, enabling them to transphosphorylate each other. This action would require close contact between the catalytic loop of one JAK molecule and the activation loop of the other.

To assess the influence of the helical orientation of the transmembrane region on EPOR signaling, extra Ala residues were inserted at position 252, after the transmembrane domain and the positive patch (^{248}SHRR^{251}) that is present at the junction between transmembrane and cytosolic domains. For each Ala insertion, a 109° rotation of the downstream helical residues is predicted to occur, so that insertion of 3 Ala would bring the dimeric interface close to the original position (327° clockwise), while insertion of 1 and 4 Ala would rotate the helix towards the opposite face. When signaling of the EPOR1A–4A mutants was examined, it was determined that EPOR3A signaled like the wild-type receptor, while EPOR1A and EPOR 4A showed impaired signaling at physiologic EPO levels and at levels of receptor expression seen in erythroid progenitors. EPOR2A responded to EPO with an intermediate activity [36]. In such EPOR3A constructs the 'switch' residues required for activation of JAK2 would be 0.5 nm farther down from the membrane, but nevertheless signaling appears intact. On the background of one Ala insertion (EPOR1A), compensatory insertion of two extra Ala in the transmembrane domain upstream was able to restore full signaling [36]. The effect of compensation, revealing helical continuity, was lost when the 2 extra Ala were inserted before the transmembrane domain or within the first predicted transmembrane residues, suggesting that the first few residues of the predicted domain might not be helical or in register with the rest of the transmembrane domain. The interruption of helical continuity between the start of the domain (residue 226) and residues 231–234 is likely due to the fact that the first residues are not α-helical and may form a helix-cap [20].

An interesting difference has been noted between EPOR and the growth hormone receptor. Both seem to require dimer re-orientation for activation. In the case of the latter, insertion of Ala that would rotate the cytosolic domain also results in constitutive activation of the receptor [39]. For example, insertion of four extra Ala residues after the transmembrane domain (T4), or insertion of 1 Ala (J1) after the polar stretch that initiates the cytosolic domain (SKQQ, equivalent to ^{248}SHRR^{251} in EPOR) leads to constitutive receptor activation [39]. Those insertions are predicted to rotate the α-helical juxtamembrane domains towards an opposite interface. The 4Ala insertion in EPOR impaired activation by EPO (as the 1A also did), but did not induce constitutive activation [36]. This difference might be due to the fact that EPOR is activated by EPO in an asymmetric manner and that for activation, a 'rotation-only' conformational change might not suffice in the absence of a 'scissors'-like displacement.

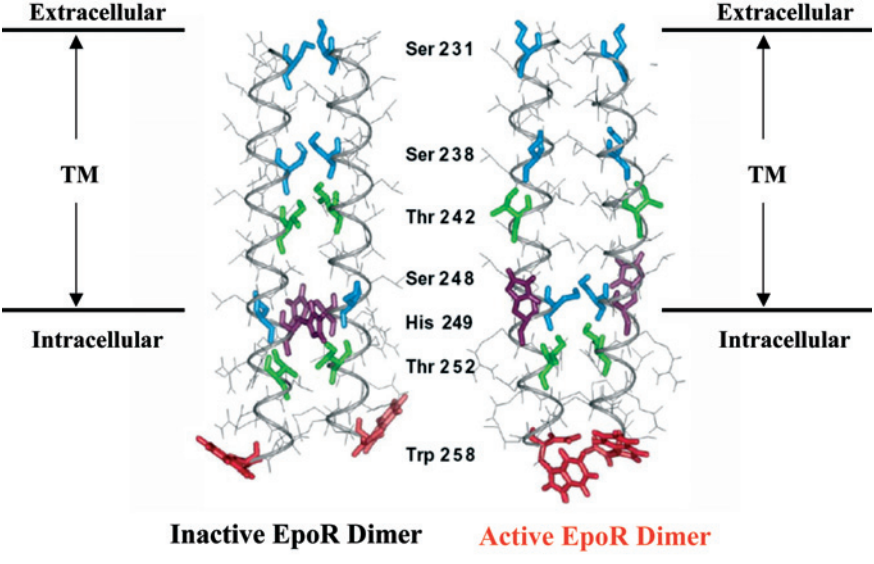

Figure 4. Model for the inactive and active transmembrane dimerization of EPOR. Independent computational searches identified two low energy structures for dimers containing the transmembrane sequence of EPOR. In one (left) dimerization is stabilized by hydrogen bonds between Ser, Thr, and His residues, and the cytosolic juxtamembrane W258 ('switch' residue preceding Box 1) is predicted to be outside the dimer interface. In the other (right), dimerization is stabilized by a Leu-zipper type of interaction, with several Leu residues in the interface. In this conformation, the cytosolic juxtamembrane switch residue W258 would be part of the dimeric interface. The latter structure was proposed to recapitulate the active EPOR conformation since coiled coil fusion proteins where this conformation was imposed were constitutively active, while the former was not.

Coiled coil-imposed orientation-specific dimerization

By replacing EPOR extracellular domain with a dimeric coiled coil, fusion proteins were engineered where the helical transmembrane domains were constrained into seven possible relative orientations [49]. One dimeric transmembrane conformation that imparted full activity to the cytosolic domain of the receptor was identified and shown to signal through JAK2, STAT5 and STAT3 proteins, and MAP kinase. In this dimeric conformation, transmembrane residues Leu241 and Leu 244 are predicted to be in the dimeric interface [20, 49], along with the hydrophobic motif 'switch' residue W258, which precedes Box 1 and is required for activation of JAK2 [24, 36] (Fig. 3). Another dimeric conformation appears to induce partial activation, with marginal JAK2 phosphorylation and preferential MAP kinase, but not STAT signaling. Independent computational searches identified two low energy left-handed dimers of the transmembrane domains, one where hydrogen bonds between Thr, Ser, and His residues stabilize the dimer (Fig. 4) and another where the dimeric interface is stabilized by Leu-zipper type of interactions and where W258 of the juxtamembrane region would be brought in the interface (Fig. 4). The latter corresponds to the active predicted structure of the coiled coil-EPOR fusion proteins cc-EPOR-III and cc-EPOR-VI, which induce activation of EPOR and where the dimeric interface contains 'switch' residue W258. The caveat of these results is represented by the symmetric nature of dimerization promoted by coiled coils, which is different from the asymmetric activation of EPOR by EPO. Nevertheless, the predicted position of particular residues in the individual coiled coil-EPOR fusion proteins was confirmed by Cys mutagenesis and dimer formation [20].

In conclusion, EPOR can be activated by the ligand EPO, by EPO-mimetic peptides [50], by disulfide bond formation between receptor monomers at extracellular (R129, E132, E133) [18, 47] or juxtamembrane residues [20, 21], by transmembrane domain mutations [21], by binding to the gp55 viral proteins [42], by fusions with coiled coils [49], or by rare agonistic monoclonal antibodies in certain conformations [51]. For the latter, it is relevant that only four of 96 monoclonal antibodies directed to the EPOR extracellular domain showed agonistic activity on cell proliferation, and of the four agonistic antibodies, only one induced robust erythroid differentiation [51]. Thus, all available evidence suggests that only some specific dimeric conformations are permissive for activation. The productive conformation would be required at several levels to fully activate JAK2; to access cytosolic receptor tyrosines for phosphorylation; and to exhibit the correct geometry of the phosphorylated tyrosine residues to promote recruitment of SH2- and PTB-domain containing signaling proteins [17]. Such conformational restrictions would be difficult to understand if the transmembrane and cytosolic domains of cytokine receptors would be completely flexible and unstructured [46]. Evidence presented for EPOR, growth hormone receptor, and gp130 [37, 39, 48] that the transmembrane and cytosolic juxtemembrane regions are in fact rigid and α-helically oriented provide a structur-

al framework for understanding why certain conformations are fully active, others inactive and some show an intermediate level of activation.

EPOR dimer reorientation activates JAK2

The major target of activation for EPOR is JAK2. The key event that must occur for activation is represented by a change in position of the EPOR 'switch' residues, which would in turn change the relative positions of JAK2 proteins, enabling them to transphosphorylate each other (Fig. 2B). This would require close contact between the activation loop of 1 JAK molecule and the catalytic loop of the other. The first event leading to activation is phosphorylation of activation loop Y1007, which places the activation loop in the active conformation [52].

No crystal structure exists for a JAK protein or for a receptor-JAK complex. For the JH1 domain in isolation, X-ray crystal structures have been solved for JAK2 and JAK3 and, as expected, the classical kinase fold has been found for these domains [53, 54]. However, the key to understanding how JAK are activated by receptors will be based on the structures of the FERM-SH2 and pseudokinase domains and on exactly how a conformational change provoked

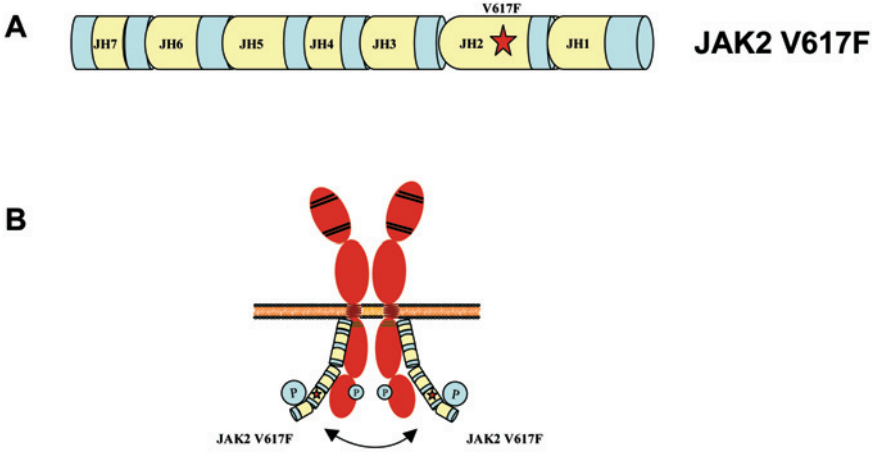

Figure 5 Preformed EPOR dimers are required for oncogenic signaling by JAK2 V617F in polycythemia vera. (A) The pseudokinase domain of JAK2 is predicted to block (prevent) autoactivation of the JH1 domain. A point mutation in the pseudokinase domain (V617F) renders the enzyme active, presumably by removing an intramolecular inhibitory interaction between the kinase and pseudokinase domains. This mutation is prevalent in human myeloproliferative neoplasms such as polycythemia vera, essential thrombocythemia, and primary myelofibrosis. (B) The mutated protein JAK2 V617F scaffolds to the cytosolic domain of EPOR, which is a preformed dimer. Receptor binding allows recruitment of JAK2 V617F to the membrane and further promotes activation by bringing to mutated proteins in close proximity. Unlike the wild type JAK2, which is not active when bound to an unliganded cytokine receptor, JAK2 V617F becomes fully active and initiates signaling.

by the receptor is transmitted to the JH1 kinase domain. Recall that the FERM domain is absolutely required for binding of JAK2 to the region encompassing Box 1 and Box 2.

Once activated, JAK2 is predicted to phosphorylate the cytosolic residues of EPOR, and of signaling proteins attracted to the receptor (such as STAT5, p85 subunit of phosphatydylinositol-3- kinase, and others), as well as proteins that would be attracted by the phosphorylated tyrosines and other motifs of JAK2 itself [55]. Notably, JAK2 contains a large number of tyrosine residues, some of which were shown to be phoshorylated after EPOR activation by EPO [56]. A FERM domain tyrosine, Y119 appears to exert, upon phosphorylation, a negative effect on the association of JAK2 with EPOR [57].

Preformed EPOR dimers are required for oncogenic signaling by the JAK2 V617F in polycythemia vera

Human myeloproliferative neoplasms, polycythemia perap, essential thrombocythemia, and primary (idiopathic) myelofibrosis are characterized by cytokine independent expansion of the myeloid compartment [58], especially the erythroid, megakaryocytic, and granulocytic lineages. A cardinal feature of these diseases, especially of polycythemia vera and essential thrombocytopenia is represented by *in vitro* cytokine-independent colony formation, for example EPO-independent erythroid colonies [59].

The major molecular determinant of myeloproliferative neoplasms is the presence of the *JAK2* V617F mutation, where the pseudokinase domain residue V617 is replaced by a phenylalanine (Fig. 5A); this mutation is present in >95% of patients with polycythemia vera and in >60% of patients with essential thrombocytopenia and primary myelofibrosis [60–63]. The V617F mutation appears to constitutively activate the kinase activity of JAK2 [64–66], most likely by removing an inhibitory effect of the pseudokinase domain on the kinase domain [67]. Indeed, previous studies suggested that the JH2 domain prevents self-activation of the JH1 domain [68]. In bone marrow reconstitution experiments, expression of JAK2 V617F in hematopoietic stem cells by retroviral transduction leads to polycythemia vera and primary myelofibrosis phenotypes [60, 69, 70]. For the essential thrombocytopenia phenotype, it appears that low levels of JAK2 V617F are required [69], as recently established by Pf4-Cre transgenic mouse models [71].

Saturation mutagenesis at position V617 indicated that large non-polar amino acids Trp, Leu, Ile, and Met also cause activation of JAK2 [72]. The strongest of these mutants was V617W, which was the only that stabilized Tyr phosphorylated suppressor of cytokine signaling 3 (SOCS3) [72], a mechanism by which JAK2 V617F overcomes inhibition by SOCS3 [73]. While JAK2 V617W would predictably be pathogenic in humans, the substitution of the Val codon, GTC, by TTG, the codon for Trp, would require three base pair changes, and for this reason, it might be unlikely to occur.

Expression of JAK2 V617F in hematopoietic cells expressing cytokine receptors leads to constitutive activation of STAT5, STAT3, MAP-kinase, and PI-3-kinase and hypersensitivity to ligands. To induce constitutive signaling, JAK2 V617F requires an intact FERM domain, indicating that it must be bound to a cytokine receptor. Introduction in the JAK2 FERM domain of a point mutation (Y114A), known to disrupt interaction with cytokine receptors [27] impairs constitutive signaling of JAK2 V617F in hematopoietic cells [74]. Furthermore, truncated JAK2 proteins that only contain the JH2–JH1 domains (pseudokinase and kinase) do not signal even when the V617F mutation is introduced in JH2 [75].

At high expression levels (six- to 10-fold higher than endogenous levels), JAK2 V617F leads to cytokine-independence of cell lines such as the IL-3-dependent Ba/F3 (murine proB) cells [60, 62, 66]. In such settings, it is presumed that JAK2 V617F binds to an endogenous cytokine receptor, such as IL-3 receptor β chain, which may be limiting, given the competition between JAK2 V617F and wild-type JAK2. However, at low levels of expression, comparable to or two-fold higher than endogenous levels, expression of JAK2 V617F does not suffice to induce autonomous growth of Ba/F3 or 32D cells [75, 76]. Expression of a dimeric type I cytokine receptor, such as EPOR is required for JAK2 V617F to induce autonomous growth under these settings. EPOR cytosolic tyrosine residues required for maximal STAT5 activation are required for promoting signaling by JAK2 V617F. In contrast, a mutated EPOR where residue W258 was mutated to Ala was able to support signaling by JAK2 V617F [75]. Recall that this 'switch' residue is crucial for activation of wild-type JAK2 by the EPO-activated EPOR. The model emerging from these studies suggests that scaffolding of the FERM domain of JAK2 V617F onto dimeric receptors such as EPOR would induce close apposition of two JAK2 V617F proteins. Unlike the wild-type JAK2, which remains inhibited when appended to dimeric receptors, JAK2 V617F becomes fully active and phoshorylates tyrosine residues on the receptor cytosolic tails, triggering ligand-independent signaling (Fig. 5B).

Conclusion

Activation of EPOR occurs when the native cell-surface dimeric conformation is reoriented towards a dimeric conformation, which is productive for signaling. The conformational change appears to involve both a relative rotation of monomers and a possible scissors-like displacement. Besides the physiologic ligand EPO, several other molecules can activate the receptor. A combination of structural and functional studies support the notion that only certain dimeric interfaces allow downstream signaling, that the unliganded receptor is an inactive dimer stabilized by transmembrane domain interactions and that the relative geometry induced by ligand-binding to the extracellular domain is important for the quality of signaling triggered by the cytosolic domains.

Several mutations at the junction between the extracellular and the transmembrane domain lead to constitutive receptor activation, indicating the critical role of this hinge region in regulating receptor activation. On the cytosolic juxtamembrane side, several residues, especially W258, were defined as critical for switching JAK2 catalytic activity on after ligand-activation of the receptor. Although the present body of knowledge about the mechanisms of activation of EPOR is extensive, the complete structure of the receptor-JAK2 complex in the presence and absence of EPO would be necessary for definitively ascribing dimer geometry to signaling output. Nevertheless, sequences around the start of the transmembrane domain may offer interesting targets for screening for small molecule regulators of EPOR signaling.

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Clinical utility

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
Edited by S.G. Elliott, M.A. Foote, and G. Molineux

Pharmacokinetics of erythropoiesis-stimulating agents

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Introduction

Erythropoietin (EPO) is a 30.4 kDa glycoprotein hormone secreted by the kidneys in response to tissue hypoxia, which stimulates red blood cell production. To regulate erythrocyte production, recombinant human EPO (rHuEPO) activates the EPO receptor (EPOR) and stimulates the proliferation and differentiation of erythrocytic progenitors in the bone marrow, leading to reticulocytosis and increased erythrocyte numbers and hemoglobin concentration in the blood. Epoetin alfa, the first commercial form of rHuEPO marketed in the United States of America (USA) and European Union (EU), and epoetin beta marketed outside the USA are both expressed in Chinese hamster ovary (CHO) cells. Both epoetins have a 165-amino acid sequence identical to human urinary EPO, contain three sialic acid-containing *N*-linked and one *O*-linked carbohydrate chains [1] leading to the same biologic effects as endogenous EPO [2–4]. No important differences in clinical efficacy are apparent between epoetin alfa and epoetin beta, and they are generally used interchangeably [5]. Darbepoetin alfa is a hyperglycosylated rHuEPO analog with five amino acid changes and two additional *N*-linked carbohydrate chains, which has the same mechanism of action as rHuEPO. Darbepoetin alfa, however, has a three-fold increased serum half-life [6–8], and increased *in vivo* potency [9], allowing for more convenient modes of administration, including extended dosing intervals [10, 11]. A large methoxy-polyethylenglicol polymer chain was integrated into the epoetin beta molecule through amide bonds between the *N*-terminal amino group of ALA and the σ-amino groups off Lys (Lys45 or Lys52) by means of a succinimudyl butanoic acid linker [12], resulting in a PEGylated epoetin beta molecule that is marketed in EU. As with other PEGylated therapeutic proteins, the PEGylation of epoetin beta reduced EPOR binding affinity. This biologic disadvantage is counter balance with a half-life of approximately 130 h in humans and allows for extended dosing intervals, similarly to darbepoetin alfa. In this chapter, we review the clinical pharmacokinetics of the marketed protein-based erythropoiesis stimulating agents (ESAs).

Mechanism of action

The mechanism of EPO synthesis is regulated through the blood partial pressure of oxygen in the kidney and other EPO-producing organs [13]. Under conditions of hypoxia, *EPO* gene transcription is activated when hypoxia-inducible factor (HIF) binds to a hypoxia-responsive enhancer (HRE) located in 3' flanking sequence of the *EPO* gene. After EPO is produced, it acts on erythroid precursor cells through EPOR, which is a transmembrane protein that is a member of the type I cytokine receptor superfamily. Two regions of EPO contain two separate EPOR-binding sites [14, 15], and EPOR is activated when one rHuEPO molecule homodimerizes two EPOR molecules on the surface of the erythroid blast-forming unit (BFU–E) and colony-forming unit (CFU–E). Each CFU–E cell contained approximately 1,000 EPOR per cell [14, 16], and approximately 5–10% of these must be continuously occupied with ligand for the cells to survive and differentiate fully into erythrocytes [17, 18]. A high-affinity (1 nM) and low-affinity (4 μM) EPOR binding sites have been characterized, however, their clinical significance is not well understood [19, 20]. ESA binding to EPOR can lead either to internalization or subsequent dissociation of the molecule from the receptor. Once internalized along with the receptor complex, the ligand may either be degraded, or it may be recycled intact to the cell surface and released [21–23].

EPOR homodimerization activates a number of signal transduction pathways, including Janus kinase/signal transducers and activators of transcription (JAK-STAT), phosphatidylinositol 3-kinase (PI3K), and RAS-mitogen-activated protein kinase (MAPK) [24, 25]. Current research suggests that EPO exerts its control over erythropoiesis by preventing cell apoptosis (programmed cell death) thus allowing more red cell precursors to survive, proliferate, and/or induce erythroid-specific proteins [26]. Some of the apoptotic tendency of erythroid cells is attributed to proapoptotic molecules produced by hematopoietic cells, macrophages, inflammatory cytokines, and stromal cells. EPO prevents apoptosis by upregulation of antiapoptotic *Bcl* proteins (Bcl2, BclXL) and downregulation/inactivation of apoptotic proteins, such as BAD, a prodeath Bcl2 family member, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) forkhead and caspases [27–32].

Darbepoetin alfa and PEGylated epoetin beta stimulate erythropoiesis by the same mechanisms as endogenous EPO and rHuEPO. *In vitro*, the affinity of darbepoetin alfa and PEGylated epoetin beta for EPOR is three- to five-fold and 50- to 100-fold less than that of rHuEPO, respectively; however, the increase in mean residence time of darbepoetin alfa and PEGylated epoetin beta results in a prolonged period of time above an erythropoietic threshold that exceedingly compensates for the reduced receptor affinity, yielding an increased *in vivo* activity [7, 9, 33]. However, in dialysis patients with stable hemoglobin concentrations, no difference in efficacy has been observed between darbepoetin alfa and PEGylated epoetin beta [34].

Regulation of erythropoietin

The primary site of EPO synthesis in adults is the peritubular cells of the kidney [5, 24, 25]. The liver is a secondary site of EPO production, with synthesis occurring in both hepatocytes and fibroblastoid interstitial cells [35]. Astrocytes and neurons within the central nervous system have been reported to express EPO [36]. No preformed stores of EPO exist, and plasma EPO concentrations are maintained at a constant concentration by the homeostatic turnover, which consists in the basal production and elimination of the hormone [37]. Within a healthy individual, the serum EPO concentration tends to be controlled tightly; however, large interindividual variability is evident from the normal range, 5 to 35 IU/L [37]. Maintenance of normal serum concentrations of endogenous EPO requires the synthesis of about 2–3 IU/kg/day, or approximately 1,000–1,500 IU/week for a 70 kg man [38]. Sex differences [38] and regular-to-moderate athletic training [39] did not appear to affect endogenous EPO serum concentrations.

The blood flow in the kidney has a circadian rhythm in normal individuals [40], therefore, the endogenous production of EPO was expected to have diurnal variations with the highest levels of endogenous EPO in the evening and at night [41, 42]. The circadian rhythm was first quantified by Hayashi et al. [43], who reported a 10% amplitude of the baseline EPO production rate. Similar results were obtained by Olsson et al. [44], who also evidenced an asymmetric time course of endogenous EPO, with a broad peak in the evening and night, and relatively narrow trough in the morning.

The overexpression of EPO occurs in a number of adaptive and pathologic conditions, which needs to be considered when the ESA pharmacokinetics is investigated. In response to acute hypoxic stress, such as severe blood loss or severe anemia, EPO production rate can increase 100- to 1,000-fold. Numerous studies have shown an exponential increase in serum EPO, with increasing degrees of anemia [5, 45], although the maximal bone marrow response to such stimulation is only a four- to six-fold increase in red cell production rate [46]. Overproduction of EPO with accompanying erythrocytosis may be an adaptive response to conditions that produce chronic tissue hypoxia, such as living at high altitude, chronic respiratory diseases, cyanotic heart disease, sleep apnea, smoking, localized renal hypoxia, or hemoglobinopathies with increased oxygen affinity [24]. Paraneoplastic production of EPO from tumors and cysts, including renal carcinomas, benign renal tumors, Wilms' tumors, hepatomas, liver carcinomas, cerebellar hemangioblastomas, adrenal gland tumors, and leiomyomas, can also result in high plasma concentrations EPO.

In chronic kidney disease, up to 60% of patients have hemoglobin concentrations <11 g/dL before beginning dialysis [47]. Multiple mechanisms contribute to the low hemoglobin concentrations [25], but the most important is the inability of the diseased kidneys to produce an appropriate EPO response for the given degree of anemia or an inability to meet the increased red blood cell demands of uremic patients [48, 49]. In addition, the uremic state itself

appears to blunt the bone marrow response to EPO, perhaps through polyamines, inflammatory cytokines, and/or parathyroid hormone mediators [25, 50]. It is of interest that serum EPO concentrations in chronically anemic dialysis patients increase to some extent in response to acute hypoxic stress (from either acute bleeding or systemic hypoxemia), suggesting that kidney failure does not result in a complete inability to produce EPO [51, 52].

The anemia of cancer is also of multifactorial etiology [37]. As with other anemias of chronic disease, including those associated with chronic infection and inflammatory disorders, there is decreased production of endogenous EPO [53], cytokine-induced suppression of bone marrow function, disordered iron absorption and metabolism [54], and decreased erythrocytes survival. Furthermore, the amount of endogenous EPO transiently increases up to six-fold within the 48 h after the administration of chemotherapy and returns to baseline within a week [55–57]. In the case of myeloablative chemotherapy, severe thrombocytopenia might contribute to a significant loss of red blood cells. It was demonstrated that ^{51}Cr-labeled control red blood cells administered to rats that had received 60 mg/kg of carboplatin were substantially lost during development of thrombocytopenia, probably due to internal hemorrhage, but not related to an increase in cell lysis susceptibility or reduction in red cell survival time [58]. Finally, the anemias associated with infant prematurity, pregnancy, allogeneic bone marrow transplantation, and HIV infection are often characterized by inappropriately low EPO concentrations [35].

Pharmacokinetics

Absorption

Due to gastrointestinal enzymatic degradation of proteins, protein-based ESAs are not administered by the oral route; either intravenous or subcutaneous routes are used. The route of administration is determined according to indication, patient, and healthcare provider preference, as well as regional considerations that affect medical practice. After a single dose of subcutaneous rHuEPO, absorption is slow, leading to peak serum concentrations at 5–30 h and a longer terminal half-life (24–79 h) than that obtained after intravenous administration. These results indicate the presence of flip-flop pharmacokinetics [59] where the rate of absorption is slower than the rate of elimination. Thus, absorption is rate limiting, and the observed terminal half-life after subcutaneous dosing reflects the absorption rather than elimination rate [60–66].

After subcutaneous administration, protein therapeutics typically enter into the systemic circulation through the blood capillaries or the lymphatic system. The lymphatic system is considered to be the primary route of absorption from the subcutaneous injection site for protein therapeutics greater than about 16 kDa due to restricted vascular access afforded by the continuous endothe-

lial layer of blood capillaries [67–69]. The intercellular junctions within the lymphatic vessel wall create cleft-like openings that provide an alternative pathway from the interstitium into the lymph and indirectly into the systemic circulation by the thoracic duct [70]. The relative roles of the blood and lymphatic absorption pathways after subcutaneous administration of rHuEPO and darbepoetin alfa have been directly assessed using lymph cannulated animal models. In sheep, the cumulative amount of rHuEPO and darbepoetin alfa recovered in peripheral lymph was 84–90% of the administered dose, respectively, indicating almost complete absorption from the subcutaneous injection site (interdigital space) and minimal clearance during transit through the lymphatic system [71, 72]. A rat model with continuous lymph collection from thoracic lymph duct evidenced that <3% of the rHuEPO dose was recovered in the lymph. Therefore, the rHuEPO subcutaneous absorption in rat occurred mainly through direct uptake into the blood capillaries without substantial involvement of the lymphatic pathway [73]. These results are supported by studies using an immunocytochemical approach where it was shown that insulin, and even molecules as large as albumin, can penetrate blood capillary endothelium by the transcellular route [74]. It has been argued the discrepancies between the results obtained from rat and sheep model might be attributed to the utilization of different anatomical injection sites and doses administered [73]. However, it has been shown that the systemic availability of darbepoetin alfa from the interdigital (106%), shoulder (92%), and abdomen (85%) subcutaneous injection sites was almost complete relative to the intravenous group. However, the rate of absorption was 0.15, 0.08, and 0.02 h^{-1} for the interdigital, shoulder, and the abdomen injection sites, respectively, which showed significant variation on the absorption rate across injection site [75].

In humans, the absorption process after subcutaneous administration of rHuEPO is complex, with at least two apparent pathways of absorption. A dual absorption model describes the pharmacokinetics of rHuEPO after subcutaneous administration of 20–160 kIU to healthy subjects [44]. In this model, the faster process corresponded to a direct absorption from the injection site to the extravascular compartment, characterized by a zero-order process that lasted for 0.7 h, followed by a slow first-order process of entry into systemic circulation with an absorption rate of 0.034 h^{-1}. The delayed and long lasting (1.5 days) zero-order absorption corresponds to the absorption *via* the lymphatic system, which requires a lag-time of almost 3 h to account for the transit time from the injection site to the point of lymph collection [44]. A similar model was used before to describe the pharmacokinetics of rHuEPO after subcutaneous administration in rats [76], monkeys [77], and healthy volunteers [78]. In these studies, approximately a 67%, 65%, and 79% of the absorbed dose in rat, monkeys, and healthy volunteers, respectively, was attributed to entry into the circulation by the lymphatics. These values are slightly lower than the values reported in sheep [71]. In addition, it has been suggested that the fraction of dose that is absorbed through the lymphatic system in humans is increased at doses higher than 300 IU/kg [44].

The subcutanteous absorption of darbepoetin alfa in humans is slow, with peak concentrations reached at 34–58 h post-dose, followed by a generally monophasic decline. Similarly to rHuEPO, darbepoetin alfa also displays flip-flop pharmacokinetics, with a longer half-life after subcutaneous dosing than after intravenous dosing. The mean terminal half-life of darbepoetin alfa, 70 h, was associated with large between patient variability, consistent with variability observed for other ESAs [79]. Nevertheless, the typical profile of the dual absorption does not become evident from the darbepoetin alfa subcutaneous pharmacokinetic profiles, which has been characterized using a simple first-order absorption model [80]. One potential explanation for this finding is that the rate of absorption through the two different pathways becomes similar and, consequently, the dual absorption profile becomes just a first-order absorption process. A 20–30% reduction in the darbepoetin alfa absorption rate per decade of age was reported. The magnitude of this finding is consistent with the estimated effect of age on the rHuEPO absorption rate and reflects the longer terminal half-life and the larger exposure of both drugs in older patients. It has been hypothesized that the age-dependent reduction in lymphatic flow rate could be the physiologic reason behind this relationship [80]. The data available also suggest that the pharmacokinetic profile of rHuEPO and darbepoetin alfa after subcutaneous administration is similar in adults and children; however, subcutaneous absorption in children may be more rapid than in adults for both drugs [61].

It has been shown that PEGylation may increase the absorption half-life of subcutaneously administered agents [81]. The absorption process of PEGylated epoetin beta after subcutaneos injection was indeed slow, with peak concentrations reached at 42–120 h post-dose, followed by a generally monophasic decline. As observed for other ESA, PEGylated epoetin beta also displays flip-flop pharmacokinetics after subcutaneous administration. Actually, the terminal half-life after a single subcutaneous injection to healthy volunteers ranged from 102–216 h, which is slightly higher than the terminal half-life observed after intravenous administrations (70–122 h). Similarly, in patients with chronic kidney disease not receiving dialysis, the mean terminal half-life after subcutaneous administration was 142 h, which is about 84% higher than the terminal half-live observed after intravenous administration, 77 h. These results are consistent with the multiple ascending dose study in healthy volunteers and in patients with chronic kidney disease on dialysis, although the difference in terminal half-live between intravenous and subcutaneous administration were narrower [82]. As for darbepoetin alfa, the typical profile of the dual absorption does not become evident from the pharmacokinetic profiles obtained after subcutaneous administration of PEGylated epoetin beta [83]. No effect of age on PEGylated epoetin beta absorption has been reported.

After subcutaneous administration, absorption rates of rHuEPO vary according to the administration site, most likely reflecting regional differences in blood and lymph flow [84]. Macdougall et al. administered 125 IU/kg subcutaneously of radiolabeled rHuEPO to healthy volunteers in sites in the arm,

abdomen, and thigh, demonstrating more rapid and extensive absorption when rHuEPO was injected into the thigh compared with the abdomen or arm [85]. The AUCs(0–∞) measured after injections into the arm and abdomen were 89% and 77%, respectively, of the AUC(0–∞) measured after injection into the thigh. Jensen et al. [84], however, concluded that the only notable difference in pharmacokinetics between epoetin beta when injected at 100 IU/kg into the thigh compared with the abdomen was a longer mean residence time (32.7 *versus* 26.2 h, respectively), which they attributed to slightly slower absorption. Similar results were obtained for PEGylated epotin beta. Actually, the maximum concentration (C_{max}) after subcutaneous injections of 3 μg/kg into the arm and abdomen were 95% and 86%, respectively, of the C_{max} measured after injection into the thigh. Similarly, the AUCs(0–last) after subcutaneous injections of 3 μg/kg into the arm and abdomen were 96% and 94%, respectively, of the AUCs(0–last) measured after the injection into the thigh [83]. Overall, the absorption rate of ESA varies according to the site of administration, however, the magnitude of the differences between the sites of administration was relatively small and not considered to be clinically relevant as the pharmacodynamic profile did not evidence any difference across the site of administration.

Bioavailability

Initial bioavailability estimates for rHuEPO after subcutaneous administration ranged from 15–40% [86, 87], and were similar for epoetin alfa and beta [88]. However, most assessments of rHuEPO subcutaneous bioavailability did not take into account the nonlinear disposition kinetics of rHuEPO and/or the time dependent clearance [89], and have estimated bioavailability relative to the same single intravenous dose administration with a comparison of AUC. In this situation, there is an underestimation of the bioavailability, especially at lower doses of rHuEPO, at which the nonlinearity in clearance is most evident. To accurately determine the bioavailability of a drug cleared in a nonlinear manner, it is necessary to determine the underlying intravenous pharmacokinetics and then estimate the subcutaneous bioavailability by modeling techniques [90].

In addition, when the pharmacokinetics of subcutaneous rHuEPO and darbepoetin alfa were studied over a wider dose range in healthy volunteers, exposure was found to increase more than proportional with dose, even when accounting for the nonlinear clearance of rHuEPO [44, 80, 91, 92]. Similar results were observed in dogs where the rHuEPO subcutaneous bioavailability for the 400 IU/kg dose, 26.8%, increased to 73% at doses of 1,000 IU/kg, and to 100% at doses >2,400 IU/kg [77]. More recently, rHuEPO subcutaneous bioavailability (F) in healthy subjects was described as a function of dose using a linear, ($F, \% = 38.8 + 0.0250 \cdot \text{Dose, IU/kg}$) [93], or hyperbolic ($F, \% = 29.1 + 64.9 \cdot \text{Dose, kIU}/[63.2 + \text{Dose, kIU}]$) [44] function. The linear function was also deemed appropriate to describe the increase of darbepoetin

alfa subcutaneous bioavailability with dose (F, % = 44.8 + 0.0586 · Dose, μg) [80]. Assuming a conversion factor of 200 IU of rHuEPO per 1 μg of darbepoetin alfa, the slopes of the linear function for the subcutaneous bioavailability were very similar for rHuEPO and darbepoetin alfa. Furthermore, according to those models the subcutaneous bioavailability of darbepoetin alfa would increase from 57% to 69% when the 200 μg dose is increased up to 400 μg, while the subcutaneous bioavailability of rHuEPO would increase from 54% to 65% when the 40 kIU dose is increased up to 80 kIU.

The bioavailability of PEGylated epoetin beta in healthy subjects ranged from 40–60% in the dose range of 0.8–3.2 μg/kg, which is slightly lower than the bioavailability of epoetin and darbepoetin alfa at equivalent doses. These results are consistent with the findings observed in comparative studies conducted in rat and dog. In addition, the dose-dependent absolute bioavailability of PEGylated epoetin beta has not been reported. However, statistical analysis of a Phase I study where subcutaneous PEGylated epoetin beta was administered in ascending doses from 0.1–3.2 ug/kg evidenced a more than dose proportional increase in the AUC and C_{max}, which could be an indirect evidence of the dose-dependent absolute bioavailability in absence of saturable elimination process. Similar to other ESA, the absolute bioavailability of PEGylated epoetin evidenced a high degree of intersubject variability (53–92%) [82].

The estimates of subcutaneous bioavailability of ESA indicated incomplete drug absorption and/or degradation during the absorption process. Based on the negative correlation previously observed between the molecular size and bioavailability of heparins [94], it was suggested that the large molecular size of ESA might impede drug absorption from the subcutaneous injection site [95], and could also explain the lower absolute bioavailability of PEGylated epoetin beta with respect to epoetin beta in different studies. In addition, the apparent increase in subcutaneous bioavailability with dose for ESA might indicate saturable presystemic processes. In fact, saturable injection site loss has been reported for several proteins, and/or saturable degradation by proteolytic enzymes in the lymph may also contribute to this phenomenon [68]. Nevertheless, despite the apparent low bioavailability, subcutaneous administration of ESA produces equivalent or better efficacy to intravenous administration, and this is assumed to be due to the prolonged absorption leading to reduced receptor saturation and increased drug efficiency [96, 97].

Distribution

Serum rHuEPO concentrations rose rapidly during the intravenous infusion and then declined in a bi-exponential manner [44]. In early clinical studies, however, a single phase of rHuEPO disposition was reported after intravenous administration [87, 93], most likely because the early rapid disposition phase was not captured with the sampling schedule used [90]. After the intravenous administration of rHuEPO, peak serum rHuEPO concentrations correlated lin-

early with dose, and a dose of 50 IU/kg produced concentrations of about 1,000 mIU/mL 15 min after the end of the infusion [38]. As expected from its large molecular weight, rHuEPO's volume of distribution was similar to the plasma volume (40–60 mL/kg), suggesting confinement of EPO within the plasma circulation [44, 66, 98]. Nevertheless, many reports have shown that blood capillaries are permeable for a variety of macromolecules. Plasma and lymph concentrations of rHuEPO in rat were 6.6 and 5.2 IU/mL, respectively, after 1.5 h after intravenous administration of 400 IU/kg, respectively. The lymph concentrations were much higher after intravenous administration than those detected after subcutaneous injection, suggesting that rHuEPO is capable of redistributing from systemic circulation to lymph [73]. In addition, PEGylation seems to limit tissue penetration as the distribution volume of PEGyated epoetin beta is only half of that of epoetin beta in rat and dog [82]. Although limited extravascular distribution to peripheral compartments has been reported for ESA [44], and it is unclear what it represents, it could be nonspecific distribution into bone marrow, blood cells, or other tissue depending on availability for transport across the capillary endothelia or through the interstitial space [99, 100].

As expected from the similarity in the molecular structure between epoetins alfa and beta, similar results in the volume of distribution were found in the pharmacokinetic analysis of rHuEPO beta in healthy volunteers [101, 102]. Steady state volume of distribution (V_{ss}) of epoetins alfa or beta across different animal species, including human, can be expressed as a function of body weight (BW) as follows:

$$V_{ss}\,(mL) = 90 \cdot BW(kg)^{0.853}\ (r^2 = 0.922,\ p < 0.01)\ [103].$$

A population pharmacokinetic analysis of darbepoetin alfa in healthy volunteers indicated that darbepoetin alfa has a similar volume of distribution as rHuEPO [80]. Similar results were found in patients who were receiving continuous ambulatory peritoneal dialysis, hemodialysis, and chemotherapy [6, 104–106]. In addition, when the volume of distribution of ESA in healthy volunteers and patients receiving dialysis are compared across studies, they appear similar and close to the plasma volume [82, 87, 107–110]. The data available also suggest that the pharmacokinetic profile of rHuEPO and darbepoetin alfa after intravenous administration is similar in adults and children. However, no pharmacokinetic studies have been conducted in pediatric patients receiving PEGylated epoetin beta [82].

In addition, dose-proportional increases in cerebrospinal fluid (CSF) concentrations of EPO were observed as a consequence of the penetration of systemically administered rHuEPO into the central nervous system of patients with cancer and brain metastases [111]. The concentration of rHuEPO in the CSF increased after a period of slow equilibration (1–3 h), peaked after approximately 9–24 h, and declined bi-exponentially thereafter with similar terminal slopes in serum and CSF. The penetration of rHuEPO into the CSF

ranged from 0.02–0.31% after single doses, which is similar to results observed in rats and macaques and consistent with the data obtained from PEGylated epoetin beta in rats [82, 112, 113]. Although the mechanism involved in the rHuEPO transport across the blood-brain barrier is not well understood, several hypotheses have been suggested and included receptor-mediated transport; carrier-mediated transport; fluid phase endocytosis; non-specific or receptor-mediated adsorptive endocytosis; and transmembrane diffusion [114]. The possibility that rHuEPO plays a role in neuroprotection is the subject of considerable debate. EPO has been shown to exert cytoprotective effects on erythroid progenitor cells as well as various nonerythroid cells. These protective effects have been largely attributed to antiapoptotic signalings of EPO. In a trial of patients who had had a stroke, EPO concentrations of approximately 15 mIU/mL in the CSF correlated with improved functional outcomes [115], consistent with concentrations achieved after a single dose of 1,500 IU/kg, where the peak rHuEPO concentrations in the CSF ranged from 11 mIU/mL to 40 mIU/mL. However, Katavetin et al. have argued that injured cells undergoing apoptosis are generally too severely damaged to function properly. Therefore, simply corrupting apoptotic pathway is unlikely to be an effective strategy, because the remaining damaged cells may not function appropriately, or they may eventually undergo necrotic cell death. Evidence suggests that EPO also provides cytoprotection by ameliorating oxidative stress, the principal cellular insult. EPO may exert its antioxidative effects directly by exploiting intracellular antioxidative mechanisms such as hemeoxygenase-1 and glutathione peroxidase. In addition, EPO may act indirectly by inducing iron depletion and thereby inhibiting iron-dependent oxidative injury. Increasing red blood cells by EPO may also indirectly reduce cellular oxidative stress, as red blood cells are loaded with a substantial amount of antioxidative enzymes [116]. Further investigation regarding the mechanisms of cellular antioxidative responses to EPO would provide a better insight to cytoprotective action of EPO, and would support the development of better cytoprotective drugs in the near future.

Elimination

Despite the long clinical experience with ESAs, the mechanism(s) of their clearance have not been fully elucidated and there is a paucity of information regarding which organ(s) and tissue(s) are important in the metabolism and elimination of these drugs. Pharmacokinetic studies of rHuEPO have demonstrated dose-dependent disposition in human adults [44, 99, 117, 122], premature infants [118], monkeys [77], sheep [119], and rats [76, 120] after intravenous administration. In these studies, nonlinear kinetics could be related to the limited number of EPOR located on the finite, but expandable, number of bone marrow erythroid progenitors that may play a role in clearance [127]. In addition, possibly nonerythroid EPOR pathways located in other tissues could

be also involved in the receptor-mediated endocytosis and subsequent intracellular lysosomal degradation [5].

EPO concentrations remain abnormally high in dogs that have been sublethally irradiated, even after the cessation of hypoxia [121], suggesting that EPO concentrations were significantly "influenced by the functional state of the erythroid tissue of the marrow" [121]. Subsequently, it has been further documented that after bone marrow ablation [122–124], in patients with aplastic anemia [125], or in anemic patients with hypoplastic marrows [122, 126, 127], plasma EPO levels were disproportionately increased relative to slightly decreased hemoglobin concentrations. Conversely, individuals with hyperactive marrow owing to hemolytic anemia had disproportionately low plasma EPO concentrations [127–129] and rapid EPO plasma disappearance [130, 131].

Additional nonclinical evidence implicating the primary role of the bone marrow in EPO metabolism has been provided when after intravenous administration of ^{125}I-rHuEPO to rats, the highest organ-specific radioactivity uptake was observed in bone marrow and spleen [132, 133]. These tissues have also been shown to rapidly metabolize EPO with tissue uptake and clearance directly correlated with the number of CFU–E [134]. ^{125}I-rHuEPO uptake by these organs was transient and observed only at low doses. These data also suggest a saturable process for EPO elimination, which is consistent with nonclinical and clinical publications reporting nonlinear pharmacokinetics for EPO.

In sheep, an 80% reduction of rHuEPO clearance was observed after busulfan-induced bone marrow ablation and was associated with a markedly decreased bone marrow cellularity [135]. These findings suggest that some elimination may occur in the bone marrow. This suggestion is supported by *in vitro* studies demonstrating that EPO is rapidly internalized and degraded after binding to EPOR on erythroid progenitor cells [21, 22, 136].

Gross and Lodish investigated the trafficking and degradation of rHuEPO and darbepoetin alfa by EPOR-expressing cells in cell culture and found that rHuEPO and darbepoetin alfa were subjected to EPOR-mediated endocytosis followed by degradation in lysosomes [22]. Both drugs were degraded only by cultured cells (BsF3) that expressed EPOR, not the untransformed counterpart, and receptor binding, dissociation, and trafficking properties affected their relative rates of cellular uptake and intracellular degradation. rHuEPO had a faster EPOR on-rate than darbepoetin alfa ($k_{on} = 5.0 \cdot 10^8\ M^{-1} \cdot min^{-1}$ *versus* $1.1 \cdot 10^8\ M^{-1} \cdot min^{-1}$) and a slower off-rate ($k_{off} = 0.029\ min^{-1}$ *versus* $0.042\ min^{-1}$) resulting in an approximately five-fold higher affinity. Surface-bound rHuEPO and darbepoetin alfa were internalized at the same rate ($k_{in} = 0.06\ min^{-1}$), and after internalization 60% of each ligand was resecreted intact and 40% degraded. Therefore, rHuEPO was more likely to bind EPOR and internalize than darbepoetin alfa. As a consequence, rHuEPO was degraded faster than darbepoetin alfa through receptor-mediated endocytosis [22]. While this observation suggested that darbepoetin alfa may have reduced clearance *in vivo* because of reduced EPOR-mediated endocytosis and degra-

dation, darbepoetin alfa has other biophysical characteristics, such as increased molecular size and decreased isoelectric point, opening the possibility that the reduced clearance could be explained by other mechanisms.

Additional clinical evidence that rHuEPO elimination occurs in the bone marrow comes from studies in patients with cancer who received myeloablative chemotherapy and hematopoietic stem cell transplantation [137]. A 34% reduction in clearance and a 95% increase in terminal half-life were demonstrated for rHuEPO after administration of high-dose chemotherapy. Chemotherapy-induced bone-marrow ablation results in a decline in EPOR carrying progenitor and loss of the nonlinear part of the clearance process, suggesting that the nonlinear clearance of rHuEPO may be due to elimination by EPOR binding and endocytosis in the bone marrow. Similar changes in the pharmacokinetics of darbepoetin alfa in patients after chemotherapy were reported, with a reduction of clearance up to 40% in patients with chemotherapy-induced anemia [105, 138]. A role for an EPOR-mediated pathway in clearance is also consistent with the significant increase in rHuEPO clearance observed during the early post-bone marrow transplant period [137].

The results described above clearly differ from those reported in rats where the EPO clearance and half-life were not significantly altered by hypo or hyperplastic marrow states [139]. Piroso et al. used cyclophosphamide to induce marrow hypoplasia and phenylhydrazine or bleeding to induce marrow hyperplasia. It has been suggested that the extremely high EPO concentrations observed among the phenylhydrazine and phlebotomized groups of study animals had saturated EPOR-mediated clearance and, as a consequence, the pre- and post-ablation differences in rHuEPO pharmacokinetics were not apparent [137]. Alternatively, the role of the EPOR-mediated pathway may not be as important a mechanism of clearance of rHuEPO as initially thought [140]. Nevertheless, the EPOR-mediated pathway is also consistent with the significant increase in rHuEPO clearance observed during the early post-transplant period [137]. The significant engraftment of erythroid progenitors approximately 1 week after the transplantation increases the amount of cell surface EPOR and, therefore, the rHuEPO clearance. A clinical implication of this finding is that the administration of rHuEPO relatively early after marrow ablation to patients undergoing peripheral stem cell transplantation may stimulate erythropoiesis before significant peripheral reticulocytosis is detectable [137].

In spite of the *in vitro* results suggesting the role of EPOR on ESA clearance, evidence of an *in vivo* role for this pathway is indirect and mostly arises from chemotherapy experiments. Chemotherapy-based approaches may result in nonspecific effects to EPOR-independent clearance mechanisms. If chemotherapy impacts one or more of the EPOR-independent elimination pathways, such as destruction of macrophages or neutrophils, then their diminished number may explain or at least contribute to the decrease in ESA clearance that is observed after chemotherapy treatment. For example, chemotherapy is known to cause pancytopenia, and a recent report showed that rHuEPO was cleared *in vitro* from cell culture medium containing unfractionated bone

marrow-derived cells [141]. In addition, an rHuEPO analog with no detectable receptor binding activity was depleted from the culture medium at the same rate as rHuEPO, suggesting that EPOR-mediated endocytosis was not the primary route of rHuEPO degradation in this model system.

An alternative method to explore the role of the EPOR-mediated clearance in the elimination of ESAs is to investigate the pharmacokinetics of EPO analogs with different EPOR-binding activity. Using site directed mutagenesis [7, 142], three rHuEPO analogs were engineered: NM385, NM340, and NM294 [140]. NM385 was devoid of detectable receptor binding activity ($IC_{50} > 10$ μM), but retained similar structure and carbohydrate content as rHuEPO and, therefore, this molecule should not be cleared by the EPOR-mediated clearance pathway. Furthermore, if the EPOR-mediated pathway was dominant, NM385 would show a substantially reduced clearance and increased half-life compared with rHuEPO. Pharmacokinetic data obtained after a single intravenous dose of NM385 3 and 30 μg/kg in mice showed that, compared with rHuEPO, a 119% and 69% increase in half-life with a 99% and 34% reduction in clearance, respectively. The observation that the effect on clearance was more pronounced at lower nonpharmacologic doses is consistent with saturation of EPOR-mediated clearance at higher doses [140].

Compared with rHuEPO, the peptide backbone of the NM385 analog carries three additional negative charges. To control for the change in charge associated with the substitutions on NM385, the rHuEPO analog NM340 was examined. NM340 retained receptor binding activity ($IC_{50} = 135$ pM) and carried four additional negative charges, while retaining the same carbohydrate content as rHuEPO and NM385. Pharmacokinetic data obtained after a single intravenous dose of 30 μg/kg in rats showed that NM340 had only a 18% and 14% increase in terminal half-life and clearance, respectively, compared with rHuEPO. Hence, extra negative charges on the peptide backbone of NM385 *per se* are unlikely to explain the observed effects on its pharmacokinetic parameters. Taken together, these results suggest that EPOR-mediated pathways play some role in clearance of ESAs. Furthermore, the findings described above also show that EPOR-independent pathways play a substantial role in the clearance of ERA as none of the techniques used to decrease the number of receptor with chemotherapy or to block the EPOR pathway with analogs without binding activity were able to shut down completely the elimination of EPO.

Several pathways involved in EPOR-independent clearance have been proposed, including liver and renal clearance. *In vivo* studies demonstrated that the kidney and liver exert negligible effect on EPO *in vivo* elimination [85, 120, 143]. Urinary excretion contributed only in a minor way to rHuEPO disposition with only approximately 5% of the administered dose excreted unchanged into the urine [96, 144]. Recently, the pharmacokinetics of subcutaneous darbepoetin alfa was compared in adults with normal renal function, with chronic renal insufficiency, or requiring dialysis, and in pediatric patients with chronic kidney disease requiring hemodialysis or peritoneal dialysis [145], and renal function did not markedly affect exposure based on the area

under the serum darbepoetin alfa-time curve, nor did it markedly affect the terminal half-life of darbepoetin alfa. These findings are consistent with data from rHuEPO and, therefore, suggest that renal excretion plays a minor role in elimination of ESAs and that altered renal function does not influence the rate of absorption.

Several studies have tested the hypothesis that EPO is cleared by an asialoglycoprotein receptor (ASGR) that is expressed in the liver. While the desialylated rHuEPO is cleared by hepatocytes by ASGR-mediated endocytosis in a matter of minutes [133, 146, 147], fully intact rHuEPO is cleared only after several hours. Clearance of rHuEPO by ASGR would require a two-step process, desialylation and subsequent clearance by ASGR in liver, but no evidence exists that desialylation of rHuEPO occurs [133, 147, 148]. In addition, hepatectomy experiments in sheep did not reveal reduced clearance of rHuEPO [148], and patients with liver disease showed no difference in rHuEPO clearance rates [149]. The rHuEPO redistributed from systemic circulation to lymph does not undergo significant clearance within the lymphatics [73].

It has been shown that carbohydrate side chains of EPO are necessary for persistence and *in vivo* biologic activity of the molecule, but not for *in vitro* receptor binding or stimulation of proliferation as carbohydrate can reduce the affinity for EPOR. Darbepoetin alfa is a hyperglycosylated analog of rHuEPO, with three- to five-fold lower affinity for the EPOR compared with rHuEPO [8, 9, 22, 150] but has three- to four-fold longer serum half-life and greater *in vivo* activity than rHuEPO. *In vitro* experiments have suggested that the lower affinity for EPOR of darbepoetin alfa relative to rHuEPO may contribute to its lower clearance [22]. To understand which clearance pathway was impacted by hyperglycosylation, the pharmacokinetics of EPO analogs with different EPOR binding activity and levels of glycosylation was examined. The analog NM294 has similar receptor binding activity to rHuEPO, but with the addition of one carbohydrate chain and, consequently, four additional negative charges. Similarly, the hyperglycosylated EPO analog, darbepoetin alfa, has eight extra negative charges due to the addition of two carbohydrate chains. If EPOR affinity and not glycosylation level was the key determinant of elimination, NM294 would be expected to have similar clearance to rHuEPO, while darbepoetin alfa should have lower clearance. However, after a single intravenous dose of 30 μg/kg in rats, NM294 and darbepoetin alfa had 51% and 62% lower clearance, respectively, than rHuEPO [140]. These findings suggest that hyperglycosylation impacts an EPOR-independent clearance pathway, and support the hypothesis that EPOR-mediated clearance may not play a dominant role on ESA elimination.

The attachment of polyethylene glycol chains to therapeutic proteins can improve the pharmacokinetic, pharmacodynamics, and immunologic properties of protein pharmaceuticals [151]. The reduced clearance of PEGylated molecules can be produced by altering the different clearance pathways, including reduced renal clearance, reduced clearance by the immune system, reduced proteolysis and/or steric hindrance to receptor-mediated clearance

alterations. It has been argued that steric hindrance resulting from the PEGylation may influence the binding affinity of the therapeutic protein to cellular receptors, resulting in changes in the bioactivity of the agent [152]. In addition, these pharmacokinetic and pharmacodynamic changes may translate to greater exposure of a drug, and therefore may allow either a decrease in the dose level or a decrease in the frequency of dosing, or both [153]. PEGylation of epoetin alfa (PEG-EPO) with succinimidyl propionate esters of 5 kDa PEG resulted in a lower *in vitro* receptor binding activity and a longer half-life *in vivo* [154, 155]. In other studies, PEGylation of rHuEPO and darbepoetin alfa resulted in a 58- and eight-fold lower receptor binding activity, respectively [22]. Jarsch et al. [156] confirmed that equilibrium dissociation constants for PEGylated epoetin beta and epoetin beta were 140 and 2.9 nM, respectively. In addition, the concentrations that inhibit 50% of the EPOR-receptor binding values were 200 and 1.5 nM for PEGylated epoetin beta and epoetin beta. Compared with epoetin beta, PEGylated epoetin beta activity at the receptor level is characterized by a slower association with and faster dissociation from EPOR, which evidence a 50- to 100-fold lower affinity for EPOR-binding sites and reduced specific activity *in vitro*. No data are available to quantify the role of the steric hindrance on the internalization rate of PEGylated epoetin beta after binding to the receptor.

Pharmacokinetic data obtained after a single intravenous dose of 30 μg/kg in rats showed that the PEGylation of rHuEPO, NM385, and darbepoetin alfa resulted in 91%, 93%, and 46% reduction in clearance [140]. The reduction in clearance and receptor-binding activity correlated with the molecular weight and total mass of the PEG that was attached. While rHuEPO and NM385 carried a 30 kDa PEG, darbepoetin alfa carried a 5–10 kDa PEG. The pharmacokinetic results are also consistent with those published by Mehvar et al. who showed that after intravenous administration in mice, the half-life of 50 kDa PEG was 987 min, substantially longer than that observed for 6 kDa PEG (18 min). These findings suggest that PEGylation affects EPOR-independent clearance pathway(s), and support the hypothesis that EPOR-mediated clearance may not play a dominant role on ESA elimination.

Taken together it appears that there are at least two ESA clearance pathways: a capacity-limited clearance pathway using EPOR-mediated endocytosis by erythroid progenitor cells or other cells expressing EPOR; and a linear clearance that is EPOR-independent, reflecting other mechanism(s) involved, although still not well understood. However, most of the previous analyses where rHuEPO saturable clearance was reported were based on limited datasets, often covering a narrow dose-range, which may have prohibited a clear understanding of the nonlinear nature of rHuEPO pharmacokinetics. In fact, Veng-Pedersen et al. [117] proposed that rHuEPO is cleared solely by nonlinear, saturable mechanism(s), while Kato et al. [120] reported both saturable and nonsaturable clearance components in rats, which was also observed in sheep [157], monkeys [77], infants [158], and healthy subjects [93]. A population pharmacokinetic meta-analysis of rHuEPO in 533 healthy

subjects using data from 16 clinical studies in which a wide range of intravenous and subcutaneous rHuEPO doses were administered have helped in defining the two separate elimination pathways and understanding the influence of demographic characteristics and other covariates on the pharmacokinetic parameters of rHuEPO [44]. At low concentrations, including the endogenous concentrations observed at baseline or in ESA-untreated states, the nonlinear clearance operates at full capacity, giving a total clearance of about 0.9 L/h. As concentrations increase, the nonlinearity of pharmacokinetics becomes more important and, at the highest doses, the nonlinear clearance of rHuEPO was nearly fully saturated, so that total clearance decreased to almost one third, being mainly represented by the linear component estimated to be 0.3 L/h. At these doses or concentrations, rHuEPO pharmacokinetics is approximately dose linear. The ratio of nonlinear (saturable) and linear clearance mechanisms is similar to what has been published in humans [137], but appears to vary between species [120], making comparisons between studies in different species difficult.

The dose-dependent clearance appears to be independent of the type of rHuEPO (epoetin alfa *versus* epoetin beta) or population (healthy subjects or patients with chronic renal failure). Therefore, the pharmacokinetic models developed on the basis of healthy volunteers data may be applied in patients with anemia due renal insufficiency, but it may have limited predictive value when applied to patients receiving chemotherapy. The possible involvement of EPOR in the elimination of rHuEPO could have important consequences for the pharmacokinetics of rHuEPO in this setting as the effect of chemotherapy on progenitor cells would reduce the number of EPOR available to clear. As a consequence, the rHuEPO clearance might be decreased and the rHuEPO elimination process would become slower than the absorption process. Therefore, the flip-flop phenomena observed in healthy subjects might disappear, leading to a completely different rHuEPO concentration time profile [44]. To explore this hypothesis fully, a more mechanistic model considering the complex interaction between the erythropoietic system, EPOR turnover, ESA pharmacokinetics and pharmacodynamics and the effect of chemotherapeutic agents would be necessary. However, the nonlinear disposition of rHuEPO has been mainly characterized with the most common model in dealing with saturation kinetics, Michaelis-Menten model, with a possible linear elimination pathway. Although such a model has been successfully applied to describe the nonlinear pharmacokinetic of rHuEPO in various species, it does not well represent underlying molecular events such as receptor binding, internalization, and degradation of rHuEPO. A general model for drugs exhibiting target-mediated drug disposition (TMDD) has been previously introduced [159]. It uses receptor binding and receptor-mediated endocytosis as a primary mechanism of nonlinear drug disposition. Furthermore, if it is assumed that free drug, free receptor, and drug-receptor complex are at quasi- or rapid equilibrium, the TMDD model can be simplified to its quasi-equilibrium form removing the need to estimate drug-binding

constants such as k_{on} and k_{off} [160]. This approach has been suggested and applied to rat, monkey and human data [161]. The target mediated drug disposition model and the Michaelis-Menten model become identical when

$$\frac{R_{tot} \cdot k_D}{(k_D + C)^2} <<< 1 ,$$

where R_{tot}, k_D and C are the total number of drug receptors, the receptor affinity and the drug concentration, respectively [162].

A further indication of the possible involvement of receptor binding in the disposition of rHuEPO can be found when studying the intravenous data at lower doses and the pharmacokinetics after multiple dosing. In the first case, a correction factor was needed to explain about a 60% reduction in rHuEPO exposure for doses <20 IU/kg [44]. As the first measurements of rHuEPO concentration were usually obtained 30 min following dose administration, it may not have been possible to observe the presence of any early rapid distribution. Such distribution could be related to rapid binding, possibly to the EPOR. At higher doses, such binding could be saturated, leading to higher serum concentrations.

Some studies have reported a time-dependent clearance. For example, McMahon et al. [66] compared the single- and multiple-dose pharmacokinetics of epoetin alfa at 150 and 300 IU/kg intravenous and found that, at both dose levels, the mean clearance increased by 25% after five doses. Sans et al. found a 57–90% decrease in AUC and a 47–76% decrease in C_{max} after three subcutaneous doses of rHuEPO beta at 30, 60, or 100 IU/kg [65]. Similarly, most studies in nephrology patients have found that clearance increases and/or terminal half-life decreases after multiple dosing [107, 109, 163, 164]. Additionally, some investigators have reported that after the initial change (i.e., after 2–3 weeks), no subsequent changes occurred [163]. Conversely, other investigators reported no major change in pharmacokinetic properties from single-dose values, either after reaching a new steady-state hemoglobin value [165] or after 56 days [166]. Jensen et al. [64] found no differences in the pharmacokinetic properties of epoetin beta after long-term treatment except for a reduction in the volume of distribution. Nevertheless, upon multiple dosing, most data suggest that after several weeks of treatment, rHuEPO clearance increases about 10–30%, with an accompanying decrease in terminal half-life [61, 90]. It has been demonstrated that the PEGylated epoetin beta clearance in patients diagnosed with chronic kidney disease (0.8 L/day) increased 10–15% after 2 months of treatment [167]. These findings are consistent with a target mediated drug disposition [159] and the involvement of the EPOR-mediated pathway in the elimination of rHuEPO. As a consequence, once rHuEPO treatment starts, erythropoiesis is stimulated, the number of precursor cells is increased and more EPOR would be available to eliminate rHuEPO, which might result in a clearance increase and/or volume of distribution decrease, leading to a reduction in terminal half-life.

Summary

We have reviewed the clinical pharmacokinetics of protein-based ESA, including epoetin alfa, epoetin beta, PEGylated epoetin beta, and darbepoetin alfa. After subcutaneous administration, ESA enter into the systemic circulation through the blood capillaries or the lymphatic system. Absolute subcutaneous bioavailability is incomplete and about 40–70% for the dosing regimens used in the clinic. In addition, the rate of absorption is slower than the rate of elimination, the observed half-life after subcutaneous administration reflects the absorption rather than elimination rate. As expected from its large molecular weight, ESA's volume of distribution was similar to the plasma volume (40–60 mL/kg), suggesting confinement of EPO within the plasma circulation. Despite the long clinical experience with ESAs, the mechanism(s) of their clearance have not been fully elucidated and there is a paucity of information regarding which organ(s) and tissue(s) are important in the metabolism and elimination of these drugs. Clearly our understanding of the nature of the clearance pathways is incomplete and predicting their impact on clinical use of ESAs is unclear.

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2. *Heatherington AC. Clinical pharmacokinetic properties of rHuEPO: a review. In: Molineux G, Foote MA, Elliott S, eds., Erythropoietins and Erythropoiesis: Molecular, Cellular, Preclinical, and Clinical Biology. Birkhauser, Basel, Switzerland, 2003; pp. 87–112.*
3. *Doshi S, Perez-Ruixo JJ, Jang GR, Chow A, Elliot S. Pharmacocinétique de les agents stimulant l'érythropoïèse. In: Rossert J, Casadevall N, Gisselbrecht C, eds., Les agents stimulant l'érythropoïèse. Paris, France, 2008.*

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Use of erythropoietic stimulating agents in the setting of renal disease

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Introduction

As kidneys fail, their capacity to produce erythropoietin (EPO) typically diminishes. This deficiency of EPO is the primary etiology of the progressive anemia of chronic kidney disease (CKD). Hemoglobin concentration decreases in association with the increase in blood urea concentration (Fig. 1) and the decline in creatinine clearance (Fig. 2) of progressive renal failure [1]. The anemic state is exacerbated by a shortened red blood cell lifespan and bone marrow resistance to EPO in the setting of advanced uremia. Bleeding time is prolonged, as well, and gastrointestinal blood loss is not uncommon. In addition, dialytic therapy may consume red cells, due to repeated diagnostic phlebotomy, hemolysis caused by the hemodialysis pump, bleeding associated with hemodialysis needle insertion and removal, recurrent anticoagulation, and incomplete return of blood from the hemodialysis filter and bloodlines at

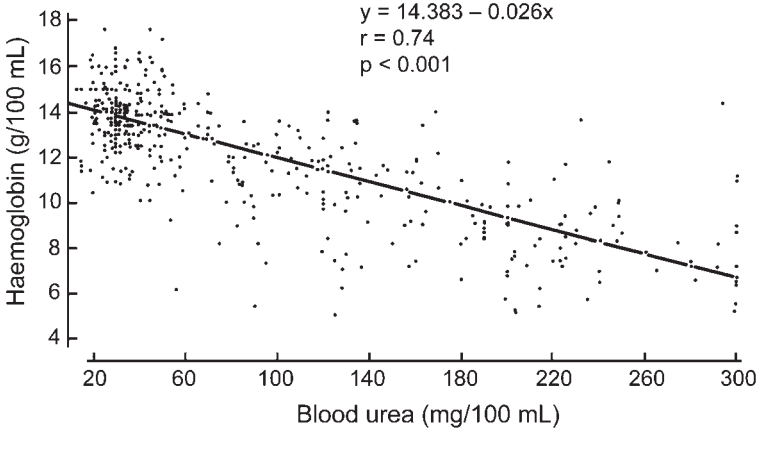

Figure 1. Relationship between blood urea concentration and hemoglobin concentration. Four hundred patients with CKD not yet requiring dialysis, 1977–1979. (From [1] with permission.)

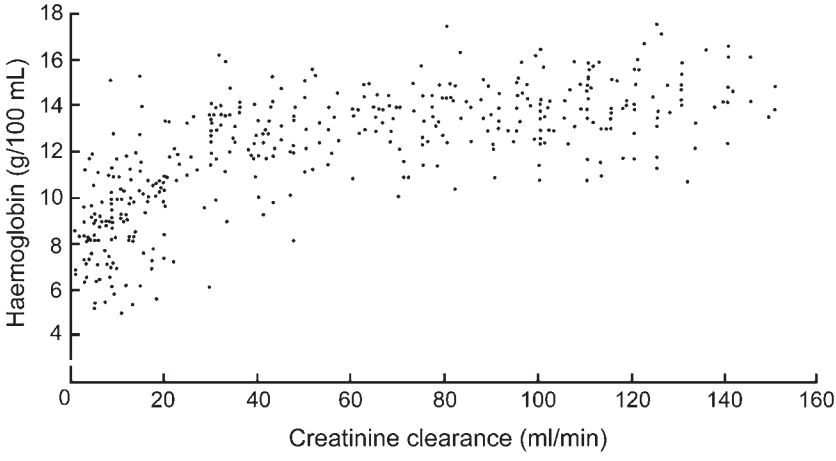

Figure 2. Relationship between creatinine clearance and hemoglobin concentration. Four hundred patients with CKD not yet requiring dialysis, 1977–1979. (From [1] with permission.)

the end of a treatment. Inflammatory cytokines and nutritional deficiencies may worsen the anemia of CKD.

If one visited a hemodialysis facility in the 1970s or 1980s, on any given day it was usual to see bags of blood suspended at multiple patient stations, with transfusions in progress. Many dialysis patients received 1–3 units of red blood cells per month [2], although some individuals had considerably higher transfusion requirements. Despite recurrent transfusions for approximately 25% of the patients, hematocrit values often oscillated within ranges <25%, causing persistent fatigue, limited capacity for exertion, and numerous other symptoms. In 1989, the first recombinant human erythropoietins (rHuEPO) were approved for commercial use. Most patients undergoing chronic dialysis treatments in developed nations and many patients with earlier stages of CKD now receive erythropoiesis-stimulating agent (ESA) therapy. Consequently, there has been a dramatic reduction in the rate of red blood cell transfusions and hematocrit values are considerably higher. In fact, dialysis professionals of more recent vintage have only seen blood transfusion as an occasionally-needed intervention for dialysis patients and not the common intradialytic process that it was in decades past.

Benefits of ESA treatment of anemia in patients with CKD

Reduction of red blood cell transfusions

A number of risks are associated with the transfusion of blood. Concern about the potential for transmission of viral infections such as hepatitis B, hepatitis C, and HIV was particularly worrisome for hemodialysis patients with chron-

ic transfusion burdens before the ESA era. Improved screening techniques have greatly reduced the risk of transfusion-transmitted HIV-1 and hepatitis C virus to approximately 1 in 2×10^6 blood units from repeated donors in the United States [3]. Of course, most individuals prefer to avoid transfusion whenever feasible, and the possibility of transmitting other unscreened infectious diseases remains (e.g., bacteremia, Creutzfeld-Jakob disease, Chagas' disease, other hepatitis viruses). Recurrent red blood cell transfusions were historically associated with iron overload, sometimes severe and requiring chelation therapy. The incidence of cytotoxic antibody formation was increased by blood transfusion and this complication limited the prospects for kidney transplantation for some patients. Table 1 shows the estimated risks of various adverse transfusion reactions, as reported in 2005 [4].

Transfusion-related acute lung injury (TRALI) has emerged as the leading cause of death due to transfusion, and its incidence may be as high as between 1 in 5,000 to 1 in 1,323 transfusions [5]. Patients with TRALI develop tachypnea, dyspnea, cyanosis, temperature elevation, cyanosis, pulmonary infiltrates on radiographic exam, and acute hypoxemia with normal central venous pressure, in a clinical constellation that appears similar to acute respiratory distress syndrome. In addition, an immunosuppressive effect referred to as transfusion-related immune modulation (TRIM) is believed to result from a decrease in cell-mediated immunity after transfusion [4].

The most striking benefit of ESA therapy in the setting of renal failure has been the reduction in need for red blood cell transfusions. In the pivotal US Phase III trial of epoetin alfa, for example, erythrocyte transfusions were elim-

Table 1. Risk of adverse transfusion events. (From [4] with friendly permission by Wiley-Blackwell Publishing.)

Serious	Estimated risk
Mistransfusion	1:14,000 to 1:19,000
ABO-incompatible transfusion	1:38,000
Death due to ABO-incompatible transfusion	1:1.8 million
Acute hemolytic transfusion reaction	1:12,000
Delayed hemolytic transfusion reaction	1:4000 to 1:12,000
Transfusion-related acute lung injury	1:20,000 to 1:5000 (5% to 10% fatal)
Anaphylaxis	1:20,000 to 1:47,000
	1:150,000
	1:1600 (platelets)
	1:23,000 (erythrocytes)
Graft-*versus*-host disease	1:1 million (Canada)
Post-transfusion purpura	1:143,000 to 1:294,000
Fluid overload	1:708 to 1:3200
	1:7000 to 1:15,000
Less serious	
Febrile nonhemolytic transfusion reaction	1:500
Allergic (urticaria)	1:250

inated within 2 months of the onset of therapy among the 333 enrollees, who had required a total of 1,030 transfusions during the 6 months before treatment [6]. In clinical practice, the need for transfusion has not been eliminated, but it has been markedly reduced. In 2005, the incidence of transfusion for dialysis patients in the US had decreased to 31 units per 100 patient-years, from about 150 units per 100 patient-years in 1989 before the introduction of epoetin alfa [7]. The cost of transfusion of a single unit of packed red blood cells in an outpatient setting, including laboratory and personnel costs, has been cited as US$469 [8]. In the year before the availability of ESA therapy, more than 600,000 blood transfusions were estimated to have been given to dialysis patients in the United States [9]. The dialysis population has grown since then. ESA therapy thus relieves a serious burden on the blood-banking system and the reduction in transfusions offsets at least a small portion of the considerable cost of ESA medications.

Relief from anemic symptoms and complications

Patients with CKD experience many manifestations of anemia (Tab. 2). A number of the symptoms listed historically had been attributed to uremia. For this reason, the extent of symptomatic relief attained after ESA treatment of anemia came as a surprise to investigators early in the development of epoetin therapy.

Table 2. Manifestations of anemia

- Fatigue
- Weakness
- Malaise, depression
- Dyspnea
- Exertional chest pain
- Cardiac enlargement
- Impaired concentration/cognition
- Impaired sleep
- Impaired libido, erectile dysfunctional
- Impaired immune system
- Bleeding tendency
- Amenorrhea
- Anorexia
- Headache
- Intolerance to cold
- Pale skin

Cognitive function

Beneficial effects on brain function among hemodialysis patients after ESA correction of anemia have been demonstrated by both EEG waveform

improvement and neuropsychiatric testing (attention, scanning, learning, memory functions) [10]. Cognitive function, including IQ scores, has been shown to improve after rHuEPO therapy in the setting of chronic ambulatory peritoneal dialysis [11].

Cardiac morphology and function

Left ventricular hypertrophy is common among CKD patients and is associated with increased mortality [12, 13]. Multiple studies of CKD patients have shown that increasing hematocrit values from ≤25% to 29–35% leads to marked reduction (11–35%) in left ventricular mass [14–18]. Study of patients on hemodialysis with asymptomatic cardiomyopathy and hemoglobin concentrations between 9–11 g/dL at entry, however, found that further increasing hemoglobin concentrations to the normal range with epoetin alfa (patients were randomly assigned to treatment to attain a hemoglobin concentration of 13.5 g/dL *versus* a control hemoglobin concentration of 10 g/dL) did not lead to further regression of left ventricular hypertrophy or dilation [19]. It may have prevented the development of *de novo* dilation.

The increase in oxygen delivery that accompanies correction of anemia improves exercise performance on treadmill testing and significantly reduces ST segment depression at maximal exercise, among hemodialysis patients with significant coronary artery disease [20–22]. Another cardiac benefit of ESA treatment is reduction of the chronically increased cardiac output associated with renal anemia [23], which has also been demonstrated after transfusional treatment [24].

It is important to note, however, that these cardiac effects have not been proven to result in improved patient survival. In fact, full correction of hemoglobin/hematocrit values to the normal range has resulted in increased mortality in two large prospective trials which are discussed in later sections.

Activity

Progressive renal failure leads to worsening exercise capabilities, likely due to several causes including uremic myopathy, disuse, and cardiovascular disease. Oxygen delivery is critical to enable activity and ESA therapy of anemia improves this capacity. Peak oxygen consumption ($\dot{V}O_2$ peak), usually measured at maximal exertion on a treadmill or stationary bicycle in studies of dialysis patients, is a surrogate marker for the cardiorespiratory capability to perform various tasks. As shown in Figure 3, $\dot{V}O_2$ peak, and hence exercise capacity, is far below normal in markedly anemic hemodialysis patients [25]. Each triangle in the figure represents average values from a separate study of dialysis patients. Increasing hematocrit to levels between 30–35% is associated with increases in $\dot{V}O_2$ peak of approximately 20–25%. Such increases enable

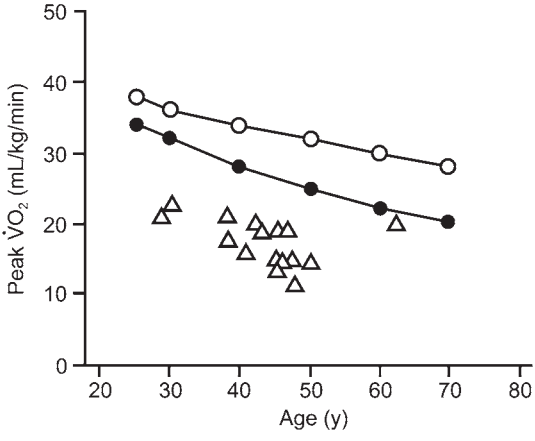

Figure 3. Average peak exercise capacity of patients receiving hemodialysis (open triangles) compared with expected levels for healthy sedentary men (open circles) and women (black circles). $\dot{V}O_2$ = oxygen consumption (From [25] with permission.)

more activities of daily living. For example, sedentary office work typically requires 5.7 mL O_2/kg/min, walking (at 4.9 km/h) requires 11.8 mL O_2/kg/min, and cooking requires 6.7–13.7 mL O_2/kg/min [26]. In contrast, vigorous activities such as playing soccer require >23 mL O_2/kg/min and are untenable for most anemic dialysis patients. Typically, a person is only able to sustain activities that consume oxygen at rates half of one's $\dot{V}O_2$ peak. For anemic dialysis patients, activities such as cooking require 40–50% of $\dot{V}O_2$ peak (moderate work), whereas walking slowly represents heavy work. $\dot{V}O_2$ peak values attained with partial correction of anemia may approach the lower limit of the normal range and such improvements ease functional limitations. In a study of eight patients, epoetin alfa therapy was used to increase hematocrit from the 18-to-24% range up to the 30-to-36% range, with significant improvements in $\dot{V}O_2$ peak and maximum aerobic exercise work capacity (watts) [27]. A larger study (n = 118) found that treatment of anemia significantly improved distance walked during stress testing, though not during a 6-min walk test [28].

Quality of life

Significant improvements were demonstrated in most quality-of-life parameters after 10 months of epoetin alfa treatment, resulting in an average hematocrit of 34%, during the open-label, pivotal, US Phase III study [29]. These measures included energy, activity level, functional ability, sleep, eating, satisfaction with health, sex life, well-being, psychological affect, life satisfaction, and happiness. A second open-label study included 484 dialysis patients starting epoetin alfa treatment [30]. Scores on the Medical Outcomes Study 36-item Short Form

Health Survey (SF-36) were compared between study entry and approximately 100 days later. Significant improvements were observed in vitality, physical functioning, social functioning, mental health, looking after the home, social life, hobbies, and satisfaction with sexual activity. The magnitudes of quality-of-life benefits were deemed clinically meaningful compared with studies of other disease states, although scores after treatment remained below the normal range for healthy subjects. A control group of patients, already receiving epoetin alfa at enrollment and whose mean hematocrit did not increase, exhibited no changes in SF-36 scores. Interestingly, regression analysis suggested that differences in hematocrit level explained only part of the change scores and the authors speculated that there could be an additional pharmacologic effect of treatment. It should be noted that the unblinded, uncontrolled nature of many of the quality-of-life studies of epoetin therapy is a less than ideal study design and the instruments used have not always been validated and rigorous.

In contrast, a double-blind prospective trial randomized patients to three groups: placebo *versus* treatment to a hemoglobin concentration of 9.5–11.0 g/dL *versus* treatment to 11.5–13.0 g/dL. This study demonstrated improvement in scores for fatigue, physical symptoms, relationships, and depression when the patients on epoetin were compared with those on placebo, though there was no significant difference in the improvement between the two epoetin doses [28]. Multiple aspects of sleep quality improve after ESA treatment [31]. In addition, epoetin therapy has been reported to decrease plasma histamine concentration and to relieve pruritus among hemodialysis patients [32].

Sexual and endocrine function

Sexual function may improve after ESA therapy due to an increase in energy, an increase in low testosterone concentration [33], and/or a decrease in elevated prolactin concentrations [34]. The latter effect has been reported to restore menses in female hemodialysis patients [35]. Other investigators have reported a lack of change in serum testosterone or prolactin concentrations, however [36, 37]. Additional reported hormonal changes include increased free T3 and free T4 [35], as well as ACTH and FSH [38].

Immune function

Patients with CKD are immunosuppressed and predisposed to infection. Partial correction of anemia repairs leukocyte respiratory burst activity, which is a critical component of phagocytosis [39], improves cell-mediated immunity [40], benefits humoral immunity/vaccine responses [41, 42], and increases expression of complement receptor 1 (CR1) on the red blood cell, which should aid bacterial clearance [43]. Normalization of hemoglobin concentra-

tion in hemodialysis patients with epoetin alfa further boosts cutaneous reactivity, increases CD8 cell counts, increases erythrocyte CR1 levels, and decreases anergy, compared with partial correction of anemia [44]. Also, decreased transfusion exposure enabled by ESA therapy may lead to diminished panel reactive antibodies against foreign HLA antigens and thereby improve a patient's candidacy for renal transplantation [45].

ESA benefits not mediated by an increase in red blood cell mass

Erythropoietin receptors (EPOR) have been reported on numerous cell types other than red blood cell precursors, including neurons, endothelial cells, retina, and cardiac myocytes. It should be briefly noted that there is exciting ongoing research into the potential direct benefits of EPO, including protection against ischemia/reperfusion injury (stroke, myocardial infarction), spinal cord injury, neuropathy (including diabetic neuropathy), and cognition [46, 47]. Although the EPO glycoprotein is >30 kDa, it has been shown to gain entry into the central nervous system. As described, quality-of-life benefits associated with ESA treatment of renal anemia have not been fully reconciled statistically to increases in hematocrit; the explanation may be that there are direct central nervous system benefits of EPO, too. It is possible that EPO may have direct detrimental effects, for example on diabetic retinopathy [48] or by causing vasoconstriction and hypertension at very high concentrations.

Adverse effects of ESA therapy in CKD

Hypertension

Blood pressure elevation accompanies the initiation of ESA therapy in as many as 35% of patients [6, 49]. Several mechanisms could theoretically underlie this tendency to hypertension and have been discussed in the literature: loss of the vasodilation induced by poor oxygen delivery to tissues, increased blood volume, increased blood viscosity, or direct epoetin-induced vasoconstriction. Increased cardiac output associated with anemia fails to decline appropriately after treatment in some patients who develop hypertension [50]. Markedly increasing epoetin dose to increase hemoglobin concentration from partial correction to normal did not have notable effects on blood pressure in two trials [51, 52], although there were significantly more reports of hypertension (30%) in the normal-hemoglobin group than in the anemic group (20%) in a third trial [53]. In clinical practice, attention to appropriate dialytic fluid removal, sodium and fluid intake, and adjustment of medication dosage has readily enabled management of blood pressure on ESA. Instances of malignant hypertension and seizures were reported during early trials, but these complications have occurred very rarely in later trials or general practice.

Thrombosis

Epoetin therapy affects hemostatic function. Many studies have been done, unfortunately often yielding conflicting results. One thorough literature review concludes that on balance the data indicate that therapy: 1) returns the prolonged bleeding time of CKD toward normal due to improved platelet aggregation and increased hematocrit (red cells are believed to displace platelets toward vessel walls); 2) transiently increases the number of circulating platelets, within the normal range; and 3) has minimal effects on the coagulation cascade (e.g., prothrombin time and partial activated thromboplastin time unchanged despite a variety of alterations in clotting proteins) [54]. While the decrease in bleeding time might beneficially diminish gastrointestinal or other bleeding, increased hemostatic activity could be detrimental to patients in other ways. Cardiovascular disease is the most common cause of death among patients with CKD and worsening of coronary or cerebral thrombosis would clearly be undesirable. For hemodialysis patients, clotting of the hemoaccess is a distressing, and expensive, complication. Whether or not ESA therapy increases access thrombosis after partial correction of anemia has been debated [55, 56], but a large, prospective study of normal *versus* low hematocrit values among hemodialysis patients with cardiac disease clearly demonstrated increased thrombosis rates of both native arteriovenous fistulae and synthetic grafts among patients randomized to the higher hematocrit target [51].

Dialytic clearance

Increases in red cell volume accompanied by decreased plasma volume theoretically could diminish the efficiency of solute removal during dialysis. Statistically significant, but small, increases in serum creatinine, phosphorus, and potassium concentrations were noted in a Phase III trial of epoetin alfa [6]. In general practice, hyperkalemia or worsening azotemia have not been issues and dialysis clearances are not significantly altered [57]. Regular attention to chemistry values, Kt/V results, and dialysis prescription have proven sufficient to avoid underdialysis during ESA therapy.

Pure red cell aplasia

A very rare, but serious, complication of treating anemia with recombinant proteins is pure red cell aplasia (PRCA). PRCA arises when patients develop antibodies to an ESA that also neutralize endogenous EPO. A severe, transfusion-requiring anemia results. The ESA must be discontinued and immunosuppressive therapy has been recommended [58]. PRCA has complicated therapy with epoetin alfa, epoetin beta, and darbepoetin alfa. There was a surge of cases in Europe between 1998 and 2002, associated with subcutaneous admin-

istration of epoetin alfa prepared by a single manufacturer, after a change in formulation. It is possible that rubber leachates from syringes acted as immune adjuvants [58]. A novel peptide currently in late stage development, Hematide, activates EPOR, but has no structural homology with EPO. Hematide rescued 10 patients with CKD (predialysis, hemodialysis, and peritoneal dialysis) who had PRCA [59].

Tumor progression

Some tumors have EPOR. ESA labeling information includes warnings that ESA therapy of anemia targeting hemoglobin concentrations above 12 g/dL has resulted in shorter times to tumor progression or death in the setting of several malignancies including metastatic breast, head and neck, nonsmall-cell lung, lymphoid, and cervical cancers.

Residual renal function

Animal studies and preliminary human trials raised uncertainty as to whether ESA therapy of anemia in CKD patients might slow the progression to end-stage renal disease (improved oxygen delivery to the kidney) or hasten deterioration of renal function (thrombosis, vasoconstriction). Larger clinical trials have not shown significant changes in the rate of CKD progression consequent to standard ESA therapy [60, 61].

Target hemoglobin/hematocrit

The initial target hemoglobin/hematocrit ranges for ESA therapy were selected empirically. Trials of epoetin alfa in the United States aimed for hematocrit values of 30–35% for several reasons: untreated hematocrit values often approximated 24% and it was felt that an increase in hematocrit roughly equivalent to a two-unit transfusion of red blood cells (i.e., an increase of 6 points to 30%) was a minimal desired benefit; there was an impression based on subjective patient responses that a hematocrit of 30% would ultimately be an appropriate point to start treatment during the course of CKD to avoid symptoms [62], and enthusiasm to correct anemia more aggressively was tempered by early cases of hypertension and seizures with rapid rises in hematocrit. Consultant hematologists contributing to study designs actually favored normal-hematocrit targets [63]. The original US product package insert for epoetin alfa specified a target range of 30–36%. Over the ensuing 19 years, some prospective trials have revealed deleterious outcomes at higher hematocrit values. Whether fortuitous or prescient, the original guidance regarding the maximal advisable hematocrit now appears appropriate.

Oxygen-carrying capacity of the blood increases linearly as the hematocrit increases. Blood viscosity, however, increases exponentially, so that a point of diminishing returns is reached for oxygen delivery (Fig. 4) [64]. These considerations suggest that a hematocrit of approximately 40% might be optimal, but they are based on viscosity measurements within glass capillary tubes, not within the dynamic environment of living blood vessels. A study of 27 patients with ischemic cerebrovascular disease indicated that oxygen delivery to the brain reaches a maximum at a hematocrit range of 40–45% [65], also suggesting that normalization of hematocrit might prove beneficial. Furthermore, observational epidemiologic studies of large databases found that higher hemoglobin/hematocrit values were associated with better survival rates among dialysis patients [66, 67] and small interventional studies of hematocrit normalization reported functional benefits and safety [31, 68–70].

Thus, it was an unexpected result of substantial import when the Normal Hematocrit Cardiac Trial (NHCT) demonstrated adverse outcomes among hemodialysis patients with congestive heart failure or ischemic heart disease targeted to normal hematocrit values (42%), compared with partial correction (hematocrit 30%) [51]. There were 183 deaths and 19 first nonfatal myocardial infarctions among 618 patients in the normal-hematocrit group *versus* 150 deaths and 14 myocardial infarctions among 615 patients in the low-hemat-

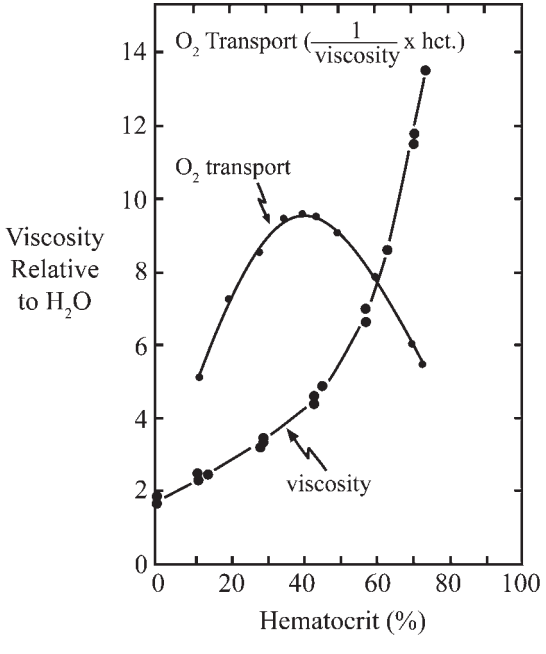

Figure 4. Effects of increasing hematocrit values on blood viscosity and oxygen delivery *in vitro*. Oxygen transport as calculated from the oxygen-carrying capacity of blood (hemoglobin concentration) and blood flow (reciprocal of viscosity). (From [64] with permission.)

ocrit group. The study was halted at the recommendation of an independent data monitoring committee after an interim analysis. The primary endpoint (time to death or myocardial infarction) results did not reach the prespecified stopping rule and were not statistically significant when adjusted for previous interim analyses [71], although the nominal P-value by log-rank test was 0.01. The latter statistic was included in the original study manuscript submitted (and was reported to the Food and Drug Administration [FDA]), but it was removed before publication after editorial input from *The New England Journal of Medicine* staff, which prompted an additional independent expert biostatistician review. The causes of death were similar between groups and death was most often due to cardiac disease. Vascular access thrombosis was more common in the normal group (39%) than in the low group (29%; $P = 0.001$). Significantly fewer patients required blood transfusion in the normal group (21%) than in the low group (31%; $P < 0.001$). Quality-of-life scores did not differ between the groups, though significantly better physical function scores were associated with higher hematocrit values. The mechanism underlying the increased mortality remains unclear. Mortality was not associated with dose of epoetin alfa and within treatment groups higher hematocrit values were not associated with higher mortality. Patients in the normal-hematocrit group received significantly greater amounts of intravenous iron, and among these patients, iron administration was associated with higher mortality, in *post hoc* analysis. The publication concluded that it is not recommended to target a hematocrit of 42% among hemodialysis patients with congestive heart failure or ischemic heart disease.

A subsequent study of 416 Scandinavian patients (predialysis, hemodialysis, and peritoneal dialysis) examined a normal-hemoglobin target range of 13.5–16.0 g/dL *versus* partial correction to 9.0–12.0 g/dL, using epoetin alfa [72]. Quality-of-life testing was administered to 253 dialysis patients. Significantly superior quality-of-life scores were reported for the normal-hemoglobin group, in physical symptoms, fatigue, depression, and frustration. Safety outcomes, including thrombotic events, were comparable between the groups.

An analysis of data from 2,858 US dialysis units (899,000 patient-years) between 1999 and 2002 found significantly lower mortality among facilities with greater percentages of patients with a hematocrit of at least 33%, after adjusting for case mix and patient characteristics [73].

Eight years after the publication of the NHCT results, two large interventional studies (CREATE [53] and CHOIR [52]) reported findings for normal *versus* lower hemoglobin concentrations among patients with CKD who did not require dialysis. The CREATE trial of 603 patients found no significant difference in cardiovascular outcomes between patients randomized to a hemoglobin target range of 10.5–11.5 g/dL and those targeted to 13.0–15.0 g/dL, prescribing epoetin beta. SF-36 quality-of-life scores were significantly better for the normal-hemoglobin group with regard to general health, mental health, physical function, physical role, social function, and vitality. No difference was seen between groups in calculated glomerular fil-

tration rate (GFR), although time to initiation of dialysis was statistically significantly longer for the lower hemoglobin group (P = 0.03). In the normal-hemoglobin group, 52% of patients received intravenous iron, compared with 42% of those in the low-hemoglobin group (P-value not provided). The CHOIR trial randomly assigned 1,432 patients to either a hemoglobin target of 13.5 g/dL or 11.3 g/dL, prescribing epoetin alfa. The primary endpoint, a composite of cardiovascular events (death, myocardial infarction, congestive heart failure requiring hospitalization, and stroke), occurred more often among the normal-hemoglobin group than among the low-hemoglobin group (125 and 97 events, respectively; P = 0.03). Fewer than 3% of patients received intravenous iron. The groups did not differ in their quality-of-life scores or in progression to renal replacement therapy. The authors state that the study did not provide a mechanistic explanation for the poorer cardiovascular outcome with the use of the higher target hemoglobin concentration.

Currently, the labeling approved by regulatory authorities for each of the ESAs states that maintenance hemoglobin concentration should be 10–12 g/dL. The most recent anemia practice guidelines from the Kidney Disease Outcomes Quality Initiative (KDOQI) recommend a target range of 11–12 g/dL [74]. The European Best Practice Guidelines (EBPG), issued before the recent large studies of normal hemoglobin concentration, recommend a target of >11 g/dL, not to exceed 14 g/dL, with the exact concentration being guided by individual patient characteristics (sex, age, ethnicity, activity, and comorbid conditions) [75].

The Dialysis Outcomes and Practice Patterns Study (DOPPS) is a prospective, observational study [76], which tracks representative random samples of hemodialysis patients from 12 nations and collects extensive information on prescriptions, comorbid conditions, and laboratory data. Table 3 shows the average hemoglobin concentrations by country among prevalent patients at

Table 3. Average hemoglobin concentration, by nation

Country	No. patients	Mean Hgb ± SD (g/dL)
Australia/New Zealand	489	11.82 ± 1.53
Belgium	481	11.86 ± 1.33
Canada	511	11.76 ± 1.32
France	552	11.73 ± 1.43
Germany	553	11.65 ± 1.38
Italy	498	11.50 ± 1.46
Japan	1,803	10.44 ± 1.24
Spain	550	11.95 ± 1.50
Sweden	524	11.86 ± 1.37
United Kingdom	358	11.61 ± 1.53
United States	1,733	11.99 ± 1.36

Prevalent cross-section of hemodialysis patients (n = 8,052) at entry into the Dialysis Outcomes and Practice Patterns Study, 2005–2007. Hgb = hemoglobin concentration; SD = standard deviation.

study entry between 2005 and 2007 (unpublished data). Consistent with the guidance, the average values are between 11 and 12 g/dL, with the exception of the mean hemoglobin value of 10.44 g/dL in Japan, where nephrologists seek lower targets.

ESA prescription

As shown in Figure 5, epoetin alfa, epoetin beta, or darbepoetin alfa were prescribed to more than 90% of hemodialysis patients in North America, Europe, and Japan between 2005 and 2007 (DOPPS data, unpublished). The proportion of patients receiving each of the three products varies widely by nation. The reader is referred to the respective product labeling instructions, plus the KDOQI [77] and EBPG [75] guidelines, for details of dosage recommendations for the specific agents. Briefly, when initiating epoetin alfa therapy for an anemic patient, the recommended starting dose is 50–100 U/kg intravenously or subcutaneously thrice weekly; for epoetin beta, 20 U/kg subcutaneously thrice weekly or 40 U/kg intravenously thrice weekly; and for darbepoetin alfa, 0.45 μg/kg intravenously or subcutaneously once weekly. Dose should be titrated to avoid increases in hemoglobin concentration >1 g/dL during a 2-week period. If the hemoglobin concentration increases too quickly, it is generally preferable to reduce dose, perhaps 25%, rather than to withhold dosing, to avoid repeated cycles of undershooting and overshooting the desired hemoglobin range.

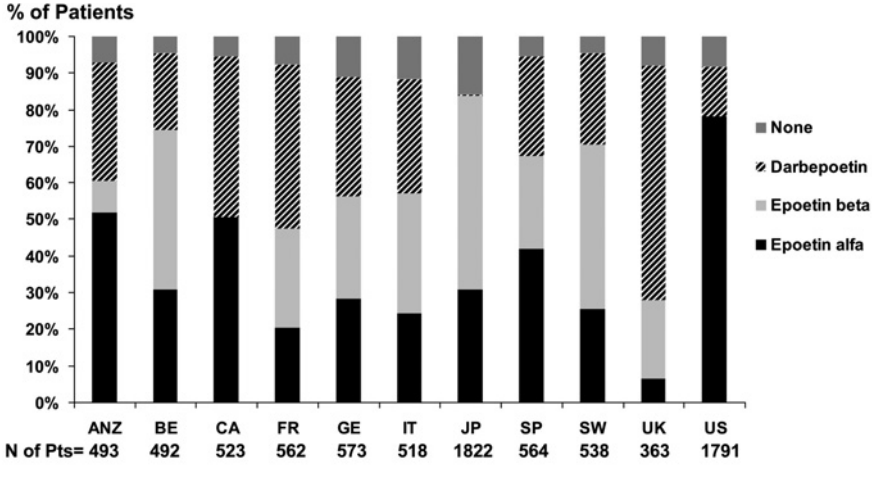

Figure 5. Distribution of ESA usage by nation. ESA prescriptions for prevalent hemodialysis patients at entry into the Dialysis Outcomes and Practice Patterns Study, 2005 to 2007 (n = 8,239). Abbreviations correspond to the nations listed in Table 3.

Route of administration

Despite lower bioavailability, subcutaneous administration of epoetin alfa allows lower doses than does intravenous dosing. There may be a threshold circulating drug concentration necessary to stimulate erythropoiesis. Drug concentration might decrease below this minimum level for a greater proportion of the week with bolus intravenous administrations than with the continued absorption that follows subcutaneous injections [78]. Another consideration is that lower nadir concentrations associated with intermittent intravenous administration might result in neocytolysis, a phenomenon wherein very low EPO concentration triggers reticuloendothelial hemolysis of young circulating red cells [79]. Two sizable, prospective trials comparing epoetin alfa dose requirements between the two routes of administration among hemodialysis patients both found that after 21 to 26 weeks the average dose is 27% lower with subcutaneous administration [80, 81].

ESAs are painless when administered intravenously during hemodialysis, whereas subcutaneous injection causes variable patient discomfort. The primary motivation for prescribing ESAs to hemodialysis patients subcutaneously is economics, as ESA therapy is expensive. (Paradoxically, in the US there are reimbursement incentives to dialysis centers that reward higher ESA consumption, and prescription via the intravenous route predominates there.) However, notwithstanding the average reduction in dose when a group of patients is converted from the intravenous to the subcutaneous route of administration, as many as 39% of individuals actually require the same or more epoetin subcutaneously than intravenously. For example, adipose tissue impedes subcutaneous drug absorption and seven of eight patients who weighed >82 kg did not have dose reductions after converting from intravenous to subcutaneous administration [82]. Hence, the greatest cost-savings might be attained by attempting both routes of administration for a given patient and then selecting the route with the lower dose requirement. In most cases this will prove to be the subcutaneous route. The half-life of darbepoetin alfa is considerably longer than the half-life of epoetin, and darbepoetin alfa dose requirements are the same for either route of administration.

Subcutaneous administration of epoetin alfa is associated with greater serial variability in hemoglobin concentration (standard deviation of 4 weekly hematocrit values = 2.02 volume %) than occurs with intravenous administration (1.37 volume %; $P < 0.01$) [82]. Many factors (e.g., body site of injection, adiposity, edema, temperature, and prior activity) alter the absorption of drugs such as epoetin, insulin, heparin, and growth hormone after subcutaneous injection, so it is not surprising that hemoglobin concentration will vary more with subcutaneous than with intravenous administration. Greater hemoglobin variability has been associated with higher mortality rates [83, 84], but it is unknown whether the magnitude of increased variability associated with subcutaneous administration of epoetin alfa is clinically significant. Predialysis and peritoneal dialysis patients lack chronic hemoaccess and are routinely treated subcutaneously.

Iron supplementation

Before the use of ESA therapy, transfusion-related iron overload was a serious complication. Stimulation of erythropoiesis with ESA therapy often leads to the opposite problem, iron deficiency, particularly for hemodialysis patients, who experience ongoing blood/iron loss associated with the procedure. Oral iron supplementation is generally believed to yield an inadequate rate of iron absorption and iron is typically prescribed intravenously to hemodialysis patients. Iron status should be assayed regularly and this is often attempted by measuring transferrin saturation and serum ferritin concentration every 1–3 months. Prescribers may wait until deficiency is detected and then infuse up to 1,000 mg in divided doses over 1–3 weeks, depending on the severity of the deficit and the iron formulation selected. A single, total-dose infusion has been described as a safe, convenient, and less expensive option [85]. Alternatively, ongoing infusions of smaller quantities of iron, e.g., with each session or weekly, are used at many centers in a strategy to prevent deficiency. Administration is prescribed more episodically for iron deficient predialysis and peritoneal dialysis patients, who lack permanent hemoaccess.

The aggregate evidence that intravenous iron supplementation decreases ESA dose requirement and aids hemoglobin maintenance is compelling. The treatment is not free of risk, however. First, intravenous iron dextran was historically associated with a low incidence of potentially lethal, anaphylactic-like reactions. Fortunately, there are alternate formulations that are safer (iron sucrose, ferric gluconate, and low molecular weight iron dextran); it has been recommended that high molecular weight iron dextran should be strictly avoided [86]. Second, patients with CKD have diminished immune function and are predisposed to infection and sepsis. Associations have been reported between increases of serum ferritin concentration >500 ng/mL and increased rates of infection [87, 88], and IV iron infusions could predispose to microbial proliferation. However, a 6-month observational study of 988 hemodialysis patients to determine risk factors for bacteremia showed no significant difference in serum ferritin concentration or receipt of intravenous iron within the past 6 months, between the 50 patients with bacteremia and the 935 without [89]. Third, the recurrent intravenous infusion of iron boluses could contribute to undesirable oxidative cardiovascular consequences, although the clinical validity of this concern has not been established. Cardiovascular disease is widely prevalent and highly deleterious in patients with CKD. Emerging data from studies in the general population suggest that binding of iron protects the heart. For example, infusion of desferoxamine during coronary artery bypass surgery, where iron exposure may result from hemolysis and cardioplegia solutions, improves postoperative cardiac function for at least 1 year [90]. Patients with type 2 diabetes and impaired coronary artery vasodilation respond favorably to desferoxamine [91]. The authors note that free iron ions available to stimulate deleterious oxygen radical reactions are typically very low (<5 micro mol/L) [91]. Experimentally, long-term iron chelation treatment

of rats with streptozotocin-induced diabetes prevents impaired relaxation of aortic rings, suggesting that iron-catalyzed oxygen radical formation contributes to the development of diabetic endothelial dysfunction [92]. A historical-cohort study of 58,058 hemodialysis patients found that doses of intravenous iron up to 400 mg/month were associated with improved survival compared with no intravenous iron, but doses >400 mg/month were associated with higher death rates [93]. Although adjustments were made for case-mix, it is not certain that these observational associations are causal – the potential for confounding by indication still exists.

Notwithstanding the above considerations, the current state of the art, KDOQI guidance, and EBPG guidance all support the use of intravenous iron to improve ESA treatment of anemia. The DRIVE study found that intravenous ferric gluconate treatment (n = 66), *versus* none (n = 66), improved hemoglobin response without causing an increase in infectious or cardiac adverse events among hemodialysis patients with serum ferritin concentration ≥500 ng/mL and transferrin saturation ≤25% [94]. Although the 6-week observation period and sample size are grossly inadequate to permit conclusions about long-term safety, the results do not signal early dangers. Large, randomized, prospective, chronic, survival studies of various doses of intravenous iron therapy would better inform optimal anemia management and are clearly lacking.

Hyporesponse to ESA therapy

It has long been recognized that there is wide variety in dose-requirements for epoetin treatment. For example, in the pivotal US Phase III trial of epoetin alfa in the setting of hemodialysis, the intravenous dose needed to maintain hematocrit values of 35% varied 42-fold (from 12.5–525 U/kg thrice weekly) among 333 iron-replete patients who at entry were deemed medically stable, without blood loss, and expected to live at least 6 months [6]. Nonetheless, patients requiring unusually high doses of ESA, or, in particular, those who previously maintained a desirable hemoglobin concentration but develop increasing dose requirements must be evaluated. Table 4 delineates causes of

Table 4. Causes of hyporesponse to ESA therapy

- Iron deficiency
- Inflammation/infection
- Bleeding
- Hemolysis
- Malignancy (particularly hematologic)
- Osteitis fibrosa (hyperparathyroidism)
- Hemoglobinopathy (e.g., thalassemia)
- Angiotensin converting enzyme inhibition
- Nutritional/vitamin deficiency
- Inadequate dialysis
- Antibody-mediated pure red cell aplasia (PRCA)

hyporesponse. For predialysis or peritoneal dialysis patients, the clinician should first verify that the patient is complying with the prescribed subcutaneous injections of the ESA. Iron deficiency, inflammation, and bleeding will account for most cases of hyporesponse and should be sought carefully. Vitamin deficiency (particularly of vitamin B_{12} or folate) may cause resistance to therapy. These deficiencies should not occur when a daily oral multivitamin supplement is taken to offset the loss of water-soluble vitamins during dialysis, but surprisingly the prescription rate of multivitamins ranges only from 4–72% across five European nations, Japan, and the United States [95]. Data are lacking to prove that angiotensin-converting enzyme inhibition or inadequate dialysis commonly or markedly inhibits ESA response, but such etiologies likely contribute occasionally. Aluminum toxicity was historically a cause of worsening anemia in CKD, but this is rarely a problem now that aluminum-based phosphate binder use has been abandoned. Some publications suggest that carnitine deficiency also contributes to ESA hyporesponse, but the data are not convincing.

Of interest, a recent retrospective case-control study suggests that hepatitis C infection *decreases* ESA dose requirements [96]. The authors speculate that viral inflammation may stimulate EPO release from the liver, but they provide no data comparing EPO levels in the absence of exogenous therapy. This new observation related to hepatitis C infection remains to be confirmed.

Novel ESA therapy: what's next?

Epoetin alfa and epoetin beta, 165-amino-acid rHuEPO, were the original ESA treatments and have established records of safety and efficacy. 'Biosimilar' or 'follow-on' recombinant versions have reached the market in some regions, as patent protections for the original compounds have expired. The miniepidemic of PRCA, which followed a subtle change in the epoetin alfa manufacturing process by one company, shows that close surveillance will be required for biologic follow-on products to assure equivalence to the original formulations. A publication describes multiple variations in the characteristics of biosimilar epoetin products procured from around the world that might impact immunogenicity/safety, including differences in pH, osmolality, protein aggregates, and even endotoxin [97].

Darbepoetin alfa, available since 2001, is a second-generation ESA, with two additional carbohydrate chains carrying increased sialic acid residues linked to the protein backbone, to prolong serum half-life and allow less-frequent dosing (weekly, every other week, and in some cases monthly). Five amino acids in the backbone were substituted to enable the carbohydrate engineering.

PEGylated-epoetin beta has entered the market in Europe and has been approved by the FDA in the United States. 'PEGylation' refers to the addition of poly[ethylene] glycol moieties, to slow clearance. The new compound has

a longer serum half-life than does darbepoetin alfa. A large, open-label, trial compared two intravenous dosing intervals (once every 2 weeks and once every 4 weeks) of PEGylated-epoetin beta *versus* standard epoetin in dialysis patients and found noninferior control of hemoglobin concentration and similar adverse event profiles across groups [98]. Monthly dosing could prove an advantage, particularly to patients who require subcutaneous injections.

Hematide is an engineered peptide, also PEGylated, that activates EPOR, although it lacks homology with EPO. The molecule has been shown to control anemia in Phase II studies of hemodialysis and predialysis patients, given either intravenously or subcutaneously every 4 weeks [99]. The incidence, if any, of antibody formation against the synthetic peptide was not reported. Hematide has proven effective in reversing ESA-induced PRCA [59].

Also in development is a very novel oral medication approach to anemia therapy. These agents competitively inhibit the enzymes that inactivate hypoxia inducible transcription factors (HIF). As a result, increased quantities of HIF are transferred to the nuclei of cells, mimicking a hypoxic state. Gene expression of EPO is stimulated, along with expression of other proteins that enhance iron availability and otherwise aid erythropoiesis. Preliminary clinical investigation has shown that EPO and hemoglobin concentrations increase after treatment with either of two HIF stabilization agents in patients with CKD [100, 101]. HIF stabilization induces the production of a number of nonerythropoietic proteins, as well; the safety profiles of the agents need to be determined with larger studies. Such oral ESA therapy might prove particularly advantageous to expand the treatment of anemia among predialysis patients and to overcome inflammatory resistance to erythropoiesis.

Asialo and carbamylated EPO analogs, which do not stimulate the bone marrow, are under development for nonanemic indications, such as ischemia-reperfusion injury states.

Also, novel approaches are being investigated to improve iron availability in the setting of inflammation, which could aid erythropoiesis in renal failure. Hepcidin, increased during inflammatory states, is a protein that diminishes iron absorption from the gastrointestinal tract and impedes release of iron from the reticuloendothelial system. Commercial efforts are underway to develop antibodies and antagonists to hepcidin and to manipulate hemojuvelin, which in turn affects hepcidin concentrations.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Abuse of recombinant erythropoietins and blood products by athletes

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History

History of doping

Ever since the beginnings of recorded time, there has been evidence that men experiment with performance enhancement. In ancient times, medicinal plants were used for doping. Now sport is coping with drugs developed with recombinant-DNA technology and genetic manipulation is being discussed.

According to Philostratus, a Greek physiologist in the third century AD, "[athletes should be]… freed from the use of clay and mud and irksome medicine" [1]. He describes Olympians raising their achievement level by eating bread soaked in opium. Centuries ago, the Incas chewed coca leaves to sustain strenuous work and Berserkers ate mushrooms containing muscarine before battle. Some of the earliest pharmaceuticals, amphetamine, strychnine, and ephedrine rapidly became interesting to athletes in search of speed. Canal swimmers are reported to have used strychnine to win races in Amsterdam. The first documented doping fatality at the Olympics shocked the 1960 Games [2] and it involved amphetamine abuse.

"There can be no doubt that stimulants are today widely used by athletes participating in competitions; the record-breaking craze and the desire to satisfy an exacting public play a more and more prominent role, and take higher rank than the health of the competitors itself" [3]. Written in 1939 by a physiologist, this quote is still apropos today and it shows how long ago the 'win at all cost' mentality was already apparent. In the 1970s, Congressional hearings in the United States documented significant abuse of stimulants and anabolic steroids [4], medical organizations took anti-doping stands, and widespread amphetamine abuse was described in professional football.

Characteristics of doping

Not all athletes dope – fortunately most do not – but those who do have a profound influence on sport. Ben Johnson set a world record in the 100-meter

dash at the 1988 Olympics in Seoul, but he was doped with stanozolol, an anabolic steroid. Until this Games-stopping event, the hope had been that doping was confined to strength sports. The Johnson affair led to vociferous cries from clean athletes for more testing and stricter penalties, and spurred sport to devote more resources to solving the problem. In retrospect, the resources were woefully inadequate but it was a start.

An axiom of drugs and sport is that some athletes will experiment with any new drug that has the potential to enhance performance. The trials are conducted secretly and of course never published. Sometimes information is shared with close partners, but generally not. Often members of the athletes' entourage are deeply involved in doping.

Doping has occurred in virtually all sports but certain ones have predilections [5, 6]. Endurance events (such as long-distance running, cycling, swimming, and skiing events) experience difficulties with various types of blood doping, including the use of erythropoietic proteins. Short-distance speed events have always had stimulant abuse. Weightlifting, throwing events, and several other sports have been plagued with anabolic androgenic steroid doping. Even shooting events have difficulties as beta-blockers improve shooting scores [7].

Development of testing

By the mid-1960s, international sport officials were growing very concerned about drugs and were actively exploring several approaches to the issue. Until then the main approach was education, but this was unlikely to deter elite athletes who were beginning to receive substantial financial rewards for success. In 1967, Prince de Mérode presented a survey of the problem to the International Olympic Committee (IOC); shortly he became chairman of the IOC Medical Commission and plans were laid to implement urine testing [8]. After experimenting with urine testing at the Winter Olympics of Grenoble in 1968, the IOC implemented widespread testing at the Summer Olympic Games of Munich in 1972. In 1984, there were only five IOC-accredited laboratories. Now there are 34 such laboratories operating in 31 countries. In the early years, the testing was mostly confined to the Olympic Games, then it spread to other competitions, and now athletes are at risk to be tested all year-around. Out-of-competition testing was implemented when it became clear that the ergogenic effects of drugs, such as anabolic steroids and erythropoietic proteins, lasted for many days after discontinuation. Otherwise simply by discontinuing the drugs before announced in-competition tests, one could evade detection. More than 200,000 samples are tested per year [9], and of these, about half are collected out of competition.

The Court of Arbitration for Sport (CAS)

The Court of Arbitration for Sport (CAS) was created by the IOC in 1983 to resolve sport-specific disputes [10]. It provides a forum for judicial resolution of disputes among athletes, national and international sport federations, national Olympic Committees, games organizers, sponsors, and others. Disputes are typically presided over by a panel of three arbitrators selected from a pool of CAS arbitrators. All CAS decisions are final with almost no possibility of appeal.

Doping cases are subject to the strict liability rule which states that "doping is… the presence in the athlete's body of a Prohibited Substance…" [11]. Thus, if a prohibited substance is found in an athlete's body, a doping violation has occurred irrespective of any other factor [10]. At times this rule results in seemingly harsh consequences as was demonstrated by the case of the young Romanian gymnast who placed first in the Women's Individual All-Round Event at the Olympic Games of Sydney. The athlete tested positive for pseudoephedrine, which was allegedly given to her by the team doctor.

The two most common subjects that are argued in CAS cases are the validity of the analytical methods used to establish that a prohibited substance has been detected in the athlete's body fluid, and the chain-of-custody of the sample. The principles of the analytical methods that may be applied, and the chain-of-custody are specified in the Medical Code of the Olympic Movement [11]. During the adjudication procedure, the laboratory director typically defends the analytical results and the sample collection officials defend the custody issues.

The World Anti-Doping Agency (WADA)

Faced with many positive test results, mounting numbers of drugs and methods to consider banning, laboratories to accredit and re-accredit, and complex issues involving new fields of science, the IOC fostered the development of a new agency, the World Anti-Doping Agency (WADA) to take over the drug-control effort. This move coincided with revelations in 1998 that a great many cyclists competing in the Tour de France were doped with recombinant human erythropoietin (rHuEPO). Since the IOC was sometimes criticized for being slow to develop adequate anti-doping programs, the WADA was structured to operate outside the direct control of the IOC.

WADA was established in 1999 and began operations in 2000. In 2004, the IOC transferred the management of the prohibited list to WADA. In addition to updating the list and the regulatory requirements of the International Standard for Laboratories annually, WADA has published a few "Technical Documents" containing recommendations and criteria on specific laboratory testing topics.

Doping by expanding the red cell mass

In the early 2000s, induced erythrocythemia was the term used to describe expansion of the blood volume by transfusion of whole blood, transfusion of packed red cells, or by administration of erythropoietic proteins. The term 'blood doping' has been used to describe any of these three methods, although there are obvious differences in the techniques and the infrastructure required to use them.

Erythropoietic proteins, other than Epogen, the first product, are referred to as follow-on biologics (FOBs) to reflect the fact that manufacturers cannot exactly duplicate the Epogen profile. The new term 'erythropoiesis-stimulating agents (ESAs)' includes Epogen and FOBs and reflects the remarkable advances in the field, and the fact that drugs other than rHuEPO can stimulate erythropoiesis. A recent comprehensive and authoritative review of ESAs and other aspects of doping discusses other compounds such as hypoxia inducible factors, desferrioxamine, cobalt, perfluorocarbons, that are beyond the scope of this review [12]. Other recent reviews cover strategies for detecting rHuEPO [13] and biosimilar rHuEPO [14]. In addition a children's book that is widely read by adults focuses on testing for rHuEPO at the Winter Olympic Games of Salt Lake City [15].

Autologous and homologous blood doping in sport

Both homologous and autologous blood products have been used by sportsmen. The typical technique for autologous transfusion involves phlebotomy, freezing the red blood cells under glycerol, allowing 8–12 weeks for hemoglobin recovery while continuing training, and the red blood cell reinfusion 1–2 days before the event. In other words, blood from one person is stored and reinfused to the same person. The use of homologous blood products avoids the regeneration period but is associated with multiple risks and adverse effects. The terms 'blood packing' and 'blood boosting' usually refer to doping with homologous and autologous blood products.

Many rumors and anecdotes suggest that a foreign airliner equipped with refrigeration devices was used to support doping at the 1976 Games of Montreal. The first documented case of blood doping occurred in the 1980 Olympics in Moscow where a Finnish distance runner freely admitted receiving two units shortly before he won medals in the 5- and 10-km [16]. At the 1984 Games in Los Angeles, seven cyclists from the US team blood doped [17]. One cyclist doped by autologous transfusion and performed his personal-best at the Olympic trials a month before the Games. By the time this news was circulated to team-mates, it was too late to use the autologous reinfusion paradigm, so with the aid of a physician, whole blood from relatives and unrelated donors was transfused in a motel room [18]. Four of the seven won medals, but so did others who did not blood dope. During the investigation, the

athletes justified the act by pointing out that the IOC list of prohibited substances did not explicitly ban blood doping. Shortly thereafter, the IOC added to the list doping with blood products. Another documented case is that of a US skier who admitted to blood doping with autologous blood in 1987 [19]. To date, the only case detected by a laboratory blood test is that of a US cyclist at the 2004 Vuelta de Espana cycling road race.

Doping with recombinant human erythropoietin in sport

Given that blood doping with transfusions is a complicated matter that requires substantial infrastructure such as blood harvesting equipment, processing devices, and refrigeration, it is understandable that the availability of a drug, even one that had to be administered intravenously or subcutaneously, would be used. Exactly when doping with rHuEPO began is not known. The secrecy and privacy that surrounds the topic precludes detailed knowledge, but it is understood that athletes and their medical entourage follow pharmaceutical developments closely. Thus it is not surprising that rumors of rHuEPO's potential for abuse by athletes first surfaced before pharmaceutical rHuEPO was introduced (1987), and that rumors of actual use circulated before the 1988 Winter Games of Calgary.

In the late 1980s, there was a remarkable cluster of up to 20 deaths among elite cyclists in the Netherlands and Belgium. The story was widely reported and there was an official investigation, but no details ever emerged. It is speculated that rHuEPO may have been involved [20, 21]. We were unsuccessful in obtaining the details through contacts in the sport community. Because the deaths occurred after the release or rHuEPO, it was widely speculated that they were due to excessive doses of it. In 1996, 'erythropoietin' and analogues were added to the IOC list of prohibited substances.

Although it was widely accepted that rHuEPO was being abused throughout the 1990s, it was not until the 1998 Tour de France that use was documented. The discovery of rHuEPO and other drugs in the trunk of an automobile just before the race led to a widespread investigation that eventually resulted in recovery of many drug products, confessions of several athletes, and evidence of widespread involvement of cycling teams and coaches [22–24]. This expose differed from other sensational drugs in sport stories (such as Ben Johnson's in 1988) in that most of the competitors were involved, not just one athlete. It convinced cycling officials that rHuEPO doping was pervasive and that users and winners were highly correlated. Further, officials in other endurance sports understood that their sports were also at risk. Some of the steps taken collectively by sport were further implementation of blood screening methods (*vide infra*), monitoring athletes' red blood cell parameters, increased funding for research on detection, formation of expert committees, and more emphasis on the role of police authorities. A most unfortunate aspect of such developments is that success is equated with doping, thereby raising

suspicions about any athlete who performs well, which has led drug-free athletes to speak out and to pressure authorities for more effective action. Another remarkable benefit of the affair is that it led the IOC to create the WADA.

Prevalence of doping with erythropoietic proteins

It is nearly impossible to estimate the prevalence of blood doping of any kind. Only one survey has attempted to estimate the prevalence of blood doping, and this study commingled transfusion doping with doping with rHuEPO. Scarpino et al. [25] interviewed 1,015 Italian athletes and reported that 7% regularly used red blood cell reinfusion or rHuEPO techniques and 25% were 'occasional' users. In the same study, coaches, managers, and team physicians also estimated the regular users at 7%. Sport hot-lines are another source of information. Throughout the 1990s, the United States Olympic Committee operated an anonymous hot line which athletes could call to request information about drugs and sport. Committee reports indicate that the number of calls regarding rHuEPO increased during the 1990s.

The lay sport literature reports widespread doping with erythropoietic proteins in endurance sports such as track, cycling, and cross country skiing. These reports are largely confined to elite athletes competing in major events. From the frequency of these sensational reports, one gains the impression that such doping is common, but prevalence cannot be estimated from such data. To date, neither college sport administrators nor the media have reported doping with erythropoietic proteins in the college sport population.

Now that a urine test for erythropoietic proteins (*vide infra*) has been deployed, some data have been forthcoming from sports organizations [9]. The WADA publishes laboratory statistics annually, including the total number of tests worldwide, and the number of A samples reported to contain rHuEPO or darbepoetin alfa (Tab. 1) [26].

Table 1. Number of worldwide doping control urine tests and A samples reported to contain recombinant erythropoietic proteins

Year	EPO	Darbepoetin alfa	N tests
2000	*	0	117,314
2001	*	0	125,701
2002	*	3	131,373
2003	51	7	151,210
2004	38	0	169,187
2005	15	1	183,337
2006	17	1	198,143
2007	22	2	225,000

Each time the United States Anti-Doping Agency (USADA) sanctions an athlete, it issues a press release that includes the athlete's name, sport, the date of the test, and the name of the event in the case of in-competition testing. USADA also publishes quarterly statistics. From 2000 to 2002, the media reported several cases of doping with erythropoietic proteins [27]. The media reports occur many months before the adjudication proceedings that could find that doping did not occur. In 2002, two cases of alleged doping with rHuEPO were heard by the CAS. In one case, the Court upheld the laboratory result and determined that the athlete had doped with rHuEPO [28]. In the second case, CAS concluded that rHuEPO doping had not been proven due to a flaw in the analysis [29]. Although no one really knows to what extent erythropoietic proteins are used by athletes, it is increasingly obvious from all sources of data that they are. Although the use currently appears to be confined to the highest echelons of sport, if the history of doping with other agents (notably steroids) is any indication, that use will spread.

The first confession of rHuEPO use was by the female winner of the 2004 Ironman Triathlon [30]. It has been followed with many more confessions by athletes, some of whom tested positive and some of whom never did.

Does expanding the red cell mass enhance performance?

The underlying theory is that increased oxygen-carrying capacity increases performance of muscle tissue by increasing oxygen supply. The ability to perform sustained aerobic exercise depends on both oxygen delivery to muscles and ability of the tissues to use it. Which of the two limits exercise capacity has been the subject of many investigations and debates [18, 31–33]. $VO_{2\,max}$, a widely accepted index of physical fitness [34], correlates with red cell mass [35], thereby, supporting the hypothesis that an expanded red cell mass enhances performance by increasing the amount of oxygen delivered to muscle. The increase in blood viscosity due to increased concentrations of hemoglobin does not limit delivery as long as the hemoglobin is approximately <20 g/L [36]. Other possible theories advanced to explain the increase exercise capacity are that the expanded blood volume leads to increased cardiac output, improved buffering capacity for the lactic acid accumulated during exercise, and enhanced heat dissipation.

A paradox exists in exercise physiology. Athlete fitness is negatively correlated with hematocrit, yet increasing the hematocrit by transfusion or by doping with erythropoietic proteins improves $VO_{2\,max}$ and performance [37, 38]. Exercise training expands plasma volume and decreases hematocrit and hemoglobin concentration, thus resulting in a negative correlation between hematocrit and fitness [38–40]. Values for hematocrit, hemoglobin, and red blood cell count are in the lower range of normal for athletes [37, 41]. Athletes with the lowest hematocrits had the highest aerobic working capacity and isometric adductor strength [38].

At first it was thought that the hemoconcentration that accompanies endurance competitions would lead to increased blood viscosity and that this would severely limit cardiac output and thereby limit the usefulness of rHuEPO. Hematocrit is highly correlated with blood viscosity [38]; however since hematocrits of 50% and more are often recorded among competing athletes, other factors must be operating. In one study of athletes competing in an ultramarathon, the immediate post-race hematocrits were not different from the baseline values, and on the day after the competition, the values were lower than baseline. Apparently replenishing lost fluid and electrolytes during very long events is sufficient to avoid serious increases in viscosity [37].

The idea that dehydration during long endurance events will lead to hemoconcentration and increased blood viscosity, and therefore result in athletes experiencing strokes and other complications of increased blood viscosity, is difficult to confirm. No media reports exist of athletes experiencing such catastrophes during endurance events. The cluster of deaths in cyclists in the late 1980s [42], could be related to increased blood viscosity, but the athletes were not competing at the time.

Autologous blood infusion increases performance

Many clinical experiments have shown that transfusions increase human performance [32, 43–46]. The most convincing study [32] used a double-blind, sham-infusion controlled, cross-over design. Highly trained elite athletes received 900 mL autologous red blood cells collected and frozen approximately 7 weeks earlier; 24 h later they experienced a 35% increase in run-time to exhaustion, a 5% increase in $VO_{2\,max}$, and a 7% increase in hemoglobin concentration. The authors concluded that the limit to aerobic activity was the transport of oxygen to muscle. In another study, this time using 10-km race-time as the outcome rather than physiological measurements, six highly-trained but not elite athletes improved their mean run-times from 33.3 to 32.1 min and their hematocrits increased 5% shortly after receiving a 400-mL autologous infusion of red cells collected 11 weeks earlier [46]. An interesting aspect of autologous infusions is that the magnitude of the improvement is related to the baseline level of fitness. Individuals who are moderately fit experience the greatest improvement while individuals who are poorly fit or extremely fit experience less improvement in maximal oxygen uptake [45]. Improvement in human performance is a consistent feature of blood transfusions provided that the amount of red cells infused is sufficient to increase serum hemoglobin concentration and hematocrit and thereby deliver more oxygen to working muscles. The improvements can be shown in race-times, fixed-run times, run-times to exhaustion, $VO_{2\,max}$, and other measures of performance. Accordingly, sport officials search for ways to deter those athletes who are determined to dope with transfusions or any other means to increase the delivery of oxygen to muscles.

rHuEPO enhances performance in healthy subjects

It is well known that when rHuEPO is administered to patients with anemia, they experience significant improvement in fatigue, physical symptoms, and physical performance [47]. In the present context, the question is whether rHuEPO enhances physical performance in healthy subjects and particularly in athletes. Since the release of rHuEPO almost 15 years ago, there have been few studies on athletes and most of these have involved its effect on red cell indices and other markers.

Two placebo-controlled studies have shown that rHuEPO increased $VO_{2\,max}$ by 6.0% to 7.7% after 3–4 weeks of subcutaneous rHuEPO (150 IU/kg/week) [48, 49]. Typically the weekly dose is administered subcutaneously in doses of 150 IU/kg/week. At a higher dose (180–210 IU/kg/week, subcutaneously), the $VO_{2\,max}$ increase was similar (7%) [50]. These studies, which used recreational athletes, establish that doses of rHuEPO that are sufficient to increase the hematocrit to nearly 50% also increase the $VO_{2\,max}$ up to 7%. Anecdotal reports, however, indicate that after a few weeks of modest doses (150 IU/kg/week), athletes lower the weekly dose to about 60 IU/kg/week. To determine the effect of such a regimen, after 3 weeks at 150 IU/kg/week, a study [49] decreased the dose to 60 IU/kg/week for an additional 5 weeks and found that the improvement in $VO_{2\,max}$ (4.7–9.7%) and increased hematocrits continued. This study and a similar low dose study [51] provide support for the anecdotal reports from athletes, and confirm that maintenance doses of 60 IU/kg/week are capable of enhancing aerobic performance for at least 3 weeks.

Adverse effects of rHuEPO in athletes

Unlike studies in patients with anemia, there are no surveys of the adverse effects of rHuEPO in athletes. Athletes tend to be healthy and that may offer some protection against the known side effects in anemic patients; however, athletes are deliberately increasing their hematocrits to levels associated with thromboembolic and other complications. Only one case report appears to link rHuEPO to a cerebral thrombosis in an athlete who admitted to using rHuEPO but was also taking other drugs [52]. Athletes who use rHuEPO are at risk to develop true iron deficiency, functional iron deficiency, or iron overload. Of course careful professional medical management could avoid these complications; however, Cazzola reports that an investigation by Italian Magistrates [53] reveals that some professional cyclists have evidence of iron overload with ferritin values in excess of 1,000 ng/mL [54]. The investigation also provided data consistent with a risk of post-treatment blunted production of endogenous EPO [54]. In addition, Berglund and Ekblom [51] have studied the effect of rHuEPO on the blood pressure of athletes. The systolic and diastolic blood pressure values at rest were unchanged after rHuEPO treatment; how-

ever, there was a marked increase in systolic blood pressure during submaximal exercise. The initial and final values were 177 mmHg and 191 mmHg. The propensity of athletes to titrate their hematocrits to high levels and to take rHuEPO without adequate medical supervision, together with their risk for iron disorders and exercise induced elevated systolic pressure, make it likely that more adverse effects are occurring than are reported in the medical literature. This under-reporting is inherent to the secretive nature of doping.

Risks and benefits

Shermer's risks and benefits analysis is that the choice to dope, especially with rHuEPO, makes sense because it is highly effective at enhancing performance, the risk of getting caught is small, the financial rewards are astronomical, and clean athletes may not be competitive [55]. Fortunately, he is able to propose ways to tip the balance of risks and benefits to favor the choice of staying drug-free.

Detecting erythropoietic proteins in body fluids

Direct and indirect tests

Sport classifies tests for doping substances as direct or indirect. A direct test identifies the substance by an unambiguous method such as gas chromatography-mass spectrometry (GC-MS), whereas indirect tests measure, for example, the serum concentration of markers that correlate with the use of a prohibited substance, without directly identifying the substance. Direct tests sufficed for many years, but now that doping has turned to endogenous steroids and glycoproteins, a variety of new strategies and indirect tests have developed [5, 13]. Indirect tests have not been used to declare that an athlete has used a substance, but it is hoped that with sufficient validation, they could become definitive.

An example of indirect test for a different class of doping agents is the detection of the use of pharmaceutical testosterone. It was a challenge with GC-MS because pharmaceutical and endogenous testosterone could not be distinguished. The problem is partly solved by performing longitudinal tests of the urinary steroid profile and by determining the $^{13}C/^{12}C$ ratio of urinary testosterone by isotope ratio mass spectrometry [56]. The glycoproteins present a special challenge because so far no MS-based method has been developed that is sensitive enough to detect either rHuEPO or recombinant human growth hormone (rHuGH) in human urine. In another example of indirect testing, Sonksen and colleagues set out to find a group of blood parameters that would indicate recent use of rHuGH. After collecting baseline blood samples, they administered rHuGH for several days and monitored serum levels of six

substances known to be influenced by rHuGH [57]. A composite score indicative of recent use of rHuGH was developed. The results showed good separation between the scores of the placebo-treated and rHuGH-treated subjects. The investigators intend to expand the studies to a much larger number of subjects and to determine the variability of the markers in various ethnic groups. The hope is to find a composite score that is so convincing that the indirect test is considered definitive. Until 2000, there was no practical direct test for rHuEPO, therefore certain sports implemented indirect tests to identify potential users of rHuEPO.

Indirect tests for doping with erythropoietic proteins

The hematocrit 'health' test

The International Cycling Union (UCI), concerned that rHuEPO had pervaded elite competition, declared that male and female athletes could not compete with a hematocrit >50% and 47%, respectively [58, 59]. The International Ski Federation (FIS) implemented a similar rule based on hemoglobin values >185 g/L (men) or 165 g/L (women). One strategy was to determine the values immediately before an event and withhold the athletes from competition if the limits were exceeded. The hematocrit cutoff values have been changing but generally they are 50% for men and 47% for women. The hematocrit test has been dubbed a 'health test' because it is considered dangerous for an athlete to compete if the hematocrit is greater than the cutoff. The term 'health test' preempts legal action because the athlete is not declared a drug user, he/she is only kept from competing, which is lifted after 5–15 days provided that the hematocrit has decreased below the limit. For example, at the 2006 Winter Olympics in Torino, several athletes were kept from competing for 5 days [60].

Aside from the health issue, the argument in favor of a hematocrit test is that by imposing an upper limit, the test prevents excessive use of rHuEPO. An argument against the hematocrit test is that it would discriminate against individuals who have naturally high hematocrits [61, 62]. Partially countering this argument is the finding that of 334 hematocrits determined on 34 professional cyclists before rHuEPO was available, the values ranged from 39–48% (mean, 43%) [63]. In addition, a protocol was developed to determine if an athlete's hematocrit naturally exceeds 50%. Postural changes in hematocrit are avoided by taking samples after sitting for 15 min. Increased hematocrits due to dehydration have not been a significant problem [64]. Despite the controversial nature of the test, there is evidence that it is having the intended effect: hemoglobin values among elite cross-country skiers increased dramatically from 1994 to 1996, and declined after the test was implemented [65].

A disadvantage of the hematocrit test is that it draws attention to the benefits of an increased hematocrit, thus tempting athletes with a natural baseline <50% to find a way to increase it. Indeed, it is common lore that some athletes check their hematocrits using portable centrifuges and self-administer saline

infusions and phlebotomy if the values are too high. They are also reported to take anticoagulants to prevent thromboembolic events.

Hypochromic macrocytes

Casoni et al. [66] studied red cell indices after administering rHuEPO every other day at an average dose of 15 IU/kg/day for up to 45 days. The parameters that changed the most were mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). The authors defined red cells with MCV >128 fl and MCH <28 pg as hypochromic macrocytes and proposed a cutoff of 0.6% to distinguish rHuEPO users from healthy controls subjects. No further work on this particular index has been published.

Serum transferrin receptor (sTfr) and ferritin

Serum concentrations of soluble transferring receptor (sTfR) and ferritin are regulated by cellular iron status, and cellular iron uptake is facilitated by transferrin receptor-mediated endocytosis. As a result of externalization of TfR during the endocytic cycle, a soluble form of TfR can be detected in serum. Thus, the major determinants of sTfR concentration are cellular iron demands and red cell proliferation rate. Since rHuEPO expands the red cell mass, it was logical to determine if sTfR and ferritin could serve as indirect markers of rHuEPO administration.

Several studies have shown that levels of sTfR increase, those of ferritin decrease, and the ratio of sTfR/ferritin increases when rHuEPO is administered [50, 67, 68]. Furthermore these changes are detectable in most subjects for up to 1 week after discontinuing rHuEPO [50]. The change in sTfR/ferritin ratio was less dramatic in subjects treated with supplemental iron [50] than in subjects who did not receive iron [67], thus the absence of supplemental iron may exaggerate the sTfR/ferritin ratio. Most users of rHuEPO are likely to take supplemental iron. The specificity of the changes in sTfR, ferritin, and sTfR/ferritin has not been evaluated in a large group of healthy normal control subjects of various ethnicities, subjects with disease, or under various conditions of iron supplementation, altitude, or training. Nevertheless these markers are relatively simple to measure and they may be useful as indirect markers of the use of erythropoietic proteins, either alone or in combination with other markers.

Multiple markers of erythropoietic activity – the Australian studies

In the year before the Sydney Olympic Games of 2000, Australian scientists performed an extensive series of studies designed to find a combination of blood markers that would indicate which athletes were using rHuEPO [48, 69]. The approach was to administer rHuEPO for 3 weeks, collect blood for analyses before, during, and for 2 weeks after administration was discontinued, and measure a variety of potential markers in serum and blood. The data were analyzed by various statistical models that provided the optimum combination of variables and the weight to attach to each. The main study compared placebo

with rHuEPO-treated subjects who received 50 IU/kg three times per week for 25 days [67]. The treatment group comprised 49 subjects from Australia and 24 subjects from Beijing, China.

The study found that five markers provided the most discrimination between the placebo and rHuEPO-treated groups. These were hematocrit, reticulocyte hematocrit (MCV of reticulocytes * number of reticulocytes), percent macrocytes, serum concentration of EPO, and serum concentration of sTfr. The total score was referred to as the 'on-score' [69]. By the end of the 3-week rHuEPO administration period the 'on-scores' of the rHuEPO-treated and placebo groups differed significantly. Serum EPO and sTfR were particularly increased compared with the control group. No differences were seen between the Australian and the Chinese subjects or between men and women.

The Australian investigators calculated the 'on-score' of about 1,200 elite athletes from 12 countries [69]. Based on these data, they calculated cutoffs for the 'on-score' values beyond which the risk of a false-positive was very low. At a meeting of experts 3 months before the Sydney Games [70], the 'on-score' was not approved as a standalone index of use of erythropoietic proteins largely due to legal and medical concerns. However, it was approved as a technique to indicate which urines should be tested by the recently developed, definitive urine test (*vide infra*). The rules stated that an athlete could not be declared 'positive' for rHuEPO unless the 'on-score' was greater than the cut-off and the urine test showed rHuEPO. Samples could be declared 'suspicious' if the 'off-score' (*vide infra*) was increased or if only one of the blood or urine tests was positive. No athletes were declared positive during the Games of Sydney but seven were reported as 'suspicious' [71].

Index of recent use of erythropoietic proteins

The Australian researchers also found that after rHuEPO was discontinued, the reticulocyte count and serum EPO were depressed while the hematocrit remained increased [69]. From these three parameters, they calculated an 'off-score' and proposed that an elevated 'off-score' be used to identify athletes who had recently discontinued rHuEPO. The 'off-score' was increased from the third to the twelfth post-administration day. Although the statistical certainty that the 'off-score' indicated recent use was quite high, the IOC expert committee did not accept the test largely because of the legal difficulty of proving that an athlete used rHuEPO on the basis of an indirect test. The 'off-score' is used today to indicate which athletes should be followed closely with longitudinal testing.

Urine EPO concentrations by immunoassay

Attempts to detect abuse of erythropoietic proteins by urine immunoassay have not been successful. Since rHuEPO and natural urinary EPO cannot be distinguished by the available antibodies [72], a diagnostic test would depend on finding urinary EPO concentrations that are far above the normal range. In addition, less than 5% of the dose is excreted in urine [73] and urinary EPO

concentrations are affected by pH, specific gravity, and exercise [74, 75]. Small increases in urinary EPO were detected by a immunoradiometric assay after large doses (200 IU/kg every other day for 10 days) [72].

Monitoring an individual's hematologic variables

Until very recently, all doping control samples were collected using the cross-sectional model, which means that the analytical results are interpreted on their face value, not compared to previous samples collected from the same person; however, if the latter is done, a linked or trend analysis can be performed. This test is a much more powerful type of analysis because results will vary from day to day between persons, however within persons, the results are generally more stable. For example, if a person has a hematocrit of 39%, it will vary by only a few percent from day to day, but if that person's hematocrit increases to 46%, even though that is well within the normal range for the whole population, for that person it is an outlier and it needs to be investigated. The WADA's Athlete's Passport, UCI Biological Passport, and USADA Project Believe, all include trend analysis.

WADA Athlete's Passport

The WADA Athlete's Passport [76] is based on monitoring an individual's selected parameters, which reflect the effects of doping indirectly, as opposed to the traditional direct detection of doping. Biologic tracing throughout an athlete's sports career should make any doping agent far more difficult to use. From a medical point of view, biologic monitoring is also likely to reveal disease, whether or not it is due to doping, making early and effective treatment possible. The Athlete's Passport will eventually improve the effectiveness of the fight against doping and protect the health of athletes. In addition, it will support the intelligent targeting of athletes for doping control. Although the concept is simple, in that different models have already been validated for widespread use in other scientific fields, such as epidemiology, legal medicine, or even veterinary monitoring, several key considerations must be taken into account before its widespread use in the field of anti-doping. While examining legal and disciplinary issues, as well as feasibility, WADA started a pilot project with the UCI in 2008; however, because of a UCI lawsuit against WADA, WADA withdrew its support of the UCI in relation to WADA's pilot project of the Athlete's Passport in the sport of cycling and will continue the Athlete's Passport pilot project with another sport. WADA's view is that the Athlete's Passport will augment, not replace traditional anti-doping testing.

UCI Biological Passport

The UCI Biological Passport is designed to maintain an individual's electronic record including urine test results and the steroid profile, blood test results and the corresponding profile; establish individual limits; detect doping indirectly; assess significant variations; and potentially use those as a basis for opening disciplinary proceedings.

USADA's Project Believe

In April 2008, USADA created a voluntary, longitudinal pilot testing program and began testing American Olympic hopefuls [77]. The volunteers agreed to provide additional blood and urine samples beyond those required by the regular in- and out-of-competition testing program. The analytical results were to be used to establish individual baselines.

Private trend analysis programs

These programs are generally operated by teams within sports. They are designed to supplement the WADA- or National Anti-Doping Organization (NADO) directed testing. They are particularly useful for sports that have major doping issues. They are private in that the data are controlled by the program rather than WADA or the NADO, meaning that the program must have careful oversight so that any aberrant results or positive results are not ignored.

Results of monitoring individual data over time

The most comprehensive study monitored hemoglobin concentration and percent reticulocytes in samples obtained from 440 female and 634 male elite cross-country skiers for 6 years [78]. For the men, the hemoglobin concentrations decreased dramatically (16.2 to 14.8 g/dL) from 1997/1999 to 2002/2003. In the same time period, the percent reticulocytes also declined significantly. The results for the women were similar. These desirable and promising results were attributed to an enhanced blood testing program and to imposing cutoff values. Unfortunately hemoglobin values increased steadily from 2002/2003 to 2005/2006 while the reticulocyte count continued to decrease. No apparent reason has been found why the hemoglobin values have been increasing. The speculation includes changes in drugs or administration details of the doping agents used by these athletes.

Direct tests for doping with erythropoietic proteins

The isoelectric focusing (IEF) test for urinary rHuEPO

A method for detecting rHuEPO (epoetin alfa or epoetin beta) in urine by electrophoresis was first described in 1995 [79]. Although this test had practical limitations, it demonstrated conclusively that the isoform pattern of urinary endogenous EPO differs from the pattern of urinary rHuEPO, and it was the first successful attempt to develop a direct test for urinary rHuEPO. At that time the only rHuEPO was epoetin alfa, although we now know that the test works equally well on epoetin beta. Similarly in 2002 Skibeli et al. [80] isolated EPO from human serum and showed, using gel electrophoresis, that endogenous and recombinant EPO differed.

A significant improvement in practical detection occurred in 2000 when Lasne and de Ceaurriz [81] described a method for detecting rHuEPO in urine based on isoelectric focusing (IEF) with immunoblotting. The method includ-

ed a novel and critical step: a second blot ('double-blot'). After the isoforms of rHuEPO are separated by IEF, the first blot is performed, then the membrane containing the transferred proteins is incubated with anti-EPO antibody [81, 82]. The second blot transfers only the anti-EPO antibodies to a second membrane, and the second membrane is incubated with a second antibody directed against the first antibody. This step markedly reduces nonspecific binding and yields clear isoform patterns [81, 82]. After the second antibody is incubated with streptavidin-horseradish peroxidase and substrate, the emitted chemiluminescence is captured with a charge-coupled device camera to produce an image of the gel. Figure 1 is an electropherogram showing the band pattern of darbepoetin alfa in a urine sample obtained from a patient who was treated with darbepoetin alfa.

Despite the fact that the IEF test is technically demanding and may take up to 3 days to complete, at this time there is a great deal of interest in it because it is a direct test that can yield conclusive results [81]. Further, the results of the test and the underlying IEF method have been accepted by the CAS [28].

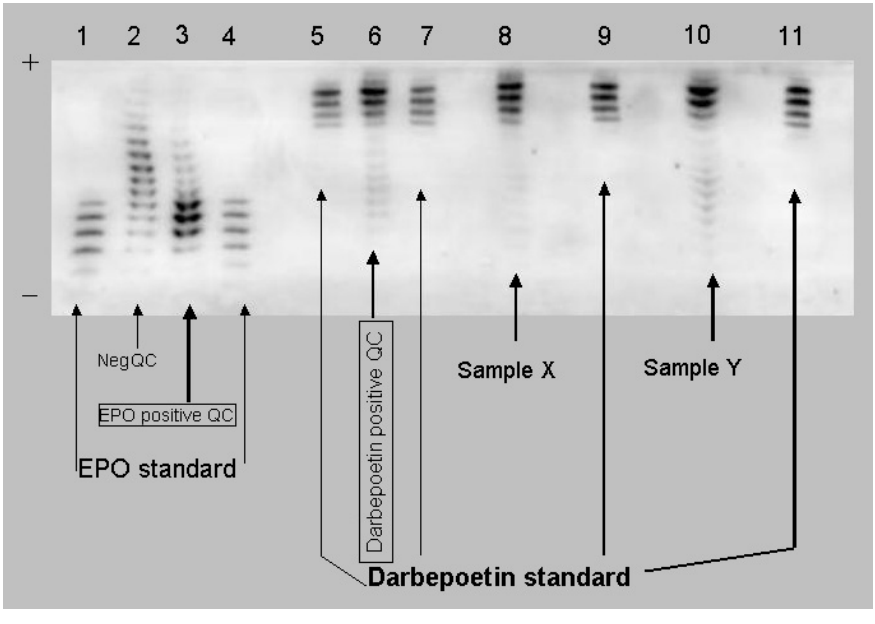

Figure 1. Electropherogram of rHuEPO and darbepoetin alfa standards; of extracts of urine obtained from drug-free control subjects and subjects treated with rHuEPO or darbepoetin alfa; and of extracts of urine from unknown individuals. The anode and cathode sides of the electropherogram are labeled + and –, respectively. The rHuEPO standard is in lane 1 and 4 and the darbepoetin standard is in lanes 5, 7, 9, and 11. The Neg QC (lane 2) is an extract of urine from drug-free subjects showing the normal pattern of endogenous hEPO. The EPO positive QC (lane 3) and darbepoetin positive QC (lane 6) are urine extracts obtained after the administration of rHuEPO or darbepoetin alfa, respectively. Samples X and Y are urine extracts from two unknown individuals (lanes 8 and 10). The bands of endogenous urinary EPO are faint or absent in lanes 6, 8, and 10.

Beginning in 2005, the WADA Technical Document TD2004EPO harmonized inter-laboratory criteria for the identification of epoetin alfa and epoetin beta, and darbepoetin alfa by IEF double-blotting and chemiluminescent detection [83]. The document was revised in 2007 [84].

Lasne and co-workers explored the interpretation of IEF data by a rational statistical approach, namely discriminant analysis [85]. Using a dataset of 116 rHuEPO-positive profiles from 26 subjects and 131 negative profiles from 131 subjects, they numbered the bands numbered according to a template of 16 possible positions. The band relative intensities were the 16 variables for the statistical analysis. The method was tested with data from an administration trial of low doses (6.7–10 IU/kg) after high-dose (265 IU/kg) injections; 71 profiles from one subject. With 10-fold validation, they had no false positives with 100,000 simulations. They concluded that they achieved improved detection of low doses.

Investigators are working on means to improve the IEF test, to expand its use to all WADA-accredited laboratories, and to further define criteria for determining if rHuEPO is present. In addition, investigators are working on ways to detect erythropoietic proteins with mass spectrometry.

Darbepoetin alfa detected at Winter Olympic Games of Salt Lake, 2002

As the IOC-accredited laboratory designated to perform the testing at the Games of Salt Lake, our laboratory began implementing the IEF technique for detecting erythropoietic proteins about 8 months before the Games. Since darbepoetin alfa was likely to be approved by the FDA before the Games, we were aware that athletes might have access to it at the time of the Games. Accordingly, we obtained a urine from a patient being treated with darbepoetin alfa. As shown in Figure 1, the electropherogram of this urine confirmed that the isoforms of darbepoetin alfa were more acidic than those of rHuEPO [86] because the former migrated to the more acidic region of the IEF gel. This sample was important because IOC laboratory regulations require that analyses include a reference standard, a known positive quality control urine, and a known negative quality control urine.

During the Games of Salt Lake, our laboratory analyzed 598 urine samples for the drugs on the IOC List of Prohibited Substances. In addition, 1,222 blood samples were obtained from athletes competing in the endurance sports of cross country skiing, biathlon, Nordic combined, long-track speed skating, and short-track speed skating. These blood samples were tested at the venues by international sport federations before competition for hemoglobin and reticulocytes.

If the reticulocytes exceeded 2.0% or if the hemoglobin concentration exceeded 16.5 gm% for women or 17.5 gm% for men, the athlete returned after competition and an additional blood and urine were obtained for EPO analysis at the laboratory. Of the 1,222 blood samples, 133 (10.6%) had reticulocytes >2%, and eight samples (0.6%) had increased hemoglobin concentrations [87]. In total, the laboratory received 77 combined blood and urine samples and of these, the samples from three athletes met our criteria for dar-

bepoetin alfa. All three athletes were cross country skiers. Together they had won eight medals.

According to the procedure in effect at these Olympics, before announcing test results to the public, a committee reviews the laboratory findings and two hearings are held. The hearings, which are attended by the representatives of the athlete with or without the athlete, delve into the details of the testing. If the testing results are upheld, the IOC Executive Board reviews the case and makes the final decision to announce the findings to the public. In addition, the athlete has the right to appeal the IOC decision to the CAS. As there is little to lose, most athletes do appeal to the CAS. The darbepoetin alfa cases were adjudicated by the CAS in 2003 and 2004. As a result, the three skiers returned all eight medals, and the athletes who crossed the finish line behind them were moved up in the rankings.

Epoetin alfa and darbepoetin alfa detection studies

Athletes do not reveal their doses or dosing regimen, thus the only experimental approach is to administer likely doses and analyze the urine at various times after the last dose. The early work of Wide et al. [79] established that their gel electrophoresis assay detected urinary rHuEPO for up to 24 h after administering rHuEPO at 60 IU/kg/week for 7–9 weeks. We have used the IEF method [81] to determine the approximate time-course of detection of epoetin alfa in urine. We gave 150 IU/kg/week to 15 healthy young subjects for 3 weeks. If the hematocrit exceeded 48%, the next dose was withheld. After discontinuing the epoetin alfa, all urines were positive by IEF for 2 days and about 50% were positive for 4 days.

We have also determined that darbepoetin alfa is detectable in urine for up to 12 days. A modest single dose (0.40 μg/kg) was detected in eight of nine subjects for 2–4 days post-administration and in some subjects for up to 12 days. Thus, the detectability of darbepoetin alfa appears to be similar or longer than that of rHuEPO, which is consistent with darbepoetin alfa's longer serum half-life [88].

One limitation of detection-time investigations is that they do not necessarily mimic the dosage regimens used by athletes. Typically athletes become aware of the retrospectivity of a test and adjust their doping schedules so as to evade detection. In the case of a rapidly evolving new test, such as the IEF test for urinary rHuEPO, athletes are undoubtedly searching for test-beating options as diligently as laboratory scientists are working on improvements. Perhaps the athletes who got caught using darbepoetin alfa at the 2002 Games of Salt Lake did not expect the test to detect it.

The effects of microdose recombinant human erythropoietin regimens in athletes

Ashenden and co-workers assessed the plausibility of anecdotal reports that microdosing with rHuEPO can allow athletes to escape detection by doping control tests [89]. Two men first received high initial doses of rHuEPO (six

injections of approximately 260 IU/kg injections between days 0 and 11) with a single treatment of iron (100 mg), aiming for 170 g/L hemoglobin. For the next 3 weeks, microdoses (<10% of initial dose) were injected every 2–3 days and adjusted based only on hematologic parameters, e.g., reticulocyte counts, hemoglobin values remained increased. Most urine samples collected >24 h after a microdose tested negative, as well as some samples collected 12–18 h after a microdose. The urine test showed the reappearance of endogenous EPO during microdosing. The authors recommend that authorities supplement the urine test with an approach with greater retrospectivity and confirm that tests must be done out-of-competition to be effective.

Lamon and co-workers estimated the detection window of darbepoetin alfa after one subcutaneous injection (4,769 IU or 40 μg or 70 IU/kg) in three men as close to 7 days, approximately twice as long as for rHuEPO (epoetin beta) (4,000 IU or approximately 70 IU/kg) [90]. The detection window was shortened in subjects with a naturally increased or stimulated EPO production rate. The authors recommend revising WADA criteria for darbepoetin alfa from IEF band intensity ratio to the position and specific distribution of the bands in the most acidic area of the gel, a less conservative set of criteria.

Retrospectivity of the urinary IEF test

The ability of the urinary IEF test to control the abuse of erythropoietic proteins among athletes is a complex function of dose, dosing regimen, detection times, urine collection time relative to last dose, and whether the test is announced in advance or a surprise (short-notice or out-of-competition testing). Sport administrators control the testing time and it is generally agreed that unannounced short-notice testing is the most effective deterrent. The athlete controls the dose and dosing interval, and the ability of the test to detect erythropoietic proteins is related to the urinary pharmacokinetics of the protein and the inherent sensitivity of the test. A ‘good’ test is one that can detect the erythropoietic proteins for as long as the beneficial effects of the drug are present. The interplay of these factors is only beginning to be explored.

The pharmacodynamic effects of erythropoietic proteins that are particularly pertinent in the context of detecting users and that have been studied in healthy subjects, as opposed to patients with anemia, are the effects on hematocrit, hemoglobin, and $VO_{2\,max}$. The effect on $VO_{2\,max}$ is the most relevant because it is a direct measure of performance. rHuEPO administered at doses of 150–230 IU/kg/week for approximately 3 weeks produces an increase of $VO_{2\,max}$ of 6–8%, as expected [48–50]. Only two studies have monitored $VO_{2\,max}$ after rHuEPO was discontinued. In the first, rHuEPO was administered for 25 days (150 IU/kg/week) and 4 weeks after the last dose the $VO_{2\,max}$ determined was not distinguishable from the $VO_{2\,max}$ of the placebo group [91]. It has been suggested that athletes may use a low-dose maintenance regimen that is sufficient to maintain the hematocrit just beneath the 50% ‘health’ test threshold [49, 81]. To explore the effects of a low-dose regimen on $VO_{2\,max}$, Russell et al. [49] administered rHuEPO for 3 weeks at a dose of

150 IU/kg/week, then lowered the dose to 60 IU/kg/week for an additional 5 weeks. They determined that the $VO_{2\,max}$ in week 12, 4 weeks after the last dose, was still increased (3.1% to 4.5%). The hematocrit remained increased for about 17 days after the last dose. Although these results need to be confirmed, the data on low doses of rHuEPO suggest that for a urine test for rHuEPO to be effective it should be capable of detecting rHuEPO for 3–4 weeks after discontinuing a dose of 60 IU/kg/week. Such data are not available.

Another approach to the retrospectivity issue is to assume that the effect on $VO_{2\,max}$ lasts as long as the hematocrit or hemoglobin remain increased. While this assumption has not been directly investigated, it is reasonable to assume that the duration of action on $VO_{2\,max}$ will correlate with hematocrit and hemoglobin. In the three studies [49, 50, 91] that measured hematocrits after rHuEPO was discontinued, a fixed dose of rHuEPO (80–230 IU/kg/week) was administered for 3–5 weeks. During the rHuEPO administration phase, the hematocrit steadily increased until a plateau of approximately 50% was reached at 12–14 days. The hematocrit-time graphs of these studies show that the hematocrit is unchanged (approximately 50%) for 12–20 days after rHuEPO is discontinued. The hematocrit was last measured on days 24, 28, and 30 post administration day; for each study, the hematocrit was still greater than that of the placebo group. Thus, performance as measured by $VO_{2\,max}$ and the hematocrit appear to follow more or less parallel time courses, again consistent with 3–4 weeks of enhancement after the last dose. An athlete could be enhanced and yet have a negative urinary IEF test.

Between lab comparisons of IEF results

Lundby et al. [92] administered rHuEPO to eight subjects, split each urine into two aliquots, and sent them to two WADA-accredited laboratories for EPO analysis by the WADA-approved IEF method. The subjects received rHuEPO every other day for 2 weeks (boosting period), followed by 1 weekly treatment over the next 2 weeks (maintenance period). Both laboratories reported negative results as expected for the eight baseline urine samples, however Laboratory B reported no positive results in the 52 samples collected during boosting and maintenance period and for the 2 weeks after. The results from Laboratory A were positive in all eight samples collected during the 2 weeks of boosting period, in six of the 16 samples collected during the maintenance period, and in two of the 20 samples collected after discontinuation of rHuEPO. Although none of the samples analyzed in Laboratory B fulfilled WADA criteria for reporting positive, five of 18 samples collected during the maintenance period were classified as ‘suspicious’. Unfortunately, no electropherograms were presented so the reader could not evaluate the quality of the analyses, the characteristics of the ‘suspicious’ samples, or compare the data from the two laboratories. This study is the first between-laboratory study that has been published thus there is no way to know how common these discrepant findings are. At the time that this chapter was written, considerable controversy had arisen over the veracity of the findings.

Other erythropoietins (follow-on biologics)

Until relatively recently, there was only one rHuEPO and only one manufacturer. Now the numbers of both rHuEPOs and manufacturers are proliferating. The term 'follow-on biologic' or biosimilars indicates that they are not identical to each other due to factors such as the cells of origin, and specific processes of production, purification, and formulation [93]. Athletes will experiment with these agents as soon as they become available. If laboratories are going to keep up with this fast-moving field, they need detailed information on the new products, a reference standard of the product, and at a minimum, a urine sample collected from a subject known to be receiving the substance. Ideally, the laboratories would administer the substance to subjects to obtain data on the isoform patterns and the pharmacokinetics of detection. These requirements are not easy to fulfill, particularly in the United States where ethics committees generally require that the drug be available as an FDA-approved product. If not, the investigator must file an application for an Investigational New Drug (IND), a lengthy, complicated, and expensive process. Alternatively the investigator may collaborate with colleagues who are working under an FDA-approved protocol.

rHuEPO (Epogen) has been and will continue to be one of the most important drugs that was ever developed. It has remarkable efficacy in the clinic and has improved the lives of a great many people with anemia and renal disease. Now that the original patents have expired a host of follow-on biologics with erythrocyte-stimulating properties are here or coming [93]. In addition, fusion proteins, second- and third-generation EPOs, EPO dimmers, and many more are in various stages of development. All show some degree of erythropoiesis stimulation, and therefore can be justified as legitimate pharmaceuticals being developed to help patients. The anti-doping movement has no way to know if someone is purposely developing these substances to aid and abet cheaters. We hope their use for legitimate medical purposes vastly overwhelms the uses for doping.

Epoetin alfa, epoetin beta, and darbepoetin alfa

The current IEF test [81] is able to detect administration of epoetin alfa, epoetin beta, and darbepoetin alfa, which enables sport to control these erythropoietic proteins provided that the urine is collected when sufficient amounts of the material are present. Much work is needed on the time-course of detection after various doses have been administered. A few such studies are underway.

Epoetin omega

This epoetin has been administered to hemodialysis subjects with anemia [94] and some information is available on its structure [95]. It is produced in baby hamster kidney cells and is less acidic than epoetin alfa or epoetin beta. In the current IEF assay, a reference standard of epoetin omega migrates to the most basic area of the electropherogram. Further, we recently encountered a urine

sample with isoforms consistent with epoetin omega. The isoelectric profile of epoetin omega shows that, compared with epoetin alfa, it has several additional bands in the basic region [96].

Epoetin delta

Epoetin delta (Dynepo) was released in 2007 in Europe for the treatment of the anemia of chronic renal disease and for end-stage renal disease with dialysis. It is the first rHuEPO on the market that was produced in cultured human cells. Its electropherogram profile is readily distinguished from that of epoetin omega and the combination of epoetin alfa and epoetin beta [96].

PEGylated epoetin beta (CERA)

Continuous erythropoietin receptor activator (CERA) is a third-generation erythropoiesis stimulating agent [97, 98]. It is used to correct anemia and maintain hemoglobin concentrations in patients with renal failure. CERA is epoetin beta that has been PEGylated. Its detectability in urine has not yet been demonstrated and there is some concern in the anti-doping context that its high molecular weight may prevent it from being excreted into urine.

Hematide

Hematide is a PEGylated synthetic dimeric peptide that binds to and activates the EPO receptor (EPOR) and causes proliferation and differentiation of erythroid progenitor cells [99, 99–101]. Its chemical structure is unrelated to any of the erythropoietins. In a Phase I study, Hematide produced a dose-dependent increase in hemoglobin concentration and reticulocyte numbers. Currently, it is in a Phase III clinical trial [101]. No means to detect Hematide in humans has been described yet.

Future of detecting rHuEPO and follow-on biologics in body fluids

After less than 7 years of only having to contend with rHuPO and darbepoetin alfa, the WADA criteria for reporting the finding of a recombinant form of EPO have to be modified to cover the follow-on biologics. The delta and omega epoetins and many more that we have studied in the laboratory have more basic bands than epoetin alfa and epoetin beta. Although no published studies show electropherograms after administration to humans, we can be quite sure that the extra bands will appear in clinical samples as extra bands in the basic region. These electropherograms will not fit the current WADA criteria for reporting and they will have to be distinguished from exercise patterns. Already laboratories are observing such patterns. Currently, they must be classified as ‘negative’ even though there is compelling evidence that some are true positives. This frustrating situation may be overcome by revising the WADA TD on EPO criteria, however it is more likely that additional criteria such as longitudinal blood indices will be added to the definition of a positive.

Characterization by SDS-Page mobility

Kohler and co-workers took a new approach based on sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) gels, using two internal standards (darbepoetin alfa and recombinant rat EPO) to calculate the relative mobility of endogenous EPO and recombinant analogs [102]. The analysis of urine samples from a reference group of 53 volunteers (30 men, 23 women) and from one volunteer who received epoetin delta (man, single subcutaneous injection, 50 IU/kg, 4,000 IU) led to significant discrimination between endogenous and rHuEPO

Characterization by mass spectrometry

Experiments conducted by Stubiger et al. [103] with reference standards of epoetin alfa, epoetin beta, and darbepoetin alfa show that they can be characterized and differentiated by mass spectrometry, based on exact molecular weight determination on intact glycoproteins by matrix-assisted laser desorption/ionisation (MALDI) and time-of-flight (TOF) mass spectrometry. Application to the testing of human samples would begin with enrichment from body fluids. MALDI-TOF analysis of enzymatically desialylated molecules improved mass spectral resolution by decreasing microheterogeneity. De-*N*-glycosylation also improved resolution, but not as much.

LC-MS-MS test for rHuEPO in horses

Because animal athletes are used by humans for sport, entertainment, or gain, it is no surprise that the abuse of recombinant EPO has surfaced in both equine and canine races [104–106]. Administration of rHuEPO to horses causes the production of antibodies that may cross-react with endogenous EPO and cause anemia and even death. Antibody detection is the simplest approach to a test. For confirmation purposes, Guan and co-workers have proposed a liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) to identify rHuEPO and darbepoetin alfa in equine plasma [107]. After immunoaffinity enrichment with anti-rHuEPO antibodies linked to magnetic beads and trypsin digestion, LC-MS-MS analysis allows identification of proteotypic peptides. The limit of detection was 0.1 ng/mL. One research horse received rHuEPO on two occasions and it could be detected for 24–48 h afterwards. Another research horse received darbepoetin alfa weekly for 7 weeks and it could be detected for 120 h after the last injection. The first test from this group could not differentiate rHuEPO from darbepoetin alfa [108]; however the second test could [107]. Both recombinant erythropoetins were found in the serum of four racehorses. The authors present the method as sensitive and reliable. They believe in its deterrence value. Applying tests and sanctions more often would improve effectiveness.

EPO gene manipulation

International sport organizations are mindful that gene manipulation might be used in the future to produce athletes with exceptional characteristics. At the

first meeting of the IOC Gene Therapy Working Group, gene therapy was defined such that it would not enhance athletes' performance ("…transfer of genetic material to human somatic cells for the treatment or prevention of disease or disorders") [109]. Further, the group opined that gene manipulation is not on the immediate horizon, but that methods such as proteomic analysis might be developed so as to be prepared when gene-based doping becomes a reality.

Participants in the third Gene Doping Symposium organized by the WADA and Russian sport authorities in Saint Petersburg agreed that there was no evidence that gene doping was being used now (2008); however, some investigators have been approached by athletes. Further, it was noted that serious conceptual and technical problems continue to plague clinical investigators, and patients have experienced severe and unanticipated setbacks and adverse events, including death and the induction of leukemia. WADA is currently spending approximately 25% of its research budget on detection of gene doping.

One example of the research on 'gene doping' with EPO is a report that cDNA in primate (cynomolgus macaque) muscle is detectable by the IEF test [110]. The test was applied to serum instead of urine. It distinguished endogenous EPO from the product of *in vivo* gene transfer (transfer of homologous cDNA into skeletal muscle by injection of recombinant adeno-associated virus).

Detecting homologous blood transfusion

Nelson and co-workers proposed to prove homologous blood transfusion by quantitation of blood group antigens [111]. This approach exploits the likelihood of differences in minor blood group antigens probed with 12 antisera used with a secondary antibody directly conjugated with fluorescein to label IgG-coated red blood cells. The method was applied to blood samples from 25 patients who had received blood transfusions, to determine percentages of antigen-negative and positive red cells. Mixed red cell populations were detected in 22 of 25 transfused patients. The three patients who were 'negative' for transfusions turned out not to have received their scheduled transfusions. The technique can detect a second population represented by <5% of red cells. Apart from doping, mixed cell populations can arise as a result of disease, or after bone marrow or peripheral blood stem cell transplantation, or through blood transfusion. Rare occurrences are hemorrhage between women and fetus, intrauterine twin-twin transfusion, or in the rare tetragametic chimeras. Increasing the number of antisera used in the test would increase the test's effectiveness.

Voss and co-workers validated a flow cytometry approach to detecting homologous blood transfusion, using eight primary antibodies (an improvement over the 12 in Nelson's method) and two secondary antibodies [112]. For each primary antibody, Voss et al. established linearity, specificity, recovery,

precision, robustness and interday-precision. Signal amplification resulted in better separation of mixed populations. Their method can detect a second population represented by 0.3–2.0% of red cells, an improvement over the 5% or less in the method of Nelson et al. [111].

Arndt and coauthors reviewed the detection of allogeneic (homologous) blood transfusions by flow cytometry and highlight the power of testing blood samples taken at different times because it provides an opportunity to detect a decrease in percentage of a minor red blood cell population as an indication that it arose from transfusion [113]. After the 2004 Vuelta de Espana cycling, stage road race, flow cytometry showed the presence of a mixed red blood cells population in one athlete, who was charged with a doping violation, namely for testing positive for transfusing another person's blood. The athlete appealed before the American Arbitration Association, who denied the appeal in 2005 [114]. The athlete's second appeal before sport's highest court, the International CAS), was denied in 2006 and a 2-year suspension imposed [115]. The CAS panel had considered the athlete's theoretical explanations including a disappearing twin, unsubstantiated problems with his positive test results, and even an extortion plot by a fan of another team.

Summary

The inherent complexity of the topic stems from interface between human behavior and the many disciplines that have something to offer – chemistry, pharmacology, medicine, law, ethics, and sociology, to name a few. Ultimately if sport continues to rely on testing as the solution, it is necessary to focus resources on physical and chemical methods for detecting drugs. At the same time sport hopes to change its culture through education and espousing ethical positions.

Blood doping, the most recalcitrant and threatening problem in recent years, goes through successive phases of coming under control and growing in a new direction. Threatening because the drugs are highly efficacious, and they enhance performance in endurance sports and perhaps even sports that rely on short bursts of energy. Recalcitrant because the methods to detect erythropoietic proteins in body fluids are complex, therefore global implementation is all the more difficult. This problem is in addition to sports usual vexing problem of tracking athletes in their travels and implementing collection procedures wherever they may be.

The current IEF test together with the current WADA reporting criteria are adequate from the perspective of unambiguous identification of epoetin alfa, epoetin beta, and darbepoetin alfa, and legal defensibility, however the test is probably not sensitive enough to detect those three recombinant erythropoietic proteins for as long as performance enhancement will last. Fortunately such a degree of sensitivity is not required to contain the use of these three products: the current test simply needs to be applied widely enough in short notice, out-

of-competition testing. Meanwhile, the WADA is working on updating criteria and exploring new approaches to move ahead of the problem.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Role and regulation of iron metabolism in erythropoiesis and disease

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Introduction

Iron is an essential element for normal cellular metabolism and growth as an enzyme cofactor, heme constituent and oxygenation sensor. In excess however, free iron is toxic. Living organisms have, therefore, evolved sophisticated and tightly regulated mechanisms to control iron uptake, transport, and release. Defects in any part of this process can lead to disease. For example, excessive uptake can lead to systemic iron overload and associated toxicity. Inappropriate or inefficient transport can lead to iron maldistribution or deficiency. Red blood cells are the primary consumers of iron and the largest body iron pool (approximately 50% of the body's iron is incorporated in heme); hence, fluctuations in iron supply can have significant effects on red blood cell production and function. This chapter provides an overview of the proteins and pathways involved in iron metabolism as they relate to normal red blood cell biology and disorders of iron excess or deficiency.

Molecular coordination of iron metabolism

Iron uptake from the diet

Absorption of iron from the diet involves two discrete steps: uptake of iron by the intestinal epithelia from the lumen of the gut and transfer of iron into the blood. Dietary iron is found in the form of inorganic ferric (Fe^{3+}) or ferrous (Fe^{2+}) ions (primarily from nonanimal sources) or heme (from hemoglobin or myoglobin). Most inorganic iron is in the ferric form, which readily precipitates if the pH is raised above 3. Precipitation is prevented by association of the iron with other dietary components such as amino acids, citrate, or sucrose. Transport of ferrous iron across the intestinal mucosa is thought to be mediated primarily by the divalent metal transporter 1 (DMT1) in the duodenum [1]. Other proteins such as the zinc transporter Zip14 have been suggested to have a role in this transport [2]. Both DMT1 and Zip14 transport ferrous iron and

reduction of ferric iron is thought to be achieved by the ferrireductase duodenal cytochrome b (DcytB) [3]. However, mice lacking DcytB do not show signs of iron deficiency or abnormal erythropoiesis, suggesting there may be other proteins or components capable of reducing ferric iron in the gut [4].

Heme is the main source of organic iron in the diet and elucidating the mechanism of heme uptake is an evolving area. As heme is hydrophobic, it may diffuse across the enterocyte membrane. Alternatively, uptake may be receptor mediated. Recently heme was shown to be taken up in an energy-dependent process by the heme carrier protein 1 (HCP-1), a protein that also transports folate [5, 6]. It is also possible that heme may enter the enterocyte by associating with a cell surface receptor that mediates endocytosis. Data suggest that heme enters the cell intact [7]. Heme degradation within the enterocyte may be mediated by heme oxygenase and the released iron likely joins the same intracellular pool as inorganic iron. Once inside the enterocyte, iron may be used immediately, stored, or transported to the basolateral surface for export. Figure 1 illustrates the key molecules governing iron movement through the duodenal enterocyte into the plasma iron pool.

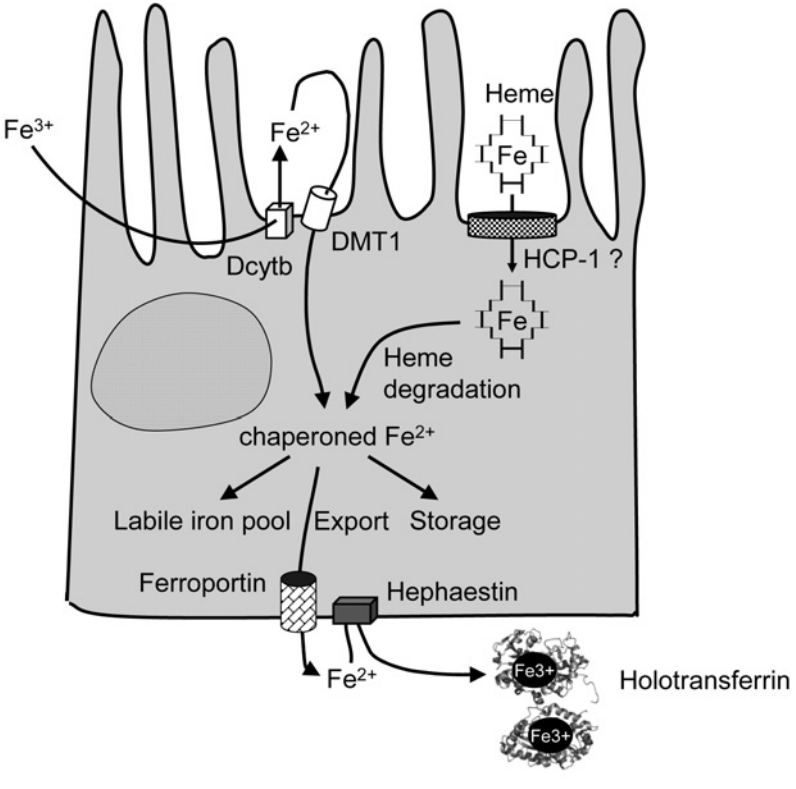

Figure 1. Molecular mechanisms of intestinal iron uptake. See text for further explanation.

Iron export

Iron is exported from the basolateral membrane of enterocytes through the multipass transmembrane protein ferroportin, which like DMT1, transports ferrous iron. In addition to a role in the enterocyte, ferroportin is also required for iron transport across the placenta and is important for iron export from macrophages [8]. Oxidation is required for iron release from ferroportin, suggesting an intimate relationship between ferroportin and an associated ferroxidase. In the enterocytes, this oxidation step is mediated largely by hephaestin, an enzyme with a copper-containing active site [9]. Oxidation in other tissues is mediated by ceruloplasmin, a copper-containing protein that is closely related to hephaestin [10]. Ceruloplasmin is present in the circulation and is also anchored to the cell surface of some cell types. Approximately 90% of the copper in serum is associated with ceruloplasmin. Both copper deficiency and aceruoplasminemia can cause anemia, emphasizing the importance of these ferroxidases in the iron metabolism pathway [11]. After oxidation of iron to the ferric form, it is loaded onto transferrin for transport to distal sites in the body.

Cellular iron uptake

Under normal conditions, most of the iron in the circulation is bound to transferrin. In this state, iron is not reactive; moreover, the association between iron and transferrin is of extremely high affinity and in practical terms, no dissociation occurs in serum ($K_D \sim 10^{-20}$ M) [12]. Uptake of iron from plasma is mediated by association of holotransferrin (fully iron saturated) with a membrane-bound transferrin receptor (TfR1). TfR1 is ubiquitously expressed and binds holotransferrin with high affinity (1–5 nM) [13]. After binding of holotransferrin to TfR1, the complex is internalized and as holotransferrin-TfR1-containing vesicles become acidified (pH ~ 5.5), iron is released [14]. The ferric iron is then reduced to ferrous iron by the ferrireductase Steap3 and transported out of the vesicle by DMT1 to be either stored or used by the cell [15]. After release of iron, the apotransferrin (no associated iron)-TfR1 complex is recycled to the cell surface where apotransferrin dissociates from the receptor and is reused. Figure 2 illustrates the molecules involved in cellular iron uptake. On average, each molecule of transferrin will make 100–200 circuits in its lifetime [16]. The transferrin compartment typically contains about 3 mg of iron at one time and turns over approximately 10 times a day [17].

Iron utilization and storage

Normal individuals have approximately 3–4 g of iron distributed in the following areas: approximately 2 g in red blood cells (hemoglobin), approximately 1 g in hepatocytes (storage), approximately 600 mg in macrophages,

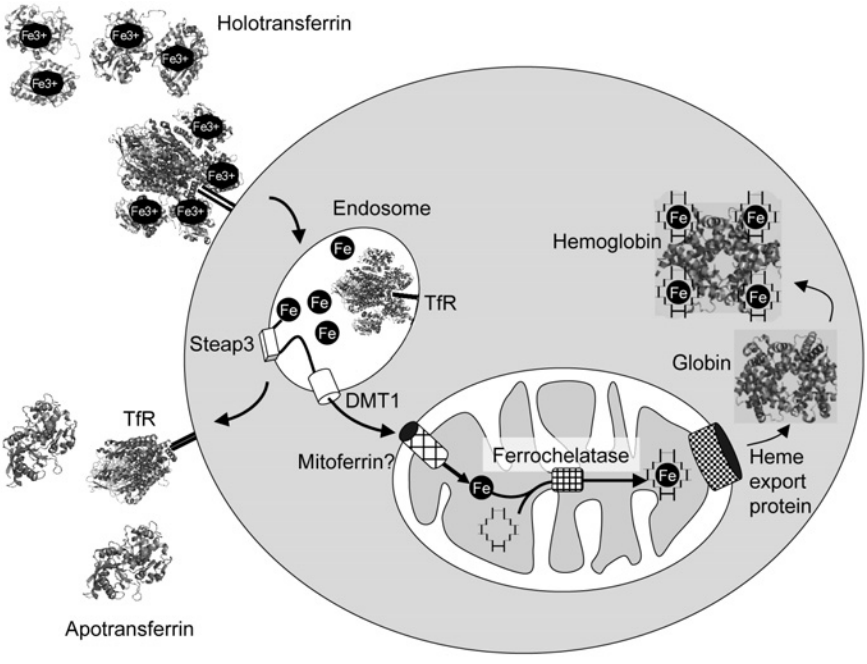

Figure 2. Iron uptake by red blood cell precursors. See text for further explanation.

approximately 300 mg in the bone marrow, approximately 300 mg in muscle (myoglobin), and approximately 3 mg associated with transferrin or other serum transport molecules [18].

Once inside the cell, iron may be incorporated into key enzymes involved in DNA synthesis, electron transport, and ATP production. The mechanisms of intracellular iron transport are not fully understood. Several theories exist to explain iron movement within the cell, such as vesicular-mediated transcytosis or small molecule- or protein chaperone-mediated transport [19]. Surplus intracellular iron within cells is stored in cytoplasmic ferritin, a spherical multimer with 24 subunits capable of storing up to 4,500 iron ions. Ferritin takes up the highly reactive ferrous iron and stores it as ferric iron in a nonreactive mineral core. The change in oxidation state is mediated by the ferritin heavy (H) chain, which has oxidative capability. The remainder of the ferritin molecule comprises the light (L) chain which appears to have primarily a structural function. The ratio of H:L chains in ferritin varies in different tissues. In those tissues where prevention of iron-mediated oxidative stress is critical, such as the heart or neurons, the ratio of H:L chains favors the H chain. This ratio is reversed in the liver, spleen, and microglia. Ferritin expression is increased in response to intracellular iron concentrations and oxidative stress [20]. Ferritin is also found in the serum and is believed to be the result of leakage from damaged or iron loaded cells or secretion in response to inflamma-

tion as an acute phase reactant. Serum ferritin is L-rich and differs from intracellular ferritin in that it is reported to contain less iron and be more heavily glycosylated [21, 22].

Iron in red blood cells

On average 200 billion new red blood cells are produced each day requiring approximately 20 mg of iron for hemoglobin synthesis. Reticulocytes acquire iron primarily through transferrin, although ferritin uptake is also possible [23]. TfRs are expressed on red blood cell precursors from the BFU–E stage through the late reticulocyte stage [24]. Hemoglobin accumulation is first apparent during the polychromatophilic erythroblast stage. Hemoglobin synthesis continues after the nucleus is ejected and stops when the mitochondria and ribosomes are degraded. Eventually each red blood cell will contain approximately 300 million hemoglobin molecules [25].

Normal adult hemoglobin is a tetramer of four globin chains (mainly $\alpha_2\beta_2$) each with an associated heme (iron and porphyrin) molecule. Heme synthesis occurs in the mitochondrial matrix. As a result, iron that is taken up from the serum by TfR1 must be transported from the endosome, through the outer and inner mitochondrial membranes, and into the mitochondrial matrix. This process is not well defined. Transport across one or more of the mitochondrial membranes may be mediated by mitoferrin [26]. In the mitochondrial matrix, iron can be incorporated into heme, used for iron sulfur cluster formation, or stored in mitochondrial ferritin [27]. Once heme is generated, it is exported out of the mitochondria for subsequent combination with globin. A number of candidate molecules may be involved in heme export, including FLVCR [28], ABCG2 (a xenobiotic transporter also known as the breast cancer [chemotherapy] resistance protein) [29], and the ABC-mitochondrial erythroid transporter (ABC-me) [30]. The process of iron uptake, transport and incorporation into heme is rapid. *In vitro* studies using reticulocytes indicate the process requires only 6–8 min at 37 °C [31]. *In vivo* studies indicate the process occurs in less than an hour [32]. Figure 2 shows the molecules involved in iron transit within the mitochondria and incorporation into hemoglobin.

Defects in mitochondrial iron uptake, transport, or metabolism may play a role in diseases such as myelodysplastic syndrome, some sideroblastic anemias, or lead poisoning [33, 34]. Although the etiology of these disorders is often unknown, they are characterized by the presence of sideroblasts in the bone marrow. Sideroblasts are abnormal erythroblasts containing iron-loaded mitochondria that cluster around the nucleus. Because of this unique morphology, they are often called ringed sideroblasts [35]. This abnormal accumulation of mitochondrial iron may be a consequence of impaired iron management or impaired porphyrin or globin coordination.

The biosynthesis of porphyrin and globin are tightly correlated with iron homeostasis in erythroblasts. The first and rate limiting step of porphyrin

biosynthesis is mediated by erythroid-specific α-aminolevulinate synthase (ALAS-E). Expression of this enzyme is increased in response to iron levels [36]. Ferrochelatase, the enzyme, which inserts iron into porphyrin, is also upregulated by the presence of an iron surplus [37]. Together this tight regulation ensures that neither free iron nor free porphyrin accumulates, since both are toxic. In addition, both alpha [38] and beta globin [39] synthesis are regulated by heme availability.

Disease states develop in conditions where the carefully orchestrated process of hemoglobin production is disrupted. ALAS-E mutation results in X-linked hereditary sideroblastic anemia due to insufficient production of porphyrin and concurrent iron accumulation [40], which is exacerbated by systemic iron overload [41]. Ferrochelatase mutation causes erythropoietic protoporphyria due to buildup of excessive protoporphorphyrin and unused iron [42]. Defective globin synthesis is exemplified by thalassemia major. Clinical symptoms of thalassemia major include anemia, liver and spleen enlargement due to excessive red blood cell destruction and iron overload due to transfusions and increased absorption. The major cause of mortality in thalassemia major patients is iron overload-related organ damage [43].

Iron recycling from red blood cells

Red blood cells circulate for an average of 120 days. As each red blood cell nears the end of its lifespan, its membrane characteristics change such that it is recognized and phagocytosed by macrophages of the reticuloendothelial system. Changes leading to recognition may include flipping or deterioration of the membrane to expose antigens (e.g., phosphatidylserine, desialylated β-galactosyl residues) [44]. Once phagocytosed, the red blood cells are digested in lysosomes. Hemoglobin is degraded and iron is liberated through the action of heme oxygenase-1 (HO-1). Iron export from the lysosome is potentially mediated by DMT1 or NRAMP1 (a member of the divalent metal ion transporter family) and may be used by the cell, stored, or exported [45, 46]. Ferroportin and HO-1 are both upregulated after erythrophagocytosis suggesting that liberated iron can be exported via ferroportin [47]. Figure 3 presents a schematic of the proteins involved in iron recycling from senescent red blood cells by macrophages of the reticuloendothelial system.

Systemic coordination of iron metabolism

Iron uptake, redistribution and storage in the body are coordinated by hepcidin, a 25 amino acid peptide concurrently identified by three groups and subsequently shown to be the protein product of the *HAMP* gene [48–50]. Under conditions of iron excess, hepcidin production is stimulated in the liver and limits iron uptake through the gut while also causing iron sequestration

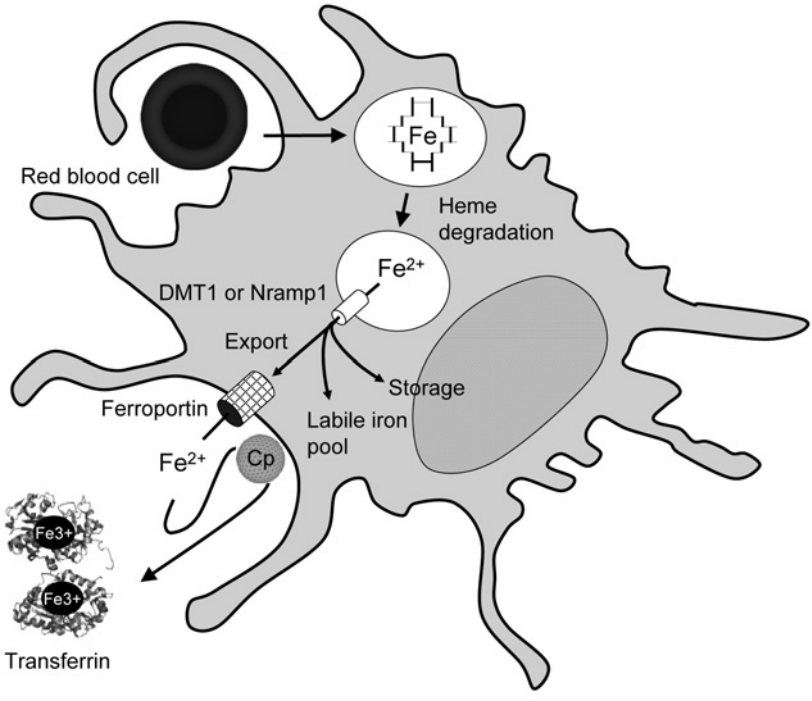

Figure 3. Iron recycling from red blood cells by macrophages of the reticuloendothelial system. See text for further explanation.

in tissues [51, 52]. Conversely, under conditions of iron limitation, hepcidin concentrations are lowered and gut uptake and release from the tissues is increased [53]. Hepcidin mediates these effects by binding to ferroportin and stimulating its internalization and degradation [54]. In macrophages of the reticuloendothelial system, degradation of ferroportin results in iron sequestration. In the gut, hepcidin activity inhibits release of dietary iron into the circulation.

Hepcidin is expressed mainly in the liver, with some expression in the heart, kidney, adipose tissue, spinal cord, pancreas, hematopoietic cells, retina and brain [49, 55–59]. Hepcidin was originally cloned as the product of a gene up-regulated by iron in mice [50]. It was identified in parallel by other groups as a protein present in blood ultrafiltrate from patients with renal failure [48] and in the urine of healthy donors [49]. Insight into its controlling role in iron regulation came from the fortuitous observation that mice with a disrupted *USF2* gene also lacked expression of hepcidin in the liver [60]. As a result, the mice were unable to regulate iron uptake, and they developed an iron loading condition indistinguishable from hereditary hemochromatosis in humans. Subsequent studies using a hepcidin-specific knockout mouse confirmed that hepcidin alone can account for the original phenotype [61].

Transgenic overexpression of hepcidin in mouse liver resulted in severe iron-deficiency anemia [62].

Mutations in the human hepcidin gene have been identified which result in juvenile onset hemochromatosis, a severe form of hereditary hemochromatosis resulting in extensive iron loading early in life [63]. Hepcidin has been shown to be overexpressed in liver adenomas associated with glycogen storage disease and linked to an iron-refractory anemia [64].

Hepcidin expression changes in response to fluctuations in amounts of systemic iron. Modulation of hepcidin transcription appears to be mediated by a number of proteins that may associate to form a large iron sensing complex [65]. These proteins include hemojuvelin (HJV), HFE, transferrin receptor 2 (TfR2), and a subset of bone morphogenetic proteins (BMP) and BMP receptors [66–69]. Activation of this protein complex or a subset of the proteins in this complex induces phosphorylation of SMAD family members, leading to an increase in hepcidin transcription [68]. Hepcidin expression is also regulated by infection, inflammation, or erythropoietic demand [69, 70]. Several inflammatory cytokines upregulate hepcidin expression, including interleukin (IL)-6 and IL-1 [71, 72]. Studies using IL-6 indicate that activation of the JAK/STAT3 pathway is involved in triggering hepcidin transcription [73]. The proteins involved in sensing erythropoietic demand that may in turn influence hepcidin expression have yet to be determined but are the subject of much attention. Hepcidin regulation by erythropoietic demand is distinct from regulation by iron or inflammation as erythropoietic demand downregulates hepcidin transcription whereas increases in iron or inflammation upregulate hepcidin transcription. Much of the understanding of hepcidin regulation has been pieced together over the last few years. The next few years may bring more understanding of how these different types of regulation fit together to control normal and abnormal iron metabolism.

Disorders of iron metabolism

Iron overload disorders

Iron overload can occur through two main mechanisms. Hereditary iron overload (hereditary hemochromatosis) is caused by genetic mutations affecting iron homeostasis. Secondary iron overload is generally a consequence of iron deposits from red blood cell transfusion. Iron loading occurs first in the liver, causing cirrhosis and subsequent end-stage liver disease [74]. Other organs affected are the pancreas, heart, and anterior pituitary, leading to diabetes, cardiomyopathy, and hypogonadism, respectively. Both hereditary and secondary types of iron overload lead to an increased mortality risk due to iron-induced cardiomyopathy, which can be illustrated most clearly in thalassemia major patients with secondary iron overload, who in the absence of iron chelation therapy have a life expectancy of less than 20 years [75].

Hereditary hemochromatosis

Hereditary hemochromatosis results from a number of genetic mutations leading to dysregulated iron uptake and distribution within the body. Increased uptake results in gradual iron loading over time, and is generally diagnosed when serum iron concentrations are inappropriately high and symptoms have arisen due to organ damage caused by excess iron deposition.

There are five types of hereditary hemochromatosis: 1, 2a, 2b, 3 and 4, which correspond to genetic defects in HFE, HJV, hepcidin, TfR2, and ferroportin, respectively. As described above, HFE, HJV, and TfR2 are all suggested to be components of the iron-sensing complex that regulates hepcidin transcription. As would be expected from defects in the proteins involved in hepcidin transcriptional induction, hereditary hemochromatosis types 1 to 3 are all characterized by inappropriately low levels of hepcidin relative to the degree of systemic iron overload [76].

Hereditary hemochromatosis type 1 (*HFE* mutation) is the most widespread form of hereditary hemochromatosis. Approximately 15% of individuals of northern European descent are heterozygous for defects in HFE and 0.5% are homozygous [77]. Despite the number of individuals carrying these genetic lesions, only a very small percent of homozygous individuals (approximately 1%) develop symptoms severe enough to be detected. If disorders do present, the age of onset is 40–60 years for men and after menopause for women [78].

Hereditary hemochromatosis types 2a (*HJV* mutation) and 2b (hepcidin mutation) are the most severe forms of hereditary hemochromatosis [79]. Individuals carrying these mutations start to experience the effects of iron loading within the second or third decade of life. Due to the early age of onset, these conditions are also known as juvenile hemochromatosis. Unlike hereditary hemochromatosis type 1, juvenile hemochromatosis affects both males and females equally [80].

There are few examples of individuals with hereditary hemochromatosis type 3 (*TfR2* mutation); however, the clinical features appear to be similar or perhaps slightly more severe than those observed for type 1 hereditary hemochromatosis and if present with mutations in *HFE* can lead to juvenile hemochromatosis [81].

Hereditary hemochromatosis type 4 (ferroportin mutation) is also termed ferroportin disease. Ferroportin mutations yield two distinct functional outcomes: loss-of-function mutations result in loss of iron export capabilities, and gain-of-function mutations result in uncontrolled iron export due to loss of hepcidin responsiveness. Iron uptake and deposition characteristics vary according to the type of mutation. Loss-of-function mutations lead to iron loading in macrophages, whereas gain-of-function mutations lead to iron loading in parenchymal cells, similar to hereditary hemochromatosis types 1–3.

Diagnosis and treatment of hereditary hemochromatosis

The first indicator of hereditary hemochromatosis types 1–3 is an increase in the percentage of transferrin occupied by iron (transferrin saturation or Tsat). If Tsat is >60% in men or >50% in women and there is no other cause for increased serum iron (e.g., medication or ingested iron), there is a high probability of hemochromatosis. Diagnosis can be confirmed by measurement of serum ferritin, which can be used as a surrogate of body iron stores [82]. Normal ferritin values are <300 ng/mL for men and <150 ng/mL for women. Iron excess can be qualified in terms of serum ferritin values as mild (between normal values and 500 ng/mL), moderate (500–1,000 ng/mL) or severe (>1,000 ng/mL). Treatment of hereditary hemochromatosis types 1–3 and disease caused by gain-of-function ferroportin mutations involves removal of 500 mL of blood once or twice weekly, depending on the degree of iron overload [82]. Each treatment removes approximately 250 mg of iron. Iron overloaded individuals may have an excess iron burden of 30–40 g, requiring several years of regular treatment to bring iron levels into the normal range. Iron chelation is not typically used for the treatment of hereditary hemochromatosis.

Secondary iron overload

Secondary iron overload after transfusion occurs in numerous conditions including thalassemia, sickle cell disease, myelodysplastic syndrome, and hemolytic and sideroblastic anemias. It can also be a significant issue in anemic patients with cancer who require multiple transfusions. In patients with secondary iron overload, iron is derived from transfused red blood cells, which are degraded in macrophages at the end of their lifespan. Because the liberated iron cannot be used to make new red blood cells due the underlying disease, the transfusion-derived iron is stored. The requirement for multiple transfusions causes accumulation of iron to toxic levels that leads to organ damage. On average, each unit of blood introduces 200–250 mg of iron [83] and no mechanism exists to upregulate iron excretion to compensate.

Treatment of secondary iron overload

Secondary iron overload cannot, in general, be addressed by phlebotomy since the patients are anemic. For this reason, the only current treatment option is chelation therapy. Although iron chelation can dramatically increase life expectancy, it has some drawbacks in terms of dosage, compliance, and toxicity. Given the fact that one iron ion requires 1, 2, or 3 molecules of chelator to remove it, daily doses are very high. Side effects differ with the chelator, but examples include neutropenia and agranulocytosis, gastrointestinal side effects (nausea, vomiting, diarrhea), hearing loss, retinal damage, and arthritis [84].

As proteins involved in iron transport and distribution are identified and characterized, novel therapeutics to modulate the activity of these proteins and alleviate secondary iron overload without the use of chelators are being explored.

Iron limitation, anemia, and ESAs

Iron limitation is a frequent cause of anemia and is quoted as the most common cause of hyporesponsiveness to erythropoietic stimulating agents (ESAs) [85]. Approximately 150 mg of iron is necessary to fuel an increase of 1 g/dL in hemoglobin and hence an adequate and continuous supply of iron is required. Red blood cell production may be limited by either absolute iron deficiency, which occurs when body iron stores are exhausted and dietary uptake is insufficient, or functional iron deficiency, which occurs when iron cannot be mobilized from iron stores. Patients with iron-limited anemia may have one or both of these conditions. Although related in outcome, these types of iron deficiency have very different presentations and etiologies. They may also benefit from different treatments.

Absolute iron deficiency

Absolute iron deficiency is a function of increased iron loss (most commonly blood loss), increased iron demand (e.g., growth, pregnancy), or limited iron supply (e.g., poor nutrition, malabsorption). It is an endemic problem in all areas of the world, affecting approximately 20% of the population [86]. Iron deficiency is associated with reduced motor and mental development in children and frequently manifests as anemia in adults. Hemodialysis patients are at particularly high risk for iron deficiency due to blood loss from repeated blood draws, blood loss during dialysis, gastrointestinal bleeding, and poor uptake from the diet. These losses may total 1–3 g/year. Other disorders with occult blood loss such as cancer also have a high incidence of iron deficiency anemia.

Diagnosis of iron deficiency anemia

Before automated blood counters and clinical laboratory analyzers were available, diagnosis of iron deficiency was based primarily on identification of abnormal red blood cell morphology on a peripheral blood smear. The first visual sign of iron deficiency anemia is the appearance of hypochromic (reduced hemoglobin content) and microcytic (smaller in size) erythrocytes mixed with normochromic and normocytic erythrocytes. The iron deficiency anemia diagnosis could be confirmed by examination of stainable iron stores in bone marrow biopsy, if necessary.

With the advent of automated blood cell counting, parameters such as mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) can be accurately quantified, producing standardization in diagnosis and reducing reliance on manual techniques. Because these indices measure the mean values of the circulating red blood cell population and red blood cells have an average life expectancy of 120 days, the patient may be iron deficient for several weeks before a change in MCV or MCH can be detected. To obtain a more real-time assessment of the health of the bone marrow and the state of erythropoiesis, reticulocyte parameters can be evaluated.

Immature red blood cells that are newly-released into the circulation (reticulocytes) contain residual RNA that can be detected by staining techniques. Automated cell counters can be used to measure the number and characteristics of these red blood cell precursors. Reticulocyte numbers have been used to account for reticulocyte growth, a first step in erythropoiesis [87] and reticulocyte mean corpuscular hemoglobin (CHr) is gaining acceptance as a means of detecting iron limited erythropoiesis [88]. Other reticulocyte parameters, such as reticulocyte mean corpuscular volume (MCVr), may also be useful but they have not been explored. Reticulocyte testing provides an additional cost burden to diagnosis of iron deficiency. For this reason, identification of a parameter which could supply similar information but does not require reticulocyte detection would preferable. One potential candidate for this is the percentage of hypochromic red blood cells (%HYPO). Because this parameter evaluates the number of cells at an extreme of the distribution curve as opposed to evaluating the mean of the population, it is easier to detect small changes. Hence, although %HYPO does not give information on the age of the iron deficient cells, it may provide a good assessment of an ongoing mild iron deficiency in a cost-effective way. This test is not used in routine clinical practice but is being tested in exploratory settings [89, 90].

Serum iron parameters can be used to predict iron deficiency and are routinely used in combination with red blood cell parameters. The serum iron indices most commonly used are Tsat and serum ferritin, with Tsat serving as a transient measure of available iron and ferritin, in this case, reflecting accumulated iron stores. Based on data from the Centers for Disease Control and Prevention, normal ranges of serum ferritin vary from 12–300 ng/mL for males and 12–150 ng/mL for females and normal ranges of Tsat vary from 20–50%. Absolute levels of these parameters used to diagnose iron deficiency anemia may vary. According to the Kidney Disease Outcomes Quality Initiative and the National Comprehensive Cancer Network guidelines, an individual is iron deficient when serum ferritin is <100 ng/mL and Tsat is <20%.

Another serum iron parameter that may aid diagnosis of iron deficiency anemia is the concentration of soluble transferrin receptor (sTfR), which is believed to be shed from cells with increased TfR production due to intracellular iron deficit. This parameter is rarely used in isolation, but can be used in combination with serum ferritin (sTfR/ferritin ratio, or R/F ratio) to evaluate body iron stores and determine whether iron supplementation is required [91].

Treatment of iron deficiency anemia

The first line of treatment for iron deficiency is oral iron administration (150–250 mg of elemental iron daily). If oral iron is not efficacious, intravenous iron can be considered. Both forms of iron have associated complications. Oral iron is poorly absorbed and may cause gastrointestinal disturbances. Intravenous iron may induce immune complications, myalgias, arthralgias, headache, and a rare incidence of anaphylaxis. It has also been speculated to increase risk of infection, is associated with increased oxidative products, and may in itself be a risk factor for some disorders such as atherosclerosis [92, 93].

In classical iron deficiency anemia with no inflammation, the amounts of erythropoietin are normal or increased, making ESA treatment unnecessary; however, in patients with iron deficiency anemia who are receiving hemodialysis and who do not have increased amounts of erythropoietin due to impaired or absent kidney function, combination treatment of an ESA and intravenous iron has been shown to improve patient response [94]. In this setting, oral iron has been shown not to be efficacious, presumably due to iron malabsorption as a function of kidney disease.

Functional iron deficiency

Functional iron deficiency occurs when circulating iron is insufficient to satisfy demand. In contrast to absolute iron deficiency where body stores are entirely absent, functional iron deficiency can occur in the presence of adequate iron stores. Functional iron deficiency can result from ESA treatment where demand is greatly increased over a short time frame. ESA treatment can boost the rate of erythropoiesis two- to three-fold [95], and under these conditions, the iron available in serum cannot be replenished quickly enough to fully meet demand and erythropoiesis may be restricted by lack of iron. This type of functional iron deficiency is transient. To compensate, iron uptake and mobilization are induced by suppressing hepcidin levels [69].

Another cause of functional iron deficiency is inflammation. Patients with inflammation show decreased serum iron and increased amounts of hepcidin [96, 97]. Mouse models lacking hepcidin do not show a serum iron decrease when stimulated with inflammation, suggesting that hepcidin is required for induction of functional iron deficiency by inflammation [69]. It has been speculated that functional iron deficiency during inflammation is a means of depriving invading organisms of iron [98–100]. Functional iron deficiency is believed to be a contributing or causative factor in the anemia of inflammation. Anemia of inflammation, sometimes referred to as anemia of chronic disease, is a broad term used to describe anemia which is inflammatory in origin.

Inflammation-induced functional iron deficiency may limit response to ESAs. In patients with chronic kidney disease, the study of inflammatory

markers has demonstrated that a link exists between inflammation, anemia, and relative resistance to ESA therapy [101–107]. Other inflammatory patient populations, such as patients diagnosed with autoimmune conditions, show a correlation between inflammatory status and response to ESA therapy [108]. A subset of patients with cancer, chronic heart failure [109] and those receiving treatment in intensive care units [110] are also predicted to have an anemia of inflammatory origin. Definitive studies correlating inflammation with ESA responsiveness have not been conducted in these patient populations.

Diagnosis of anemia of inflammation

Distinguishing anemia of inflammation from iron deficiency anemia and mixed anemia (containing components of both) is complicated since most of the commonly used diagnostic parameters are influenced by inflammation (present as part of the acute phase response). The ratio of sTfR concentration (a marker of erythroid activity) to the log of the ferritin (Ft) concentration has been described as a means of distinguishing between inflammatory and iron deficiency anemia [111]. Either sTfR or ferritin alone can give information about iron stores or iron mobilization, but both are impacted by inflammation. sTfR concentrations are high in patients with active erythropoiesis or iron deficiency but low or normal in patients with inflammation (blunted erythropoiesis). Conversely, ferritin can be increased due to inflammation or to increased iron stores. Although both ferritin and sTfR are confounded by multiple interpretations when considered independently, they can effectively differentiate between iron deficiency and inflammation when used in combination. Anemia of inflammation is reported to be characterized by a low sTfR/log Ft ratio (values <1), while a high ratio is indicative of iron deficiency anemia [111, 112].

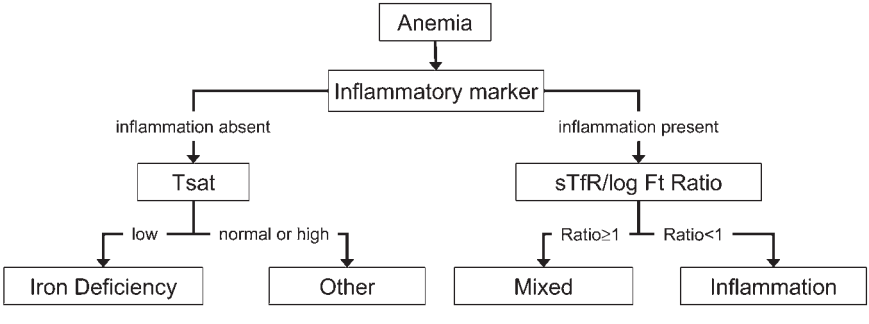

Figure 4. A potential diagnostic scheme to identify patients with iron deficiency anemia (IDA), anemia of inflammation (AI), anemia of mixed origin (some components of IDA and AI) and anemia of other origins (other).

A diagnostic scheme using inflammatory status, Tsat, and sTfR/log Ft (modified from that proposed by [112]) is presented in Figure 4. This scheme illustrates how existing tests could be used to distinguish anemia of inflammation from iron deficiency anemia and mixed anemia. This diagnostic decision tree relies on first detecting the presence or absence of inflammation and then the presence of iron deficiency. A good candidate for the inflammatory marker may be C-reactive protein, a liver-expressed protein that is upregulated in response to inflammation. Another potential candidate is serum hepcidin although a broadly-available and technically accessible assay would first be required [113, 114]. Further distinction between anemia of inflammation and mixed anemia is achieved using sTfR/log ferritin. Iron deficiency is identified in noninflamed patients using Tsat. This type of approach has not been validated and substantial patient data with treatment outcomes will be needed to define the cutoffs for each of the parameters used in the scheme. Tests for other markers clarifying the relationship between iron metabolism and anemia of inflammation are actively sought, including the measurement of hepcidin, which may simplify diagnosis of anemia of inflammation [113].

Treatment of anemia of inflammation

Anemia of inflammation is a multifactorial disease: in addition to functional iron deficiency, it is characterized by inflammatory impairment of endogenous erythropoietin production, reduced responsiveness of marrow cells to ESAs, and shortened red blood cell lifespan [115, 116]. Administration of ESAs can compensate for decreased EPO production and decreased progenitor responsiveness; however, the reticuloendothelial iron block remains as an obstacle to a complete therapeutic response. Treatment with oral iron provides little benefit as uptake is blocked by the increased hepcidin in these patients. The use of intravenous iron may generate short-term response to ESAs, but the bulk of the parenteral infusion may be rapidly sequestered in the reticuloendothelial system potentially creating reactive iron stores in macrophages. Some studies have suggested that intravenous iron treatment and sequestration may be a risk factor for cardiovascular disease and other disorders [117].

New therapeutic strategies for the manipulation of iron

As understanding of the molecules and mechanisms involved in iron metabolism increases, a number of therapeutic opportunities may emerge. Treatment options for iron excess and deficiency have changed little over the years and equate to either removing (phlebotomy, chelation) or administering iron. These treatments have a number of drawbacks including poor patient compliance, undesirable side effects, and limited efficacy. The hope of future therapeutics is the advent of molecular therapies that will enable the controlled redistribu-

tion of iron, which would prevent inappropriate accumulation in conditions of iron excess and allow adequate mobilization to satisfy demand in conditions of iron deficiency. It would also avoid iron sequestration in potentially dangerous locations. Potential therapeutics for hereditary hemochromatosis may include antagonism of enterocyte transport proteins or chelation of iron in the gastrointestinal tract to inhibit iron uptake from the diet. Administration of hepcidin or a hepcidin agonist could potentially reverse or limit progression of hereditary hemochromatosis as has been suggested by mouse models [118]. For iron overload disorders in general, modulation of proteins involved in iron sensing or transport may provide therapeutic potential.

Treatment opportunities to address functional iron deficiency include direct neutralization of hepcidin or modulation of proteins that regulate hepcidin expression such as HFE, HJV, TfR2, BMPs, or inflammatory cytokines. The ability to downregulate IL-6-induced hepcidin expression in multicentric Castleman's disease (an IL-6 driven disorder) has been demonstrated using a humanized anti-IL-6 antibody [119]. In addition, hypoxia-inducible factor prolyl hydroxylase (HIF-PH) inhibitors are reported to suppress hepcidin expression in addition to stimulating EPO expression [120]. Development of even a few of these possibilities would change the treatment paradigm for many iron metabolism disorders and potentially provide patients with safer and more efficacious treatment.

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Erythropoietins, Erythropoietic Factors and Erythropoiesis
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Nonhematopoietic effects of erythropoiesis-stimulating agents

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Introduction

Erythropoietin (EPO) was originally considered a hormone with action restricted to erythrocytic progenitors. The colony-forming units–erythroid (CFU–E) possess abundant EPO receptor (EPOR) molecules, and EPO is essential for their survival, proliferation, and differentiation. The mature functional human erythropoietic EPOR is a homodimer of two 484 amino acids transmembrane glycoproteins of approximately 60 kDa. On binding of 1 EPO molecule, the EPOR dimer undergoes a conformational change that is transmitted to the cytosolic domain resulting in the autophosphorylation of EPOR-associated Janus protein tyrosine kinases 2 (JAK-2). The tyrosine phosphorylated EPOR dimer exhibits intracellular docking sites for signaling proteins containing SRC homology 2 (SH2) domains [1]. The specific roles of the various then-activated enzymes, such as phosphatidylinositol-3 kinase (PI-3K)/Akt, mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and transcription factors, such as signal transducer and activator of transcription 5 (STAT-5), with respect to survival, proliferation, and differentiation are only partly understood. Clearly, however, the action of EPO requires the presence of functional EPOR and of appropriate EPOR signaling molecules. Activation of the EPOR is terminated by JAK-2 dephosphorylation and internalization of the EPO/EPOR complex [2].

Studies suggest that EPOR is expressed in a variety of nonhematopoietic tissues, including the brain, the cardiovascular bed, and the kidneys [3, 4]; however, the physiologic role of EPO/EPOR system in nonhematopoietic tissues deserves further clarification for several reasons. First, EPOR mRNA expression does not prove the translation of functional EPOR molecules. Unfortunately, the detection of EPOR protein has been rendered difficult due to the lack of appropriate antibodies for use in immunohistochemistry and Western blotting [5]. Second, while EPO/EPOR system is essential for erythropoiesis, nonhematopoietic EPOR may be dispensible under normal conditions. Transgenic mice with a constitutive genetic deletion of *EPOR* are not viable, but the animals develop normally and are fertile after tissue-specific,

re-expression of EPOR in the erythrocytic lineage [6]. Distinct tissue-protective effects of EPO outside the bone marrow, however, are probably relevant under pathophysiologic conditions of organ injury. Signaling through EPOR can prevent apoptotic cell death caused by reactive O_2 species, Ca^{2+} mobilization, and mitochondrial dysfunction resulting from stressors such as hypoxia/reoxygenation, cytotoxic drugs, or mediators of inflammation. The pharmacological use of recombinant human EPO (rHuEPO), of analogous erythropoiesis-stimulating agents (ESA) and of nonerythropoietic derivatives thereof for nonhematopoietic indications, such as neuro-, cardio-, and nephroprotection, is in the focus of nonclinical and clinical research (Fig. 1). In considering EPO a pleiotropic cell survival factor, however, the question of whether administration of the drugs may promote tumor growth [5, 7] must be addressed.

We summarize *in vitro* and *in vivo* studies of the role of EPOR in nonhematopoietic tissues. The available data are considered with respect to the following questions:

- Is EPOR mRNA detectable?
- Is EPOR protein detectable?
- Can EPOR signaling be demonstrated?
- Do effective ESA concentrations match the physiological and/or pharmacologic levels attained in patients treated with ESA?

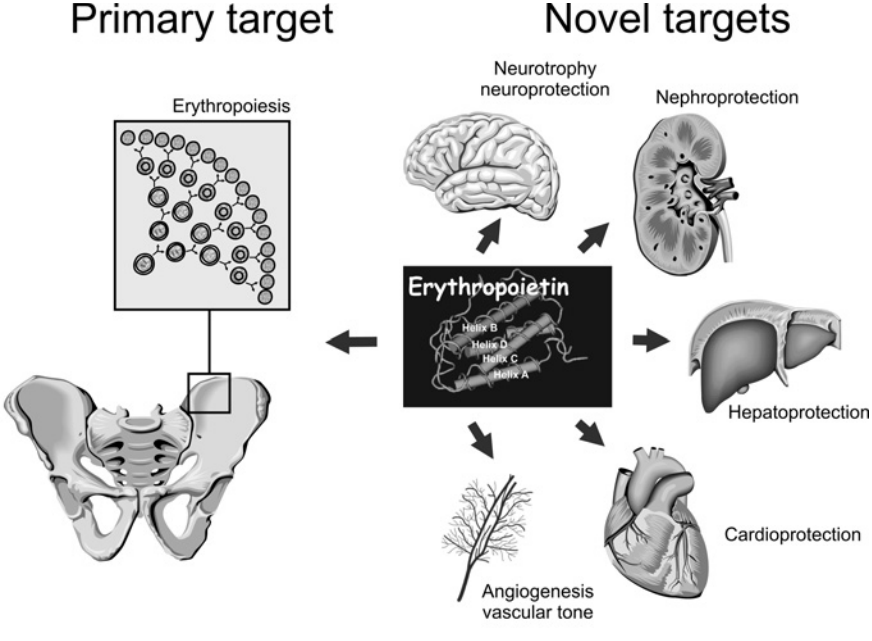

Figure 1. Targets of EPO, respectively its derivatives and analogs. Apart from stimulating erythropoiesis, EPO has tissue protective potential in various other organs.

Effects of ESA in the central nervous system

First evidence for the expression of functional EPOR by neuronal cells was provided 15 years ago with the findings that rat PC12 pheochromocytoma and mouse basal forebrain SN6 cells have EPO-binding sites, and that EPO stimulates the release of monoamines from PC12 cells, augments choline acetyltransferase activity in primary cultures of mouse neurons, and supports the survival of septal cholinergic neurons in rats with fimbria-fornix transactions [8, 9]. Subsequently, investigators detected EPOR mRNA and EPOR protein in defined areas of the brain of rodents, monkeys, and humans, with EPO-binding sites being detectable mainly in the hippocampus, capsula interna, cortex, and midbrain [10]. EPOR mRNA is expressed in the central nervous system during human fetal development at about 7 weeks, and increases from 8–24 weeks [11]. Simultaneously, the *EPO* gene is expressed in brain, where EPO can exert cytoprotective functions separate from the remainder of the body [12]. EPO has been reported to stimulate the generation of neurons from mammalian forebrain neuronal stem cells [13]. In addition to neurons and astrocytes, brain capillary endothelial cells express EPO-binding sites [14]. It was earlier claimed that EPOR can be detected within and around human brain capillaries [15], but the anti-EPOR antiserum used for the study was later shown to be unspecific [16–19]. Thus, the localization of EPOR protein in the brain requires reinvestigation [18].

Most *in vitro* studies aimed at demonstrating effects of EPO on neuronal cells were performed with very high EPO concentrations (≥0.5 IU/mL) with few reports showing positive effects at physiologically low EPO concentrations [20–22]. The concentration of endogenous EPO in the cerebrospinal fluid is normally <0.001 U/mL [23], but the actual concentration in the brain microenvironment is not known, as EPO derived from neuronal and glial cells may act in an autocrine and paracrine way. This fact must be remembered when proposing that EPO can protect neuronal cells against toxic agents and hypoxia/reoxygenation injury. EPO rescues hippocampal and cerebral rat cortical neurons in primary culture from nitric oxide (NO)-induced death [24]. Preconditioning with EPO protects neurons from NO and N-methyl-D-aspartate (NMDA) injury [25]. Preconditioning is not necessarily required for neuroprotection, because EPO prevents apoptosis of neurons when applied simultaneously with hypoxic stress [26]. Inhibition of glutamate-induced damage of cultured neurons may be the primary mechanism by which EPO protects hippocampal and cerebral cortical neurons from death due to hypoxia [27]. Of potential clinical interest is the finding that EPO can protect rat primary hippocampal neuronal cells in culture from damage induced by β-amyloid [28], as this protein contributes to Alzheimer's disease. The neuroprotective action of EPO involves the JAK-2, PI-3K/Akt, MAPK, and NFκB pathways [25, 28] (Fig. 2).

In vivo evidence for the neuroprotective potential of the EPO/EPOR system was provided with the findings that the infusion of EPO into the lateral ventricles of Mongolian gerbils rescues hippocampal CA1 neurons from ischemic damage, while infusion of EPOR causes neuronal degeneration and impaired

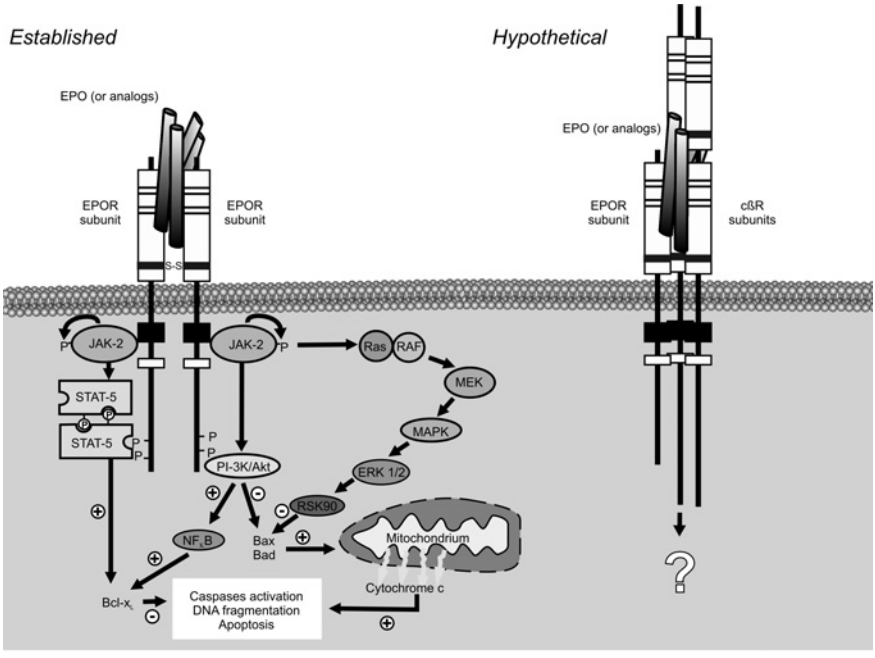

Figure 2. Scheme of the established homodimeric EPO receptor (EPOR) and the heteromeric receptor proposed to function in nonhematopoietic tissues. The antiapoptotic action of EPO on binding to EPOR involves autophosphorylation of JAK-2 (Janus kinase 2), tyrosine phosphorylation of EPOR, homodimerization of STAT-5 (signal transducer and activator of transcription 5), induction of Bcl-x_L, activation of PI-3K (phosphatidylinositol-3 kinase), the G-protein Ras and the sequential activation of the serine kinase RAF, MEK (syn. MAPKK) and MAPK (mitogen activated protein kinase). As a result Bax and Bad are prevented from increasing mitochondrial membrane permeability and cytochrome c release and the activities of caspase 3 and other caspases otherwise executing apoptosis are silenced. Whether EPO and its derivatives and analogs can exert anti-apoptotic effects on binding to a putative heteromeric receptor composed of EPOR and cβR (common β-subunit of the IL-3, IL-5, and GM-CSF receptor) in nonhematopoietic tissues is under debate.

learning ability [24]. EPOR mRNA concentrations increase in the periphery of the cerebrocortical infarct, the so-called ischemic penumbra, in rats with permanent middle cerebral artery occlusion [29]. In this model, the infusion of EPO into the cerebral ventricles reduces the ischemia-induced place navigation disability, cortical infarction and thalamic degeneration. The protective effect of EPO involves the induction of Bcl-x_L, an anti-apoptotic member of the Bcl-2 family [30] (Fig. 2). Using mice with a conditional *EPOR* knockdown, it has been shown that the brain-specific deletion of *EPOR* is associated with reduced cell proliferation in the subventricular zone and a specific deficit in post-stroke neurogenesis through impaired migration of neuroblasts to the peri-infarct cortex [31]. Neuronal cells from mice with a partial *EPOR* knockout restricted to organs outside the hematopoietic tissues are more sensitive to hypoxia/glutamate toxicity than normal neurons *in vitro* and *in vivo* [32]. Thus, EPOR

appears to be required for the development and for repair processes of the central nervous system. Less conclusive evidence is available in proof of the idea that these effects are mediated by a putative heteroreceptor consisting of EPOR and the common cytokine beta receptor (βcR) [33] (Fig. 2). EPOR/βcR hypothesis is mainly based on the observation that erythropoietically inactive carbamoylated EPO (CEPO) provides neuroprotection in animal models [34].

The brain EPO/EPOR system initially was thought to be completely separate from blood-borne EPO [4], because it seemed unlikely that a large glycoprotein like EPO could readily cross the blood-brain barrier. Brines et al. [15] showed that the systemic administration of rHuEPO reduces brain lesions due to focal ischemia, trauma, or experimental autoimmune encephalitis in rodents. Furthermore, the authors reported that 5 h after intraperitoneal administration of biotinylated rHuEPO, biotin was detectable by immunohistochemistry, which was initially localized around capillaries and later (after 17 h) to scattered neurons [15]. Note, however, that the possibility cannot be excluded that the biotin-labeled EPO was degraded in endothelial cells leaving the liberated biotin to move into the brain. In another study, the systemic application of rHuEPO (5,000 IU/kg, administered intraperitoneally) reduced the volume of infarction in rats 24 h after middle cerebral artery occlusion [35]. The neuroprotective effects of systemically administered rHuEPO in experimental animals with acute cerebral ischemia, crush injuries or encephalomyelitis has been confirmed in several other studies [12, 36].

With respect to the potential use of rHuEPO or its derivatives and analogs as neuroprotective agents in humans, the question as to what extent the drugs can cross the blood-brain barrier has remained a critical issue [37]. Possibly, the drugs get easier access to the brain after a hypoxic lesion of the blood-brain barrier. Another point of interest relates to likely differences in the transfer of drugs with a different molecular size. When either epoetin alfa (5,000 IU/kg) or an equivalent dose of its hyperglycosylated analog darbepoetin alfa (25 μg/kg) were administered intravenously to rats, both drugs were measurable in the cerebrospinal fluid for up to 8 h with the rate of penetration amounting to 0.09% for epoetin alfa and 0.08% for darbepoetin alfa [38]. Interestingly, intravenously administered asialo-rHuEPO, which has a very short plasma half-life of 1.4 min and does not stimulate erythropoiesis, exerts neuroprotective effects in cerebral ischemia, spinal compression, and sciatic nerve crush in rodents [39]. Whether the small cyclic EPO mimetic peptides (EMP; composed of about 20 amino acids) are of therapeutic value in brain injury has not been disclosed. Such studies may be promising, because EMP may cross the blood-brain barrier more readily, and one compound (EMP1) has been shown to inhibit Ca^{2+}-induced glutamate release from cultured rat cerebral cells of hippocampal neurons [27].

The fact that systemically administered EPOR ligands are neuroprotective in experimental animals has led to consideration of rHuEPO or derivatives thereof as therapeutic option in human brain diseases. A pioneering double-blind, placebo-controlled, randomized, proof-of-concept study on 40 patients with

acute stroke used magnetic resonance tomography to show a strong trend for reduction in infarct size on rHuEPO treatment [23]. After intravenous administration of 33,000 IU rHuEPO, plasma EPO concentrations amounted to about 5 IU/mL after 3 h, while in the cerebrospinal fluid, the concentration was about 0.02 IU/mL (normal < 0.001 IU/mL). The rHuEPO-treated patients exhibited a markedly improved neurological recovery 1 month after the stroke [23]. 39 men diagnosed with chronic schizophrenia were recruited for a double-blind, placebo-controlled, randomized, multicenter EMP proof-of-principle (Phase II) study to test the hypothesis that EPO is useful as a neuroprotective add-on strategy in schizophrenia. The patients were treated with 40,000 IU rHuEPO intravenously or placebo weekly for 12 weeks. Compared with baseline, the schizophrenia-relevant cognitive function score improved in both groups, presumably reflecting the nonspecific stimulatory effect of the study environment. However, the improvement was significantly greater in the rHuEPO than in the placebo group. The hemoglobin values were relatively stable at a low rate of venesections, indicating a small hematopoietic response [40]. rHuEPO, respectively its derivatives and analogs, are presently considered for neuroprotection in other neurological diseases such as multiple sclerosis, Alzheimer's disease, or Parkinson's disease [37, 41]. A placebo-controlled study of the effects of rHuEPO (40,000 IU) on self-reported mood and cognitive function in healthy volunteers has resulted in behavioral effects similar to those seen on acute administration of serotoninergic antidepressants [42].

In conclusion, EPO appears to provide neuroprotection by two means: systemic EPO stimulates erythropoiesis and, thereby, increases the blood O_2-carrying capacity and brain oxygenation [12]; and brain EPO promotes neurogenesis and the survival of neurons on hypoxic and toxic stress [36]. The local production of EPO in the brain will allow for sufficient bioavailability of the survival factor independent of the transfer across the blood-brain barrier. The neuroprotective action of EPO has prompted clinical trials to use ESA in humans with stroke, schizophrenia, and neurodegenerative diseases [37].

Effects of ESA in the cardiovascular system

Effects of ESA on vascular cells

Although the rHuEPO therapy-induced increase in arterial blood pressure seen in some patients with chronic renal failure results primarily from the increased blood viscosity and the abolishment of tissue hypoxia-associated vasodilation, it is clear that EPO can stimulate the production of endothelial cell-derived modulators of the vascular tone, including both vasoconstrictive mediators, such as endothelin, and vasorelaxant products, such as NO. EPOR mRNA is expressed in human vascular endothelial cells in culture [43]. EPO stimulates the *in vitro* proliferation and migration of endothelial cells, and it induces a proangiogenic phenotype of endothelial cells and neovascularization [44, 45].

The angiogenic potential of EPO may be disadvantageous under certain conditions. For example, the question has been raised whether EPO could promote the retinopathy in diabetes mellitus patients [46]. On the other hand, EPO itself is an important survival factor for photosensor cells [47].

Like erythropoietic cells, cultured human umbilical vein endothelial cells (HUVEC) respond to rHuEPO with the rapid tyrosine phosphorylation of cytosolic proteins and the translocation of STAT-5 [48]. A differential display analysis has shown that rHuEPO upregulates four groups of genes in HUVEC, including genes that encode proteins controlling vascular function (thrombospondin-1), gene transcription (c-myc purine-binding transcription factor PuF), mitochondrial function (cytochrome c oxidase subunit 1) and regulators of signal transduction [49]. High doses of EPO (≥100 IU/mL) have been shown to reduce the incidence of apoptosis in bovine pulmonary artery endothelial cultures treated with bacterial lipopolysaccharide (LPS) [50]. EPO has been reported to protect rat cerebral microvascular endothelial cells in primary culture from apoptosis due to O_2 and glucose deprivation [51]. The protective effect requires PI-3K activation, and it uses the phosphorylation of STAT-3, STAT-5, MAPK, and the fork head transcription factor FOXO3a. Note that in all of the cited endothelial cell culture studies, the concentration of EPO greatly exceeded that reached in the blood of patients treated with rHuEPO.

In addition to acting directly on the resident vasculature, EPO promotes the mobilization of myeloid $CD34^+$ hematopoietic and endothelial progenitor cells into the blood stream. The primary role of endothelial progenitor cells has been demonstrated in studies on transgenic mice lacking *EPOR* in nonerythroid lineages. On exposure of the mice to normobaric hypoxia (10% O_2 for 3 weeks), mobilization of endothelial progenitor cells and incorporation into the pulmonary endothelium was greatly impaired, which resulted in pulmonary hypertension [52]. The increased number of circulating cells detected after the administration of rHuEPO or darbepoetin alfa in humans may prove useful for augmenting the neovascularisation of ischemic tissues [53, 54].

Vascular smooth muscle cells also express EPOR mRNA *in vitro* [55]. When applied in concentrations that are by several orders of magnitude higher than those reached in the clinical setting, rHuEPO induces *in vitro* vascular smooth muscle contraction [56] and vasoconstriction [57]. EPO signaling in vascular smooth muscle cells is Ca^{2+}-dependent and involves the activation of the phospholipase C cascade and of oncogenes (Myc, jun, fos) [58] that promote DNA replication and cellular growth. However, it seems unlikely that EPO plays a major role in vascular smooth muscle biology *in vivo*, because circulating EPO will rarely penetrate through intact endothelium.

Effects of ESA on the heart

EPOR mRNA and EPOR protein have been detected in cultures of human [59] and rat [60] cardiomyocyte cell lines and in samples of human heart [61]. With

respect to reports of EPOR protein in the human heart it must be noted, however, that for Western blotting and immunohistochemistry, antiEPOR antibody was used that was subsequently shown to be unspecific and to interact with several other proteins including heat shock proteins [16–19]. On the other hand, animal studies have provided strong evidence that EPO activates cardiac protein kinases [62], opens potassium channels [63], and inhibits apoptosis of cardiac myocytes and fibroblasts [64]. When human atrial trabeculae isolated from patients undergoing coronary artery bypass grafting were exposed to 90 min hypoxia followed by 120-min reoxygenation the addition of rHuEPO (≥5 IU/mL) to the reperfusion medium increased the force of contraction, which required PI-3K and MAPK activities and was associated with reduced caspase 3 activity [65]. Transgenic mice with a tissue-specific EPOR-knockout outside the bone marrow exhibit greater myocardial damage on ischemia/reperfusion than wild-type mice, suggesting that the endogenous EPO/EPOR system plays a protective role in the heart [66]. Treatment of experimental animals with ESA reduces the myocardial infarct volume and improves contractile properties after ischemia reperfusion injury [60, 67–70]. Moreover, the administration of rHuEPO (1,000 IU/kg 3 times a week) for 4 weeks after coronary artery constriction has been shown to prevent cardiac remodeling and dysfunction, and to improve hemodynamic parameters [71]. CEPO, which is devoid of erythropoietic activities but appears to confer cytoprotection outside the bone marrow, has been assigned cardioprotective properties on ischemia/reperfusion *in vivo* [72]. Further nonclinical studies of the cardioprotective potential of EPO and its derivatives and analogs have been summarized elsewhere [73].

Well-designed large clinical trials are required to demonstrate benefits of the administration of rHuEPO, derivatives or analogues thereof, in humans with acute or chronic coronary syndrome and cardiac failure. In a single-center, investigator-initiated, prospective study on 10 patients with a first acute myocardial infarction, the administration of a single dose (300 μg) darbepoetin alfa was found to be safe and well tolerated [74]. Treatment with drug produced a strong increase in the number of circulating $CD34^+/CD45^-$ cells within 72 h. The left ventricular ejection fraction did not differ when compared with a control group of patients [74]. Previous studies were performed on anemic patients with congestive heart failure, and the improved condition after ESA therapy has been primarily related to the increased red blood cell concentration and, hence, tissue oxygenation [75].

Effects of ESA on renal cells

EPOR mRNA has been detected in tubular and mesangial cells from human and rodent kidneys [76]. Binding studies with radiolabeled EPO provided some evidence for the presence of EPOR, although unspecific antiEPOR antibody was used for Western blotting. In addition, EPO was shown to stimulate

DNA synthesis and cell proliferation in cultures of murine proximal tubular cells [76]. High concentrations of rHuEPO (200 IU/mL) protect human proximal tubular epithelial cells (PTEC) in primary culture from hypoxia-induced apoptosis [77]. Furthermore, rHuEPO (≥10 IU/mL) has been shown to prevent the apoptotic cell death associated with oxidative stress in the human PTEC cell line HK-2 [78]. EPO inhibits tubular cell caspase-3 activation, with upregulation of Bcl-x_L and XIAP (X-linked inhibitor of apoptosis protein) in tubular cells. These effects are dependent on JAK-2 signaling and the phosphorylation of Akt by PI-3K [78].

Single injections of high doses of rHuEPO (3,000 IU/kg) have been shown to protect against subsequent ischemia/reperfusion injury of the kidneys in rats [79]. rHuEPO treatment accelerates renal tubular regeneration with cisplatin-induced acute renal failure [80]. When rHuEPO was given subcutaneously as either a 3-day pretreatment (1,000 IU/kg and day) or as a single bolus (1,000 IU/kg) upon reperfusion to mice subjected to bilateral renal artery occlusion for 30 min, the protection was greater by the pretreatment regime than by the single bolus at the time of reperfusion [81]. The protective potential of darbepoetin alfa was studied in a classic remnant kidney rat model [82]. Animals were subjected to 5/6 nephrectomy followed by either saline or 0.1 μg/kg darbepoetin alfa once weekly. The authors observed a persistent activation of the pro-survival Akt signaling pathway in endothelial and glomerular epithelial cells in the darbepoetin-treated animals, which was accompanied by a significant reduction of apoptotic cell death in renal tissue [82]. Darbepoetin alfa treatment ameliorated endothelial damage, attenuated the composite tissue injury score, which includes vascular sclerosis, glomerulosclerosis, tubulo-interstitial damage, and preserved renal function. Based on the observation that low-dose darbepoetin alfa treatment confers vascular and tissue protection it has been suggested that the use of rHuEPO or analogs may have utility in preventing ischemia-related progressive renal vascular injury and organ failure [82].

Effects of ESA on hepatic cells

Similar to their effects on kidneys ESA appear to protect the liver from ischemia/reperfusion injury. When rats were pretreated with 1,000 IU rHuEPO intravenously before liver ischemia (30 or 45 min) followed by reperfusion, there was less hepatic apoptosis on EPO treatment compared with control animals [83, 84]. Thus, it may be worthy to evaluate the therapeutic properties of ESA in preventing an ischemia/reperfusion injury of the liver, including the preservation of livers for transplantation [85]. Furthermore, ESA are apparently hepatoprotective in acute liver failure due to inflammation, as has been demonstrated in darbepoetin alfa-treated mice challenged with D-galactosamine and *E coli* LPS [86]. Although the site of action of darbepoetin alfa was not clear from this study (hepatocytes?, endothelial cells?, leukocytes?),

the observed reduction in liver damage and improved recovery call for a clinical trial with ESA in patients with acute liver injury.

Effects of ESA on tumor growth

Relevance of tumor oxygenation

EPO may influence the growth of solid tumors directly by stimulating erythropoiesis, thereby increasing tumor oxygenation, and indirectly by modulating local immune responses, angiogenesis and cell proliferation (Fig. 3).

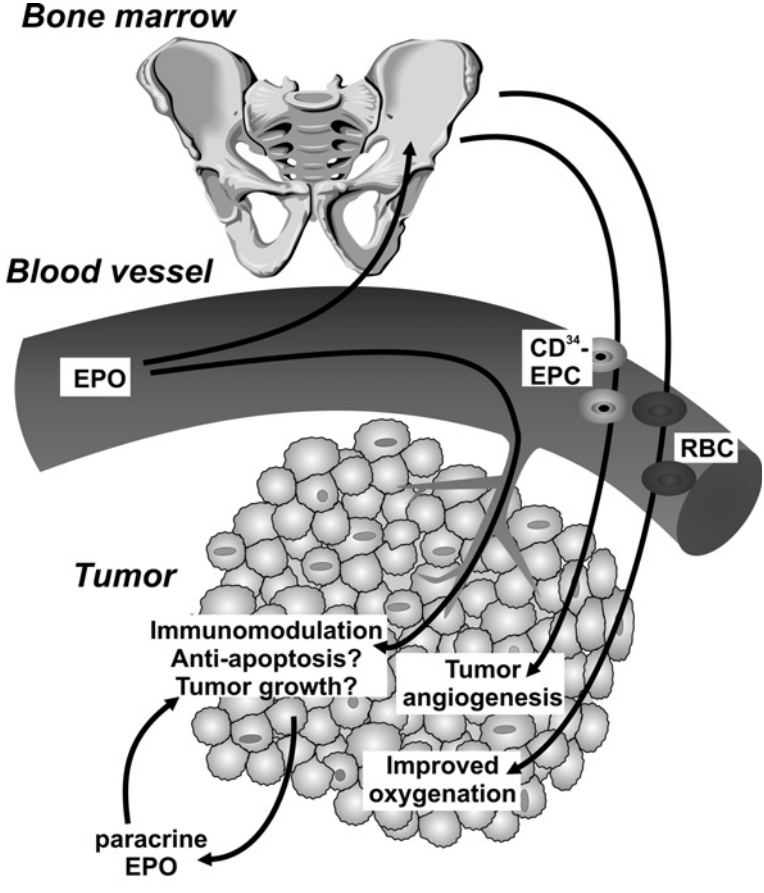

Figure 3. Possible role of EPO in tumor biology. EPO increases red blood cell concentration, blood O_2 capacity and tumor oxygenation. EPO and its pharmacological analogs may stimulate angiogenesis through mobilization of CD34-positive endothelial progenitor cells (CD^{34}-EPC) and through local effects on the endothelium. Whether endogenous EPO and exogenous ESA (erythropoiesis stimulating agents) can promote tumor growth directly via functional EPOR is a controversial issue.

Oxygen availability is central in controlling energy homeostasis, tumor cell survival and metastasis [87]. Several studies have considered the importance of the correction of anemia in tumor patients which leads to an increase in the O_2 supply and, thus, the sensitivity towards chemo- and radiotherapy [88]. For example, the cytotoxic efficacy of cyclophosphamide is greater in rats treated with carboplatin, if anemia is prevented by the administration of rHuEPO [89]. rHuEPO has been shown to suppress the growth of Lewis lung carcinoma in synergy with cisplatin, though not with mitomycin C or cyclophosphamide [90]. Darbepoetin alfa has been proven to increase blood hemoglobin concentration and tumor oxygenation and to reduce tumor mass in murine models of Lewis lung carcinoma in synergy with cisplatin, although darbepoetin alfa does not modulate tumor growth directly [91].

Direct effects of ESA on tumor cells

Because EPOR mRNA is present in essentially every organ, it is also detectable in cancer tissue. A study has shown that *EPOR* is not overexpressed in human cancers. A gene amplification analysis of 1,083 human solid tumors has revealed that amplification of the *EPOR* locus is rare with frequencies similar to other nononcogenes [92]. In addition, *EPOR* transcript amounts in human tumors are low compared with bone marrow and equivalent to, or lower than, the one in matched nonmalignant tissues [92].

Another important question is whether tumor cells translate EPOR protein and whether the EPOR is functional. Since specific anti-EPOR antibodies suitable for immunohistochemistry or Western blotting are not available at present [16–19, 93], an encyclopedic survey of the numerous investigations utilizing such antibodies [5, 7] is not provided here. In brief, most reports claiming the presence of EPOR protein were based on studies with the commercial polyclonal antibody C-20 (Santa Cruz) that was raised against the 20 *C*-terminal amino acids of the human EPOR. The C-20 antibody binds to proteins of 35, 66 and 100 kDa that are clearly unrelated to the EPOR. In all likelihood, the 66 kDa band seen on Western Blotting is heat shock protein HSP70 [16, 17, 94] and the 100 kDa band is HSP90 [19]. Another commonly used polyclonal antibody, M-20 (Santa Cruz), which is raised against the 20 *C*-terminal amino acids of murine EPOR, reacts with murine and human EPOR but also with several other proteins [17]. Both the C-20 and the M-20 antibody falsely produce stain signals in EPOR knockout mouse tissues [17]. Other antibodies used for detection of EPOR on human tumor tissue have included the polyclonal anti-human EPOR antibody H194 (Santa Cruz), the polyclonal anti-murine EPOR antibody 07-311 (Upstate) and the monoclonal anti-human EPOR antibody MAB307 (R&D Systems). All of these antibodies crossreact with multiple proteins different from the EPOR as predicted by the apparent molecular weight [17–19]. When the *EPOR* gene was knocked out by small interfering RNA technique (RNAi) in *EPOR* transfected human osteosarcoma cells, only

the 60 kDa protein was lost, while several other bands still stained with the C-20 antibody [19]. Thus, the significance of published immunohistochemical and blotting studies of EPOR protein in biopsies from human tumors or human cancer cell lines has to be called in question.

A number of *in vitro* studies using a variety of tumor cell lines have shown that EPO does not induce EPOR signaling and does not stimulate cell proliferation [5, 7]). In contrast, some investigators have reported growth stimulating effects [5, 7, 95]. Growth stimulation was generally observed only with very high doses exceeding the concentrations measured in the plasma of healthy non-anemic humans (approximately 0.02 IU/mL) or of patients after subcutaneous administration of rHuEPO in clinical practice (approximately 0.15 IU/mL). For example, 250 IU/mL were required to show significant effects on protein phosphorylation, DNA synthesis, proliferation and migration in breast cancer cell cultures [96, 97]. Somewhat lower concentrations of EPO (0.5–1 IU/mL) sufficed to stimulate the growth of distinct human renal [98] or prostate cancer cell lines [99]. It should be noted that the negative and positive responses of tumor cells on the addition of EPO are not related to the use of serum-containing or of serum-free media. In addition, even if significant effects of EPO were shown, these were generally very small, in absolute terms. It was reported that rHuEPO (10 IU/mL) induces signaling (activation of STAT-5, Akt, MAPK) in the nonsmall-cell lung carcinoma cell line H838, but no proliferative response [100]. In contrast to erythropoietic cells, H838 cells do not ubiquitinate and degrade EPOR on binding of EPO. Even in tumor cell lines expressing EPOR mRNA, no EPO binding sites were detectable on the cell surface as assessed by application of radiolabeled rHuEPO [92, 101], which may be due to the lack of EPO mRNA translation or lack of cell-surface-trafficking factors such as JAK-2.

Liu et al. [102] have raised the question as to whether ESA may not influence the basal viability of tumor cells but may protect the cells from the cytotoxic effect of drugs like cisplatin. rHuEPO has been reported to cause resistance to cisplatin in HELA [103], U87 glioma, and HT100 cervical cancer cells [104], and to dacarbazine in melanoma cells [105]. When ovarian cancer cells (line A2780) were treated with rHuEPO in the long term a phenotype developed that exhibited enhanced EPOR signaling and increased resistance to paclitaxel [106]. This effect was drug-specific, since no change in cisplatin or carboplatin sensitivity was observed. The increased resistance towards paclitaxel was explained by a reduced expression of the pro-apoptotic proteins Bcl-2 and Bcl-10 [106]. In contrast to this observation, Carvalho et al. [107] have reported that renal carcinoma cells display a high rate of apoptosis on treatment with a combination of daunorubicine or vinblastine and rHuEPO than with either of these agents alone. Still other investigators have reported that rHuEPO treatment fails to interfere with the cytotoxicity of chemotherapeutics such as adriamycin, taxol or tamoxifen in breast cancer cell lines [108].

Since most *in vitro* investigations on tumor cell growth were performed with permanent cell lines, earlier studies by Bauer et al. [109] are of major

importance. These scientists investigated the effects of rHuEPO (up to 400 IU/mL) on the *in vitro* clonal growth of 53 human primary tumor specimens (mainly of renal and colorectal origin) in a soft agar cloning system. rHuEPO treatment was without effect in 47 specimens while stimulation was observed in two, and inhibition in five specimens [109]. Others investigated the effects of epoetin alfa, epoetin beta, and darbepoetin alfa on the *in vitro* proliferation of hematologic tumor cells from eight patients with B-cell chronic lymphocytic leukemia (B-CLL), three patients with mantle-cell lymphoma (MCL), and four patients with multiple myeloma (MM) [110]. None of the ESA induced proliferation in any of the cultures. Thus, neither cancer nor lymphoid malignant or myeloma cells in primary culture are responsive to ESA, in general.

Effects of EPO on the local immune response

Studies of the effects of EPO on whole blood cell cultures from rHuEPO treated patients with chronic kidney disease [111] and on cultures of peripheral polymorphonuclear leukocytes have suggested direct immunomodulating effects of EPO [112]. rHuEPO augments B-cell responses, manifested by stimulation of immunoglobulin production and LPS-induced proliferation of splenocytes [113]. A T cell-mediated tumor-specific immune reaction to myeloma cells has been assumed from studies showing that rHuEPO treatment induces complete tumor regression in 30–60% of mice with syngeneic myeloma [114]. This interpretation has been confirmed in subsequent studies using two lymphoproliferative murine models (MOPC-315 MM and BCL1 B-cell leukemia lymphoma) in which rHuEPO treatment reduced tumor cell growth [115]. The action of EPO as an immunomodulator is incompletely understood, however. Investigators have explained the positive effect of rHuEPO therapy on the survival of mice carrying a subclone of colon 26 adenocarcinoma by a decrease in the production of the cachexia-inducing cytokine interleukin 6 (IL-6) [116].

Effects of EPO on tumor angiogenesis

Several lines of evidence suggest that endogenous EPO may be an important survival factor for the endothelium of tumor vessels. Yasuda et al. [117] have shown that the application of anti-EPO antibody or of soluble EPOR in the transplants of uterine or ovarian tumors in nude mice results in a reduction of tumor size. In subsequent studies it was found that the intraperitoneal administration of a synthetic EPOR blocking peptide (EMP9) in mice with subcutaneous xenografts of human stomach choriocarcinoma (SHC) or melanoma (P39) inhibits tumor angiogenesis and opposes tumor cell viability, whereas the application of an EPO mimetic peptide (EMP1) promotes tumor angio-

genesis and tumor cell survival [118]. Furthermore, the application of either anti-EPO antibody, soluble EPOR or an inhibitor of JAK-2 delays tumor growth in a tumor-Z chamber model with rat syngeneic mammary adenocarcinoma cells [119]. Studies utilizing the dorsal skin-fold window chamber technique, which allows one to study tumor morphology by intravital microscopy, have shown that the coinjection of mammary carcinoma cells with either soluble EPOR or anti-EPO antibody results in reduced tumor angiogenesis and tumor growth in mice [120]. Transgenic expression of an EPO-antagonist protein (R103A-EPO) likewise inhibits tumor angiogenesis and tumor growth whereas expression of a constitutively active *EPOR (EPOR-R129C)* in the tumor cells is associated with enhanced tumor angiogenesis and growth [120]. Since tumor cells can endogenously express the *EPO* gene, tumor cell-derived EPO may promote angiogenesis *in vivo* [121]. With respect to the administration of ESA to tumor patients a deeper insight into the role of EPO for tumor angiogenesis is obviously required.

Conclusions

rHuEPO and its hyperglycosylated analog darbepoetin alfa are routinely used for the treatment of the anemias associated with chronic kidney disease or chemotherapy. Primarily in preclinical studies, rHuEPO and its derivatives and analogs prevented apoptosis and provided protection against damage caused by hypoxia, ischemia/reperfusion or cytotoxic agents in several nonhematopoietic organs and tissues, including the brain, the heart, the kidneys and the liver. Reportedly, certain EPO derivatives that are devoid of erythropoietic activity, such as carbamoylated EPO, can still confer cytoprotection in nonhematopoietic tissues.

However, many of the enthusiastic reports of the nonhematopoietic cytoprotective potential of EPO and its derivatives have remained phenomenological, in that statistically significant effects were deduced on the addition – often in very high doses – of the drugs to more or less artificial experimental setups. Furthermore, the presence of EPOR mRNA does not equal the presence of functional cell surface EPOR protein. EPOR protein was often not demonstrated, primarily because specific anti-EPOR antibodies for immunodetection are not available. This problem also holds true when reports proposing growth promoting effects of EPO on tumor tissue are critically assessed. EPOR transcripts are not increased in tumors, and ligand/EPOR binding and EPOR signaling has rarely been proven.

It remains to be investigated whether the promising results of the use of EPO in neuronal, myocardial, renal and hepatic cell culture or whole organ studies and in animal models can be reproduced in clinical practice. Properly designed, carefully blinded and placebo-controlled, clinical trials are needed to assess the efficacy and safety of these interventions in patients.

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