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CHAPTER 2

Regulation of erythropoiesis

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1. Introduction

Erythropoiesis is the pathway that produces mature red blood cells from haematopoietic stem cells. During mammalian development, erythropoiesis occurs successively in the yolk sac, the foetal liver and the bone marrow. This cellular process is characterised by commitment and differentiation steps that restrict the differentiation potential and the proliferative capacity of the cells as they go through the erythroid-specific program of gene expression. Erythropoiesis is regulated by the combined effects of microenvironmental and growth factors that promote the survival, proliferation and/or differentiation of erythroid progenitors, and nuclear factors that regulate the transcription of genes involved in the establishment of the erythroid phenotype. At the cellular and molecular levels, erythropoiesis is one of the best-studied haematopoietic lineages for the following reasons: first, the different stages of erythropoiesis can all be defined by phenotypic markers; second, erythroid diseases are well-characterised and in many cases their molecular causes have now been found; third, terminal erythroid differentiation depends on only one exogenous growth factor, erythropoietin; fourth, most if not all the transcription factors that regulate erythropoiesis are known. The understanding of this complex system may shed light on basic cellular biology and also the pathophysiology of various diseases including bone marrow failure, degenerative diseases and cancers.

Many features differentiate primitive and foetal erythropoiesis from adult erythropoiesis both at the cellular and molecular levels. This review will focus on the late stages of adult erythropoiesis starting from the common erythro/megakaryocytic precursor and ending with the mature red blood cells.

2. Description of adult erythropoiesis at the cellular level

The production of erythrocytes is the largest quantitative output of the haematopoietic system with an estimated production rate of $2x10^{11}$ erythrocytes per day. The frequency of committed granulocyte-macrophage progenitors is actually three times greater than that of committed erythroid progenitors in the bone marrow but this is counteracted by a high proliferative index in the late transitional stages of erythroid development (1, 2). The cell cycle time of pro-erythroblasts is estimated to be 6-7 hours, which is unique in the adult under steady state conditions and more closely resembles that of embryonic cells. How cell differentiation and activation of specific gene expression programs are coordinated with cell growth remains to be clarified, despite a detailed knowledge of the network of transcription factors that governs lineage gene expression (see (3) for review). Committed erythroid progenitors can be detected by their ability to form erythroid colonies in *in vitro* culture in methyl

cellulose, and are called burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) (1, 4). BFU-Es are the most immature haematopoietic cells that are already committed to the erythroid lineage. BFU-Es represent only 0.03% of bone marrow haematopoietic cells and only 40% are cycling (2), whereas CFU-Es represent 0.3% of bone marrow haematopoietic cells and most of them are actively cycling (5). There is a continuous cellular process from the earliest BFU-Es to the latest CFU-Es but the growth factor requirements for BFU-Es and CFU-Es are completely different. BFU-Es require stem cell factor (SCF) (6) and many other haematopoietic growth factors for their growth and differentiation, whereas CFU-Es are highly dependent on erythropoietin (Epo) (5). CFU-E will differentiate into the first morphologically identifiable cell of the erythrocyte lineage, the pro-erythroblast, then differentiates successively into the basophilic erythroblast, the polychromatophilic erythroblast, and the acidophilic erythroblast, which is the last nucleated cell of the mammalian erythrocyte lineage. Enucleation of the acidophilic erythroblast gives rise to the reticulocyte which matures finally into the red blood cell. This process occurs within the erythroblastic blood island in the bone marrow, in which a macrophage is surrounded by erythroblasts at all stages of maturation (Figure 1).

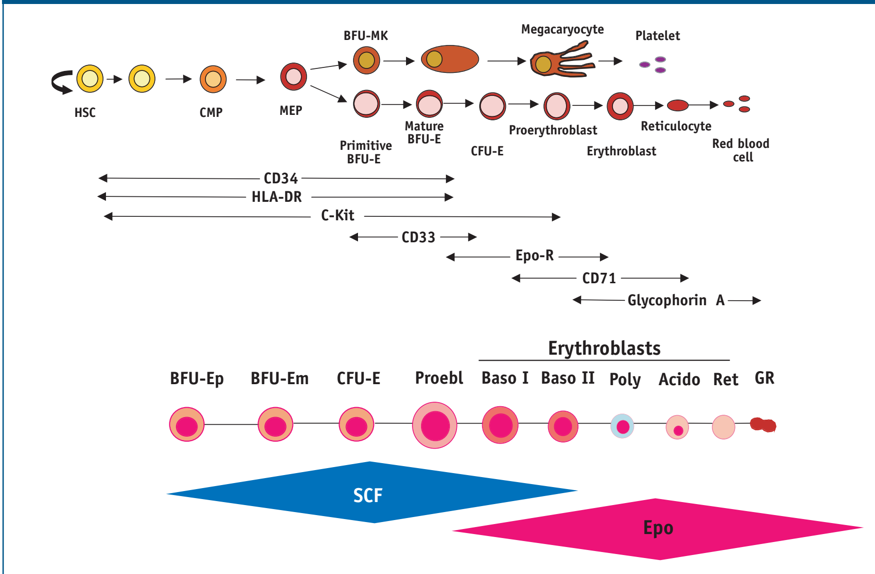
3. Description of adult erythropoiesis at the transcriptional level

During erythroid differentiation, the expression pattern of erythroid-specific genes follows a precise timing that is mainly regulated at the transcriptional level. Studies on the *cis* and *trans* acting factors that regulate the erythroid-specific genes, together with perturbations of erythropoiesis in leukaemia and inherited erythroid diseases and in experimental animal models, have greatly increased our knowledge of the transcriptional regulation of erythropoiesis. The most interesting findings of these studies are the importance of protein-protein interactions in the transcriptional regulation of erythropoiesis and its relationship with leukaemogenesis (see (3) for review).

3.1 Cis-acting sequences involved in the regulation of erythroid-specific genes

Functional analysis of erythroid and megakaryocytic specific genes has shown the importance of a sequence, 5′ A/T GATA A/G 3′, now called the GATA motif, in the lineage specific expression of these genes (7). This sequence is associated with a GT or CACC-like sequence in erythroid specific genes, whereas it is associated with an Ets binding site in megakaryocytic specific genes (7). These two associations are now considered as hallmarks of erythroid or megakaryocytic-specific regulatory regions. Another motif, 5′ TGAC/GTCAGCA 3′, is also found in the core region of erythroid expressed gene promoters or enhancers and is necessary for efficient

Figure 1: **Erythropoiesis is the process by which multipotential haematopoietic stem cells differentiate into mature red blood cells**



The requirement for erythropoietin starts at mature BFU-E and stops after the proerythroblasts stage. The expression is indicated of the different surface markers commonly used to characterise the different erythroid precursors. BFU-Ep: burst forming unit- erythroid primitive. BFU-Em: burst forming unit-erythroid mature HSC: haematopoietic stem cell; CMP: common myeloid progenitor; MEP: megakaryocyte erythrocyte progenitor.

erythroid-specific transcription of the genes that contain this motif (8). No other motif has been repeatedly found in regulatory sequences of erythroid or megakaryocytic specific genes. For each binding motif identified as recurrent in the regulatory sequences of erythroid or megakaryocytic genes, families of nuclear factors that recognise the motif have been characterised. Of importance, none of the trans-acting factors identified has an expression restricted to the erythroid or the megakaryocytic lineage indicating that the specificity of gene expression in erythropoiesis or megakaryopoiesis, as in the other haematopoietic lineages, is established by a specific combination of trans-acting factors rather than by lineage specific factors.

3.2 Trans-acting factors that regulate the expression of erythroid-specific genes

Trans-acting factors involved in erythropoiesis are shown in Table 1.

Table 1: **Trans-acting factors involved in erythropoiesis**

Trans-acting factor	DNA-binding site	Target genes	Phenotype of gene inactivation	Human pathology
GATA-1	5′ A/T GATA A/G 3′	Globin, erythroid specific membrane proteins, GATA-1, GATA-2	Anaemia and thrombocyto-penia	X-linked anaemia or thrombocytopenia, megakaryoblastic leukaemia of Down's syndrome
GATA-2	5′ A/T GATA A/G 3′	GATA-1, GATA-2	No proliferation of immature progenitors	
TAL-1	5′ CAGATG 3′	Glycophorin A, p21	Anaemia, no HSC	T-cell acute leukaemia
EKLF	5′ CCNCACCC 3′	Globin	Anaemia	
p45NF-E2	5′ TGAC/GTCAGCA 3′	Enzymes of the haem biosynthetic pathway, globin	Thrombocyto-penia	
ZBP-89	5′ CCNCACCC 3′	Globin, erythroid specific gene, GATA-1	Anaemia and thrombocyto-penia	
Gfi-1B	5′ TAAATC(A/T)GCA 3′	p21, SOCS1-3, BclxL	Anaemia	

DNA-binding sites and known target genes of the indicated transcription factors together with the phenotype of their gene inactivation in mice and the human pathologies associated with gene mutations are shown.

3.2.1 GATA-1 and GATA-2

The GATA family of transcription factors consists of six transcription factors, GATA-1 to -6, that can bind the consensus sequence 5′ A/T GATA A/G 3′. These proteins contain two conserved zinc fingers motifs (ys-X_2-Cys-X_{17}-Cys-X_2-Cys) specific to the GATA family. The carboxyl terminal zinc finger is responsible for binding to DNA whereas the amino terminal zinc finger stabilises the DNA/GATA interaction. Outside of the zinc finger regions, the conservation between GATA factors is low but each factor is conserved between species. As regards erythropoiesis, only GATA-1 and GATA-2 have been shown to play a role both in erythrocytic determination and in terminal differentiation (see (9-11) for review).

During erythropoiesis, there is sequential but overlapping expression of GATA-1 and GATA-2. GATA-2 is expressed early in the erythroid lineage and its expression decreases concomitantly with an increasing GATA-1 expression. Numerous studies

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have shown that GATA-2 is involved in the cellular proliferation of non-committed and committed erythroid progenitors, whereas GATA-1 is critical for terminal maturation of erythroid cells. At the molecular level, cross talk between GATA-1 and GATA-2 occurs at the transcriptional level, as GATA-2 inhibits transcription of the GATA-1 gene and GATA-1 has the same effect on the GATA-2 gene. The regulatory network between GATA-1 and GATA-2 during erythropoiesis results in the sequential substitution of GATA-2 by GATA-1 in the regulatory regions of numerous erythroid specific genes.

The X-linked GATA-1 gene is the founding member of the GATA factor family. During adult haematopoiesis, its expression is restricted to the erythroid, megakaryocytic, eosinophil and mast cell lineages. Mice that lack GATA-1 die at day 10-12 of anaemia with a blockade of erythroid differentiation at the pro-erythroblast stage. The cells die by apoptosis, indicating a role for GATA-1 in cell survival as well as maturation. GATA-1 activity during erythropoiesis is highly regulated at the transcriptional, translational and posttranslational levels. GATA-1 positively regulates the transcription of its gene through GATA sites located in the promoter and enhancer regions of the GATA-1 gene. GATA-1 possesses an alternative translation initiation site located at methionine 84 and the GATA-1s (short isoform of GATA-1) protein produced is not fully functional, as it cannot drive erythroid differentiation when expressed at normal levels. GATA-1 activity and levels must be tightly regulated to prevent continuous accumulation of GATA-1 in the cells, which may ultimately block erythroid differentiation (12).

An important way by which GATA-1 activity is regulated is by acetylation. GATA-1 acetylation by P300 (13) or CREB-binding protein seems to enhance the self-association of GATA-1 and thus its transcriptional activity. Likewise, mutation of the main site of acetylation eliminated the ability of GATA-1 to bind to all examined cellular target sites *in vivo*, including genes that are normally activated and repressed by GATA-1 during erythroid differentiation (14). On the other hand, acetylated GATA-1 is targeted for degradation via the ubiquitin/proteasome pathway. Acetylation positively signals ubiquitination, suggesting that activation by acetylation simultaneously marks GATA-1 for degradation. Acetylation alone is not enough to signal degradation, but MAPK phosphorylation of GATA-1 cooperates with acetylation for efficient ubiquitination (15).

Biochemical studies have shown that GATA-1 is phosphorylated at six serine residues in undifferentiated murine erythroleukaemia cells. Akt directly phosphorylates GATA-1 at serine 310 *in vitro* and *in vivo* and enhances its activity in erythroid cells (16). GATA-1 Ser^{310} phosphorylation is not essential for overall erythroid gene expression (major late erythroid genes) but it is absolutely required for a subset of genes like the *TIMP-1* gene (17). Finally, GATA-1 activity is in some circumstances

regulated by a caspase-mediated cleavage during terminal erythroid differentiation (see below).

GATA-1 transcriptional activity is also dependent on protein-protein interactions. Many proteins have been shown to interact physically with GATA-1 including FOG-1, LMO-2, TAL-1, Gfi-1b, PU-1, ZBP-89 and CBP. While most of these interactions may have some relevance to the transcriptional activity of GATA-1, we will focus on the GATA-1/FOG-1 interaction as it has numerous implications in the physiology of erythropoiesis (see (18) for review). FOG-1 (Friend of GATA-1) is a multitype zinc finger protein that specifically interacts with the amino zinc finger of GATA-1. FOG-1 is co-expressed with GATA-1 in the erythroid and megakaryocytic lineages and FOG-1$^{-/-}$ mice die in mid-embryonic gestation of severe anaemia with an arrest in erythroid maturation that is similar to the one observed in GATA-1$^{-/-}$ mice. There is considerable evidence that the function of GATA-1 in erythropoiesis is linked to FOG-1. One of the most important observations was that a single amino-acid change in the GATA-1 amino zinc finger, which abolishes interaction with FOG-1, is lethal in mice due to severe anaemia and is also associated with dyserythropoietic anaemia in patients. The different functions of GATA-1/FOG-1 are mediated by two distinct nuclear complexes. One, the GATA-1/FOG-1/MeCP1 complex, represses transcription whereas the other, GATA-1/FOG-1, links GATA-1 to transcriptional activation. The existence of these two different complexes with opposing transcriptional activities explains how GATA-1 simultaneously activates and represses target genes during erythropoiesis. However, the crucial question as to how some genes harbouring GATA binding sites are activated whereas others are repressed remains open (see (18) for review).

3.2.2 TAL-1, LMO-2 and LMO-4

TAL-1 (SCL), a member of the basic helix-loop-helix (bHLH) family of transcription factors, was first identified through its involvement in human T-cell acute lymphoblastic leukaemia (T-ALL), where it is found in up to 30% of cases. Studies in mice have shown that TAL-1 is required for specification, but not maintenance, of haematopoietic stem cells and for maturation of the erythroid and megakaryocytic lineages (19, 20).

At the molecular level, TAL-1 acts through both DNA-binding dependent and independent mechanisms and can activate or repress transcription. TAL-1 can assemble a pentameric complex (21) containing the ubiquitously expressed E-proteins (E2A, HEB, E2-2), LMO-2, Ldb1 and GATA-1 in erythroid committed cells and can activate the expression of terminally expressed erythroid specific genes, while in CD34^{+} haematopoietic progenitors, a GATA-2 variant of this TAL-1 complex activates c-Kit transcription (22). Alternatively, the TAL-1/E2A heterodimers, which are less potent activators than E2A homodimers or E2A/HEB heterodimers, can repress

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E2A target genes. Analysis of the complexes nucleated by TAL-1 in erythroid cells has shown that ETO2, a potent transcriptional repressor, is associated with the pentameric complex during the initial stages of erythropoiesis whereas ETO2 is no longer present in the complex during terminal erythroid differentiation. Thus, the composition of transcription factor complexes varies during erythroid differentiation, resulting in differential transcriptional output. In early erythroid cells, the TAL-1 complex associates with erythroid specific regulatory regions such as the GPA promoter or the HS-26 of the murine α-*globin* locus. This association might keep the chromatin in an open state but transcription is repressed by ETO2. Later during erythropoiesis, an elevation in TAL-1 and E2A or HEB levels changes the ratio of TAL-1 to ETO2 in favour of TAL-1, which offsets the inhibitory activity of ETO2 and triggers sustained expression of TAL-1 marked erythroid genes.
LMO-2, a LIM domain nuclear factor, has also been identified through its involvement in rare cases of human T-ALL. LMO-$2^{-/-}$ and TAL-$1^{-/-}$ mice have identical phenotypes consistent with the direct physical interaction between these two nuclear factors during erythropoiesis.
A transcriptional complex comprising LMO-2, TAL1, E47, GATA-1, and LDB1 regulates erythroid genes. While TAL1 has been shown to induce erythroid differentiation, LMO2 appears to suppress foetal erythropoiesis. In addition to LMO-2, the closely related LMO-4 gene is expressed in haematopoietic cells, but has unknown functions. LMO-2 and LMO-4 are expressed at the same level in erythroid colonies from mouse bone marrow, implying a function in erythroid differentiation. However, while LMO-2 induced erythroid differentiation, LMO-4 had no such effect. Interestingly, both LMO-2 and TAL1 were able to partially suppress myeloid differentiation, implying that they activate erythroid differentiation in uncommitted bone marrow progenitors. Both LMO-2 and LMO-4 interacted strongly to LDB1, which was required for their localisation to the nucleus (23).

3.2.3 EKLF (Erythroid Krüppel-like factor) and NFE-2

The CACCC motif associated with the GATA motif in the regulatory regions of erythroid specific genes is recognised by widely expressed nuclear factors of the SP1 family and, in some genes, by the erythroid specific factor EKLF. EKLF binds with high affinity to the CACCC site found in the promoter of the human β-globin gene. Mutation of this sequence in humans is found associated with β-thalassaemia and targeted disruption of the EKLF gene also results in β-thalassaemia in mice. Beside its role in globin gene activation and the switch from foetal to adult globin gene expression, EKLF is required for the last steps of erythroid differentiation - but not proliferation - and directly regulates genes involved in haemoglobin metabolism and membrane

stability. Despite these functions, EKLF is very different from GATA-1 or TAL-1 as it may only be required for the regulation of a few erythroid-specific genes, though these are of major importance for the function of red blood cells (see (24) for review). Besides the GATA and CACCC motifs, an AP1 like sequence that is recognised by the erythroid nuclear factor NF-E2 recurs in the regulatory regions of erythroid-specific genes. NF-E2 is a member of the leucine-zipper family of transcriptional activators and recognises the consensus sequence TGCTGA(G/C)TCA located in the regulatory sequences of a number of erythroid-specific genes, including the locus control regions (LCRs) of both β and α-globin genes. The NF-E2 complex consists of a haematopoietic-specific subunit NF-E2p45, associated with a ubiquitous small Maf-protein subunit NF-E2p18 (also known as MafK). NF-E2p18, which does not contain a transactivation domain, can bind DNA as a homodimer or heterodimer with other basic leucine zipper proteins. Consistent with its lack of transactivation domain, NF-E2p18 homodimers can act as transcriptional repressors through NF-E2 DNA-binding sites. NF-E2p45 binds DNA as an obligate heterodimer with p18 and contains a transactivation domain essential for target gene activation. The regulation of NF-E2 transcriptional activity during erythropoiesis is unique as the levels of p18 and p45 proteins do not change during erythroid differentiation, but NF-E2 DNA-binding and transcriptional activities markedly increase upon differentiation. This occurs through relocalisation of p18 from heterochromatic nuclear compartments in erythroid progenitors to euchromatic compartments where p45 is located in erythroid cells expressing the NF-E2 target genes (25).
The transcriptional factor E2f2, which regulates the transition from G1 to S phase, is a direct target of EKLF and EKLF-deficiency leads to cell cycle perturbation and defective terminal erythroid differentiation (26).

3.2.4 Gfi-1B

Gfi-1B (growth factor independence 1B) is a GFi family transcriptional repressor that contains a SNAG domain which mediates transcriptional repression and a zinc finger domain at its carboxyl terminus for DNA binding to the TAAATC(A/T)GCA recognition sequence. *Gfi-1B* disruption results in embryonic lethality due to a failure of red blood cell formation. *Gfi-1B* represses *p21*, *SOCS1*, *3* and its own genes. Enforced expression of GFi-1B in early erythroid progenitors cells induces an expansion of early erythroid progenitors but a massive apoptosis of later erythroid precursors, with a significant reduction of *BclxL* expression, although no canonical consensus sequence is found in the promoter of the *BclxL* gene. GATA-1 activation leads to the early, transient induction of Gfi-1B, followed by the late induction of Bcl-xL during erythroid maturation in G1ER cells. A constant level of GATA-1 binds to the Bcl-xL

promoter throughout the entire induction period, while Gfi-1B is transiently associated with the promoter in the early phase. The sustained expression of Gfi-1B abolished GATA-1-induced Bcl-xL expression. GATA-1 binds to the noncanonical GATT motif of the Bcl-xL promoter for trans-activation. Gfi-1B expressed at increased levels is recruited to the Bcl-xL promoter through its association with GATA-1, suppressing Bcl-xL transcription. Therefore, the down-regulation of Gfi-1B in the late phase of erythroid maturation is necessary for Bcl-xL induction (27). Taken together, Gfi-1B plays a complex role in erythropoiesis. In immature erythroid progenitors, through the zinc finger domains, Gfi-1B would *trans*-activate target genes implicated in cell proliferation for erythroblast expansion. At the onset of differentiation, Gfi-1B would regulate genes that have to be repressed for differentiation induction.

3.2.5 ZBP-89 and Id2

The Krüppel-type zinc finger transcription factor ZBP-89 is a component of multiprotein complexes involving GATA-1 and its essential cofactor FOG-1. GATA-1 and ZBP-89 co-occupy *cis*-regulatory elements of certain erythroid-specific genes, including an enhancer of the GATA-1 gene itself. Loss-of-function studies in zebrafish and mice demonstrate an *in vivo* requirement for ZBP-89 in definitive erythropoiesis but not primitive erythropoiesis, phenocopying aspects of FOG-1- and GATA-1-deficient animals. These findings identify ZBP-89 as being a novel transcription factor involved in erythroid development and suggest that it serves a cooperative function with GATA-1 and/or FOG-1 in a developmental stage-specific manner (28). Inhibitors of DNA binding (Id) family members are key regulators of cellular proliferation and differentiation. These activities are related to the ability of Id proteins to antagonise E proteins and other transcription factors. Id2 intrinsically regulates erythroid development via interaction with different target proteins (29). Overexpression of Id2 enhances erythroid development, while decreased level of Id2 impairs normal erythroid development. Id2 regulation is mediated via interacting with PU.1 and modulating the activities of PU.1 and GATA-1.

4. Regulation of erythropoiesis

As discussed, the process of making red cells is orchestrated by a complex network of transcription factors. Among these, GATA-1 plays a critical role by regulating genes involved not only in erythroid differentiation, but also in cell cycle and survival. This program of differentiation must be regulated positively and negatively to ensure a continuous but controlled production of red cells in order to provide oxygen at a physiologic level in peripheral tissues.

4.1 Role of stromal cells in the regulation of erythropoiesis

In the adult, definitive erythropoiesis occurs essentially if not exclusively within the bone marrow microenvironment. At this level, stromal and immune cells play an important role by providing factors that include integrins and/or their ligands and cytokines. These factors are capable of inducing proliferation and survival, which allows the program of differentiation of erythroid progenitors to take place (for review see (30, 31)). Erythroblastic islands, the specialised niches in which precursors proliferate, differentiate and enucleate, are composed of erythroblasts surrounding a central macrophage. Erythroid islands localise not only to regions adjacent to bone marrow sinusoids but also to regions throughout the marrow. Erythroblasts express adhesion molecules that mediate both erythroblast/erythroblast and erythroblast/macrophage interactions. Historically, Emp (erythroblast macrophage protein) was the first molecule identified that appeared capable of forming attachments via homophilic binding. Emp-null foetuses die with severe anaemia showing that Emp performs a critical role in erythropoiesis (32). The interactions VLA-4/ICAM-4 and VCAM-1/α_V integrin between erythroblasts and central macrophage contribute to island integrity. An enhanced erythroblast proliferation related to contact with macrophages has been demonstrated which occurred at all Epo concentrations which resulted from decreased transit time in the G0/G1 phase of cell cycle by a mechanism different from the anti-apoptotic effect of Epo (33).
Recent studies have shown a role of DNase II in erythroid maturation and, particularly, in the process of enucleation occurring at very late stages of erythroid maturation. Mice deficient in DNase II die of severe anaemia and show a marked decrease of circulating red blood cells associated with the presence in the circulation of definitive nucleated erythroblasts, a cell type normally present only in the bone marrow and not in peripheral blood. Central macrophages present in erythroblastic islands may represent the source of DNase II and are then responsible for the engulfment of expelled nuclei through recognition of exposed nuclei phosphatidyl serine as they do for apoptotic cells (34).
Abnormalities in macrophage differentiation can lead to perturbations in the function of the erythroid island and affect erythroid differentiation. Rb protein, a regulator of macrophage differentiation is necessary for erythroid maturation. Target disruption of Rb gene in mice leads to embryonic death with anaemia caused by failure of enucleation (35-37). Rb protein plays also a cell-intrinsic role in erythropoiesis mediated by coupling the process of mitochondrial biogenesis to exit from the cell cycle. Its absence leads to ineffective erythropoiesis, with a differentiation block at the transition from early to late erythroblasts (38).

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4.2 Terminal maturation and enucleation

4.2.1 Role of the pro-apoptotic BH3-only-like factor Nix

Erythroid cells undergoing terminal differentiation exhibit concurrent transcriptional up-regulation of the anti-apoptotic Bcl-xL and the pro-apoptotic BH3-only-like factor Nix. Nix null mice exhibit anaemia and erythroid hyperplasia. Cultured Nix null erythroid cells are hypersensitive to Epo and resistant to apoptosis induced by cytokine deprivation. These results indicate that Nix is a negative regulator of erythropoiesis through modulated apoptosis (39). Nix is also required for the selective mitochondrial clearance during reticulocyte maturation, by triggering incorporation of mitochondria into autophagosomes followed by maturation of the autophagosomes (40, 41).

4.2.2 Role of the Rac GTPases

Recent studies suggested that Rac GTPases and their effector protein mDia2 play significant roles in mouse foetal erythroblast enucleation by affecting the formation of the contractile actin ring in late-stage erythroblasts. Rac GTPases are in a dynamic on-and-off state and either inhibition or excessive activation of Rac GTPases leads to inhibition of enucleation (29).

4.3 Positive regulation of erythropoiesis

4.3.1 Positive regulation of erythropoiesis at the cellular level

To ensure a constant production of red cells, various components are required including iron for haemoglobin synthesis, and folic acid and vitamin B12 for DNA synthesis (review in (42)). Insulin growth factor 1 synergises with other cytokines to increase proliferation of early erythroid progenitors and may coordinate the production of red cells during development and increase of body mass. Steroid hormones through their nuclear receptors may also enhance red cell production but none of these molecules are involved in the fine regulation of erythropoiesis. However, androgen production may explain why haematocrit is higher in males than in females (43). Similarly, glucocorticoids may contribute to the increase of erythroid production during stress erythropoiesis (44). There is also evidence for a role of the renin angiotensin system in the regulation of erythropoiesis. Angiotensin II stimulates the proliferation of normal erythroid progenitors and a lack of angiotensin-converting enzyme may cause anaemia. In angiotensin-converting enzyme knock out mice, there is moderate anaemia and Epo synthesis is blunted, suggesting that *in vivo* angiotensin II plays rather a critical role for the regulation of Epo production by the kidney (45) (see below), consistent with the therapeutic

effect of angiotensin-converting enzyme inhibitors in secondary erythrocytosis after renal transplantation.

Cytokines play a critical role in positive regulation of erythropoiesis. They act on erythroid progenitors and precursors at all stage of maturation (BFU-E, CFU-E, erythroid precursors) to prevent apoptosis, induce proliferation, and promote or delay/inhibit differentiation. Delay in differentiation may play an important role by allowing self-renewal and expansion of erythroid progenitors before terminal maturation. As a result, production of red cells is increased. Cytokines are mainly produced by bone marrow stromal cells and endothelial cells, within the bone marrow environment, but also by immune cells including macrophages and lymphocytes and by organs outside the bone marrow, particularly the liver and the kidney which respectively produce thrombopoietin (TPO) and Epo.

Several cytokines have been described as possessing burst-promoting activity, i.e. they act on early erythroid progenitors to increase of the number of BFU-E; these include TPO, IL-3, IL-6, IL-8, IL-9, IL-11, GM-CSF (for review see (46)). However, none of them is critical for *in vivo* erythroid development. Genetic and biochemical approaches have demonstrated that, in contrast, SCF and Epo, which act on their respective receptors c-Kit and Epo-R, are absolutely required for erythroid cell production. Mice with mutations in the SCF gene (Steel (Sl) phenotype), or its receptor gene c-Kit (W phenotype) develop severe anaemia characterised by depressed erythropoiesis (47). Epo and Epo-receptor (Epo-R) knockout mice die of failure of foetal liver erythrocyte generation. Therefore, we will focus in this review on the effect of these two cytokines at the cellular and molecular levels and we will analyse the mechanisms by which they act synergistically.

a. *SCF and c-Kit*

SCF is a cytokine produced by stromal cells which interacts with its receptor (c-Kit) (48). C-Kit is expressed in the majority of $CD34^+$ haematopoietic progenitors and its expression is maintained at high levels during the stages of differentiation from BFU-E to CFU-E; at later stages of differentiation during the maturation of CFU-E, c-Kit expression progressively declines and disappears in polychromatophilic and orthochromatic erythroblasts. Thus, SCF exerts its effects on erythroid cells mainly during the early and late stages of differentiation of erythroid progenitors, and may act also on immature erythroid precursors.

Two isoforms of SCF are encoded as a result of mRNA splicing. One isoform is cleaved and released as a soluble biological form and another, which lacks the proteolytic cleavage site, remains principally membrane associated. Importantly, mutant mice that lack membrane associated SCF, but have the normal soluble form, are severely anaemic. Although not fully understood this difference may be due to the kinetics

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of c-Kit activation. C-Kit activation by membrane associated SCF results in a slow internalisation and degradation and in an increase of activation when compared to the activation induced by the soluble form.

At the cellular level, SCF induces an increase of cell survival and proliferation of early erythroid progenitors and precursors towards CFU-E, and the proerythroblast stage of maturation. At the same time SCF delayed erythroid differentiation and maturation (49). This effect allows self-renewal of early erythroid progenitors and precursors before they reach the stage where cells are no longer able to divide. At the later stage of maturation its main role is to prevent apoptosis and to induce proliferation, in synergy with Epo.

Currently, there is no evidence that SCF production is regulated by the need for red cells and oxygenation within the bone marrow. Its synthesis is probably more constitutive and although unproven, the level of available SCF may depend on its consumption by the amount of c-Kit protein expressed on erythroid precursors.

In fact, it is now agreed that regulation of erythropoiesis occurs at the CFU-E level depending essentially on the level of available Epo.

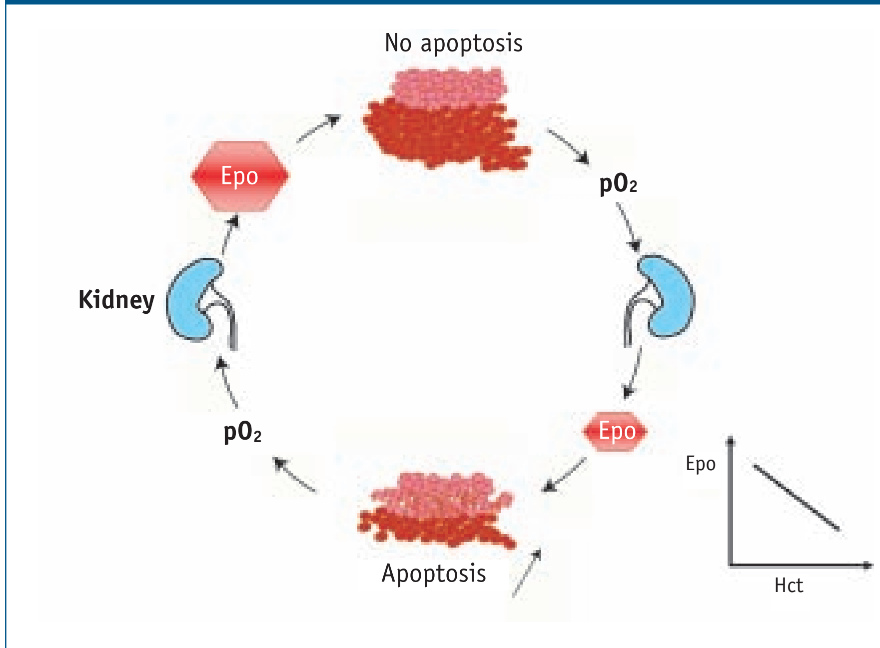
b. *Erythropoietin and erythropoietin receptor*

Haemoglobin in erythrocytes carries oxygen from the lungs and delivers it to other tissues. The number of circulating erythrocytes is the major determinant of tissue oxygenation.

Although the daily production of erythrocytes is tightly regulated, loss of blood due to bleeding or haemolysis leads to increased reticulocyte production within a few days. As the increased reticulocyte production normalises circulating erythrocyte numbers, the rate of new reticulocyte formation decreases to avoid rebound polycythaemia. Epo is responsible for this finely tuned homeostatic control of erythrocyte numbers by tissue oxygenation.

There is therefore an inverse relationship between erythropoiesis and oxygen delivery by the blood (Figure 2). The normal range of Epo serum concentrations is relatively low (5-30 mU/mL) and in physiologic conditions, a linear decline in haematocrit results in an exponential increase in serum Epo. The kidney is the major site of Epo production for definitive erythropoiesis. Epo is also produced in the adult liver, which is the main Epo producing organ during foetal erythropoiesis at the time where erythropoiesis is located in foetal liver. The renal Epo-producing cells are a subset of cortical interstitial cells located adjacent to the proximal tubules (see Chapter 1). The number of cells producing Epo increases exponentially with linear decreases in haematocrit. In adult liver the Epo-producing cells are the hepatocytes and interstitial cells, and their number also increases dramatically as

Figure 2: **Endocrine regulation of erythropoiesis**



Pink erythroid progenitors are less sensitive to Epo than red ones, and die of apoptosis at low concentration of Epo. On the right side, correlation of Epo level with haematocrit (Hct).

haematocrit decreases (for review see (50)). Hypoxia inducible factor (HIF)-1α is induced by hypoxia and interacts with the promoter of the *EPO* gene to form a transcription factor complex that positively regulates *EPO* gene expression. The rapidity of HIF-1α induction by hypoxia and its almost immediate disappearance after relief of hypoxia is a consequence of its rapid ubiquitination by a complex involving the von Hippel-Lindau protein, and then degradation by the proteasome (see Chapter 1). The binding of HIF-1α by the von Hippel-Lindau protein is dependent on the hydroxylation of two specific prolines by a proline hydroxylase. Within the transcriptional complex, HIF-1α is hydroxylated on asparagine residues by an asparaginase hydroxylase, which as a consequence inhibits HIF-1α binding to other proteins of the transcriptional factor complex. Thus, normoxic cells have sufficient oxygen for the hydroxylations of HIF-1α that lead to its rapid degradation and inactivation, while hypoxic cells do not have sufficient oxygen for these

reactions, and the nonhydroxylated HIF-1α survives and mediates the transcription of the *EPO* gene. The reports that patients with loss of function mutations in VHL and more recently in proline hydroxylase may develop polycythaemia have demonstrated *in vivo* the critical role of these proteins in the regulation of Epo synthesis (see Chapter 1).

EPO gene targeting reveals that Epo is dispensable at earlier stage of erythropoiesis including BFU-E (51). Likewise, *in vivo* administration of Epo has little influence on the number of BFU-E. In contrast, Epo administration dramatically increases the number of CFU-E and erythroid precursors up to the stage of basophilic erythroblasts, mainly by preventing their apoptosis. Erythroid progenitors and precursors exhibit a wide range of Epo sensitivities. This range of Epo sensitivities corresponds to the exponential range of serum Epo concentrations found in normal and anaemic states. At low Epo concentration only hypersensitive progenitors and precursors will survive.

Therefore, the regulation of apoptosis of erythroid progenitor and precursor cells during their stages of Epo dependence explain the rapid but tightly regulated control of erythrocyte populations in response to hypoxia, hyperoxia, and anaemia without requiring any effect of Epo on cell cycling or differentiation. In this model Epo modulates the rate of apoptosis of erythroid progenitors and precursors. Although this model was proposed more than twenty years ago, the molecular mechanisms underlying the Epo sensitivity of erythroid progenitors remain to be determined. It is possible that besides different intrinsic properties of erythroid progenitors, Epo sensitivity may be related to heterogeneity in the microenvironment in which erythropoiesis occurs.

Thus, erythropoiesis is mainly regulated in an endocrine manner, the kidney being the Epo producing gland and the target being the bone marrow (Figure 2).

4.3.2 Positive regulation of erythropoiesis at the molecular level

a. *SCF and c-Kit*

The binding of SCF to c-Kit results in dimerisation and autophosphorylation of the receptor on several distinct and specific cytoplasmic tyrosine residues that serve as docking sites for various Src homology 2 domain-containing enzymes and adaptor proteins including phospholipase Cg (PLC-g), phosphatidylinositol 3-kinase p85 subunit (PI 3-kinase), Ras GTPase activating protein, SHP2 phosphatase, Src kinases, Grb2, Grb7, and Shc. Although the precise role of each pathway is not yet fully elucidated, all of them are involved in cell proliferation and survival. However, PI 3-kinase and Src family kinases appear to play an essential role in regulating growth and survival of erythroid cells. The PI 3-kinase pathway might be activated through

both direct binding to specific tyrosine phosphorylated residues or through Src kinase activation. P85 knockout mice exhibit a reduction in BFU-E and CFU-E numbers (for review see (52)).
At the level of erythroid progenitors these pathways may act synergistically with EPO-R signalling as discussed below (for review see (53)).

b. *Erythropoietin receptor*
As discussed above, Epo is the crucial regulator of red blood-cell production and delivers essential growth, differentiation and survival signals to erythroid progenitors and precursors. Epo at the molecular level exerts its effect by stimulating Epo-R. The Epo-R belongs to the type I cytokine receptor family, characterised by a single transmembrane domain and a cytoplasmic tail lacking a kinase domain (54). The Epo-R is believed to exist in an unliganded dimeric state. On binding Epo, it undergoes a conformational change that activates the pre-bound, cytoplasmic, tyrosine kinase, Janus kinase 2 (JAK2). JAK2 is absolutely required for Epo-R activation and JAK2-knockout mice have a phenotype identical to Epo-R knockout mice. A ligand-induced receptor homodimer conformational change leads to trans-phosphorylation and activation of JAK2. Activated JAK2 phosphorylates eight key tyrosine residues in the Epo-R cytoplasmic domain, thereby providing docking sites for SH2 domain-containing downstream signalling molecules STAT5a/b, SHP1, SHP2, SHIP, p85a regulatory subunit of PI 3-kinase, Grb2, Lyn tyrosine kinase, and suppressor of cytokine signalling (SOCS) (for review see (55)). In addition to these signalling molecules, other signalling molecules that bind Epo-R have also been identified, including tyrosine kinases Syk, Tec, PLC-γ, and adaptor proteins Shc, Cbl, Crkl, IRS-2, and Gab1/2, and the nucleotide exchange factors Sos and Vav. The physiologic roles of the signalling cascade initiated via the phosphorylation of these tyrosines in Epo-R remain to be fully elucidated. Epo-R activates signal transducers and activators of transcription 5 (STAT5), Ras/mitogen-activated protein kinase (MAPK), and phosphoinositide-3 kinase (PI-3K)/Akt pathways as well as calcium entry and other signalling molecules. The role of these pathways in erythropoiesis will be briefly reviewed (Figure 3).

› **b1.** *JAK2/STAT5 and Bcl-XL*
Epo stimulation leads to a robust tyrosine phosphorylation of JAK2, which is time and dose dependent. JAK2 like all other members of the JAK kinases family has seven unique domains, termed JAK-homology (JH) domains. A catalytically active tyrosine kinase domain (JH1) and adjacent pseudokinase domain (JH2) are located at the C terminus. The pseudokinase domain has the architecture

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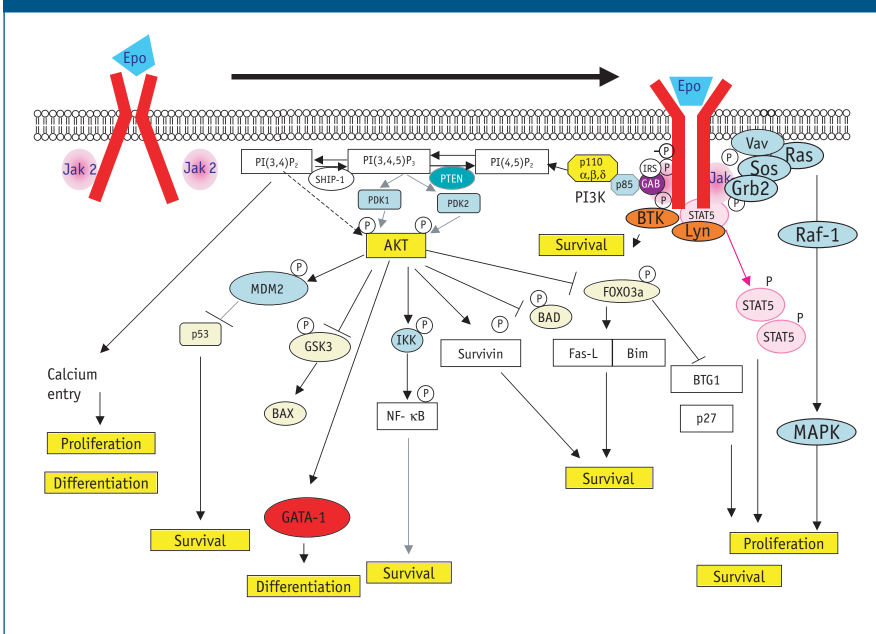
Figure 3: **EpoR and its role in cell survival, proliferation, and differentiation**



of a tyrosine kinase domain, but may have a negative regulatory role as recently evidenced in the pathophysiology of polycythaemia vera (56). JAK2 plays a pivotal role in mediating signals downstream of Epo.

JAK2 can support steady-state erythropoiesis via a route independent of Epo-R tyrosine phosphorylation that, in part, may involve MEK 1,2, and ERK 1,2 stimulation. Recent investigation of Epo-regulated survival genes shows that the "JAK2 only" axis induces down-modulation of pro-apoptotic Bim, Foxo3a and Trb2 (57). Moreover, the Epo "JAK2 only" pathway induces cell cycle regulation through induction of cell-cycle progression factors like G1 to S phase transition 1 (Gspt1), nuclear protein 1 (Nupr1), Egr1, Ngfi-A binding protein 2 (Nab2) and Myc (58).

Although in erythroid cells JAK2 is able to phosphorylate STAT1 (59) or STAT3 (60) that may play a role in regulation of stress erythropoiesis, the most important targets for steady state erythropoiesis are STAT5a/b isoforms. STAT5a and STAT5b are two isoforms of STAT5 that share 90% of homology and are one of the seven members of the STAT family, which are characterised by their critical role in transducing cytokine signalling. These proteins are latent in the

cytoplasm. They contain an SH2 domain allowing their docking to Y343 phosphorylated Epo-R and in turn their activation by phosphorylation on tyrosine residues in the transactivation domain. STAT5 is also phosporylated on serine residues but the function of these is not yet elucidated and phosphorylation may have an inhibitory effect on STAT5 transactivation. Activated STAT5 homodimers translocate to the nucleus to bind DNA and enhance gene activity involved in cell survival, proliferation, and differentiation. Mice expressing truncated Epo-R-mutants that retain solely the ability to activate STAT5 but lack all other tyrosines critical for activation of other signalling pathways live normally (61). These animals display only mild abnormalities in recovery from erythropoietic stress. Additional mutation of the Tyr 343 required for STAT5 activation, however, strongly affects stress erythropoiesis (62).
STAT5 plays an important role in maintaining a high erythropoietic rate during foetal development and during stress responses in adult mice, as shown by studies using STAT5a$^{-/-}$ STAT5b$^{-/-}$ mice (63). STAT5a/b-deficient erythroid progenitors exhibit high levels of apoptosis and are less sensitive to Epo. Indeed, a recent study implicated STAT5 in the stimulation of Pim1, Pim3, Irs2, Serphin-3G and Trb3, new anti-apoptotic effectors (57). Introduction of dominant negative STAT5 into adult erythroid progenitors also induced cell cycle arrest. It has recently been shown that STAT5 is implicated in cell cycle regulation through down-modulation of inhibitory cyclin G2, p27/Cdkn1b, B-cell leukaemia/lymphoma 6 (Bcl6), and also through induction of cyclin D2. Furthermore, during differentiation divisions of later stage erythroblasts, Epo modulation of cell cycle distribution suggests an influence of Epo in reprogramming toward differentiation (58). Expression of a constitutively activated STAT5a mutant is sufficient to complement the proliferation defect of Epo-R$^{-/-}$ JAK2$^{-/-}$ cells, enabling self-renewal and erythroid differentiation in the absence of an Epo signal (64). STAT5ab$^{-/-}$ mice show differentiation instead of renewal, causing accumulation of mature cell and gradual proliferation arrest. STAT5ab was additionally required for Epo-induced terminal differentiation. Differentiating STAT5ab$^{-/-}$ erythroblasts undergo apoptosis instead of erythroid maturation, due to the absent induction of the anti-apoptotic protein Bcl-xL. This defect can be fully rescued by exogenous Bcl-xL (65).
The anti-apoptotic role of STAT5 in Epo-R signalling, although still controversial, is probably mediated in part through its direct induction of Bcl-xL in erythroid cells. The Bcl-xL promoter region contains binding sites for STAT5 and GATA-1 that may act synergistically to positively regulate its activity (63). Embryonic Bcl-xL-knockout mice die around E12.5 because of brain defects and severe

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anaemia, and disruption of Bcl-xL in the haematopoietic lineage results in severe haemolytic anaemia (66). Although both models generate normal numbers of BFU-E, CFU-E and erythroblasts, defective maturation leads to anaemia. Exogenous expression of human Bcl-xL results in Epo-independent differentiation of wild-type murine erythroblasts, which indicates that expression of Bcl-xL alone can drive erythroid differentiation without the need for activation of other pathways. Therefore, Epo appears to be a permissive factor for the program of differentiation, which is driven by the complex network of transcriptional factors. Interestingly, Bcl-xL besides its major role as an anti-apoptotic factor in erythropoiesis, Bcl-xL is involved in the regulation of haem synthesis (67) and a marked increase in Bcl-xL expression was observed during early stages of the differentiation from a BFU-E to a CFU-E and from CFU-E to pro-erythroblasts. Bcl-xL levels increase during the early and intermediate stages of erythroid maturation, reaching a peak level of expression when the majority of erythroid cells had reached the stage of polychromatophilic erythroblasts.

› **b2.** *PI 3-kinase*

Epo also stimulates cell survival, proliferation and differentiation by activating PI 3-kinase (p85 (regulatory subunit)/p110 (catalytic subunit)) through direct recruitment of the p85 to Epo-R phosphorylated tyrosines, and also indirectly through binding to Cbl, Gab1, Gab2, insulin receptor substrate (IRS)-2 and Vav (reviewed in (68)). It may also be activated by Ras. PI 3-kinase activation is necessary but not sufficient to protect against apoptosis. Mice that do not express the p85 subunit of PI 3-kinase have markedly reduced erythropoiesis with reductions in the number of BFU-E and CFU-E precursors. By activating PI 3-kinase, Epo has an effect on the progression of the cell cycle through up-regulation of cyclin D3, E and A at the protein level, and c-Kit at the mRNA and protein level (69). Protein kinase B (PKB)/Akt, a serine/threonine kinase is activated downstream of PI 3-kinase through the generation of second messenger 3'-phosphorylated inositol lipids (PIP3), and in this process PKC activity is required (70). Many downstream substrates of PKB/Akt play an important role in regulating proliferation and survival of erythroid progenitors. They include the forkhead Foxo3a/ FKHR-L1 (71, 72) transcription factors, the proaoptotic protein Bad, and the glycogen synthetase kinase-3 (GSK3) (73).
PI 3-kinase may induce cell proliferation through the phosphorylation and thereby inhibition of transcriptional activity of Foxo3a. Among the genes transcriptionally activated by Foxo3a two play a critical role in erythroid cell proliferation, B cell translocation gene 1 (BTG1) and p27 Kip1 cyclin-dependent kinase inhibitors (74), and Ccng2 (Cyclin G2).

PKB/Akt promotes cell survival at various levels (for review see (75)). First, it phosphorylates the pro-apoptotic protein Bad. Bad sequestrates the Bcl-xL protein in the cytoplasm. Upon Bad phosphorylation, Bcl-xL is released and thus allows maintenance of mitochondrial integrity. Second, upon phosphorylation by Akt, Foxo3a is exported from the nucleus to the cytoplasm where it is sequestered by 14-3-3 proteins and thereby cannot activate genes involved in cell death including Fas-L and Bim. Third, Akt activation is involved in the positive regulation of Survivin, a member of the IAP family (inhibitor of apoptosis family) that inhibits caspase activity. Fourth, by a mechanism not fully elucidated, Akt increases the transcription of survival genes through the activation of NF-κB and CREB transcription factors. Finally, the glycogen synthetase kinase-3 (GSK3), which is involved in glycogen metabolism is also inhibited by Akt and the inhibition of its activity is able to protect erythroid cells from apoptosis upon Epo deprivation. Although its mechanism of action is not yet fully known, PI 3-kinase may be also involved in cell differentiation and its activity, at least in some cell lines, is required for Glycophorin A and haemoglobin synthesis. Akt mediates a signal downstream of Epo-R, distinct from its survival signal that supports differentiation of foetal liver erythroid progenitor cells. Activated Akt complements Epo-R signalling and supports erythroid-cell differentiation in WT and JAK2-deficient foetal liver erythroid progenitors (76). Akt may act by phosphorylation of GATA-1, which increases its transcriptional activity (17). Moreover, the PI 3K/Akt signalling pathway has been identified as a mediator of Epo-induced phosphorylation of GATA-1. Akt phosphorylates GATA-1 S310 in erythroid cells and enhances GATA-1 transcriptional activity (16).

› **b3.** *Ras and MAP kinase pathway*

Epo activates the Ras pathway through several mechanisms. Grb2, a cytoplasmic adapter protein, can bind directly through its SH2 domain to the tyrosine phosphorylated Epo receptor, or indirectly through the tyrosine phosphatase SHP2 or SHC. All these mechanisms lead to translocation of SOS, a Ras guanine nucleotide exchange factor, to Ras, resulting in its activation. In addition, Vav proteins may also play the role of exchange proteins to activate Ras. Downstream of Ras activation, Raf-1 is activated and in turn MAP kinases, and some early genes are activated including c-fos, c-myc, c-jun.

Ras activation is essential for the differentiation, proliferation, and survival of erythroid progenitors *in vitro*. In mammals, although there are 3 homologous Ras proteins, H-Ras, N-Ras, and K-Ras, only K-Ras is essential for erythropoiesis; it is particularly required for the differentiation of erythroid progenitor cells to

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late basophilic erythroblasts and for the proliferation of haematopoietic progenitors (77).
Epo activates the catalytic activity of several MAP kinases, including extracellular regulated kinases 1/2 (Erk1/2), SAP kinase (SAPK)/Jun kinase (Jnk) and p38. Erk1/2 is thought to participate in mitogenesis, whereas the roles of SAPK/JNK and p38 are more complex and not fully elucidated. The ERK1/2 pathway in erythroid cells is involved in the early proliferative phases of erythropoiesis and in the inhibition of terminal erythroid differentiation (78). JNK is a serine/threonine kinase that phosphorylates Jun proteins and activates its transcriptional activity within AP1 complexes. AP1 is implicated in the promotion of cell cycle activity, and in both positive and negative regulation of apoptosis. AP1 complexes and particularly those containing Jun-B have been implicated in the induction of apoptosis observed when growth factors are removed. In contrast, AP1 complexes containing activated c-jun have been implicated in the repression of apoptosis in growth factor-dependent cells, and expression of c-jun delays apoptosis induced in erythroid cells. JNK activity appears to be an important regulator of proliferation in immature, primary erythroid cells and erythroid cell lines but may not be required for the survival or proliferation of CFU-E or pro-erythroblasts (79). P38 knock out mice are severely anaemic. This phenotype is due to a defect in the post-translational regulation of Epo mRNA (80). The p38 MAPK signal transduction pathway may play a critical role in differentiation as evidenced in butyrate-induced erythroid differentiation of the K562 cell line, as well as in Epo-dependent differentiation of mouse erythroleukaemia SKT6 cells and of primary erythroblasts (81).

› **b4.** *Lyn and Btk kinase pathway*
Other kinases associated with Epo-R including Lck/Yes-related novel tyrosine kinase (Lyn) and Bruton's tyrosine kinase (Btk) may play a role in Epo-R phosphorylation and signal transduction (82). Lyn is a substrate for Epo signalling and has a role in erythroid differentiation. Evidence of its involvement has emerged following the isolation of erythoid cell line J2E-NR, which is Lyn-deficient and has impaired erythroid differentiation (83). Erythroblasts from Lyn-deficient mice do not express GATA-1 and EKLF, and expression of STAT5a and STAT5b are decreased. Impairment of erythroid differentiation and anaemia are observed as evidenced by an increase of BFU-E and CFU-E number in spleen (84). Reduced phosphorylation of Lyn but not of JAK2 by CD45 inhibits erythoid differentiation of cord blood $CD34^{+}$ (85). Lyn plays a positive role at both stages of erythroid development, for expansion of early progenitors and survival of late erythroid progenitors (86). Bruton tyrosine kinase, which is critical for B cell

development is also involved in Epo-R signalling. Although neither patients with X linked agammaglobulinemia (Btk deficiency) nor Btk knockout mice show any overt defect in erythropoiesis, Btk deficient erythroblast progenitors exhibit a reduction in Epo-R and JAK2 phosphorylation and have enhanced erythroid differentiation and reduced capacity for self renewal. The mechanism of action of Btk on Epo-R signalling remains to be determined, but probably occurs downstream of JAK2 and Lyn activation. Furthermore, Btk is involved in the protection of erythroid progenitors and early precursor sensitivity to death receptor stimulation (87) (see below).

› **b5.** *Regulation of intracellular calcium by erythropoietin*

Activation of Epo-R causes an increase in intracellular calcium. Specific tyrosine residues in the Epo-R are required for this calcium influx. The transient receptor potential channel proteins TRPC2 and TRPC6 are highly expressed in erythroid progenitors and may play a role in calcium entry. In erythroid cells calcium concentration increases more in the nucleus than in the cytoplasm after Epo stimulation. Calcium may regulate cell proliferation and differentiation through expression of proto-oncogenes, phosphorylation of transcriptional factors, or activation of calcineurin (55).

c. *Synergy between c-Kit and erythropoietin receptor at the molecular level*

Epo and SCF act synergistically to increase erythropoiesis by several molecular mechanisms. SCF sensitises cells to erythropoietin and therefore the requirement of Epo is lower for full completion of erythroid proliferation and survival. At the same time, SCF slows down differentiation of erythroid progenitors, which may result in expansion of erythroid cells. *In vitro*, whereas Epo alone is able to produce human erythroid cell development and maturation from progenitor cells, a markedly enhanced, synergistic proliferation and expansion of developing erythroid cells is supported by the combination of Epo and SCF. In cell lines, c-Kit and Epo-R interact physically via the box2 cytoplasmic domain of the Epo-R (88), but this interaction is not sufficient to induce erythroid formation in primary cells. Mechanistically, c-Kit stimulation by SCF does not activate Epo-R by inducing its dimerisation, but by phosphorylating tyrosine residues in the cytoplasmic domain of Epo-R. It seems that Epo-R Y343, which binds STAT5a, is implicated in c-Kit co-signalling. On the other hand, tyrosine residues that bind Src kinases in the c-Kit receptor appear to be sufficient for co-signalling with Epo-R (88).

However, Epo and SCF may have distinct and sequential effects on erythroid differentiation and no joint effect of Epo and SCF is required to quantitatively describe the proliferation or death of erythroid cells during erythroid differentiation. Overall,

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SCF promotes the early proliferation of primitive cells, while Epo primarily promotes the survival of differentiating erythroid progenitors (89).
At the molecular level, SCF and Epo synergistically enhance the magnitude and the duration of the MAP kinase (MAPK, ERK1/2) pathway, which may be important for both proliferation and for reducing differentiation of erythroid cells (78). Prolonged activation of MAPK might also play a role in preventing apoptosis. However, SCF and Epo may lead to activation of MAPK through different pathways and their downstream targets may be not identical.
The synergistic effect between Epo and SCF on cell survival may also occur at others levels through distinct signalling mechanisms. For example, SCF through PI 3-kinase activation induces a robust activation of Akt and thereby phosphorylation of Bad and its sequestration in the cytosol by 14-3-3 proteins, allowing the release of Bcl-xL in the mitochondrial membrane which protects the cell against apoptosis (see below). In this model of synergy, Bcl-xL expression is increased by the JAK/STAT pathway activated by Epo-R, and its activity is increased by the c-Kit receptor. Likewise, STAT transactivation activity is increased by the activation of the PKA/CREB dependant pathway induced by c-Kit and the recruitment of CREB binding protein CBP/p300 (90). Multiple other pathways that remain to be determined could also be involved in the major synergistic effect observed between SCF and Epo. Interestingly, pathways activated by c-Kit might be sufficiently divergent from those activated by other cytokines (TPO, IL-3, GM-CSF, etc) to explain the lack of redundancy with SCF *in vivo*.

4.4 Negative regulation of erythropoiesis

Although not fully elucidated, the cellular regulation and the molecular signals responsible of positive regulation of erythropoiesis are well characterised. However, a negative control is clearly required at several levels to avoid overproduction of erythroid cells and polycythaemia, which may lead to hyperviscosity and ultimately to thrombosis.

4.4.1 Negative regulation of erythropoiesis at the cellular level

a. *SCF and c-Kit*

As mentioned above there is no clear evidence that excess of erythroid progenitors or red cell regulates SCF production by stromal cells. In contrast, Epo concentration and Epo-R activation are the major parameters that determine red cell production.

b. *Erythropoietin production*

Epo production is determined at two levels. First, Epo production is well controlled

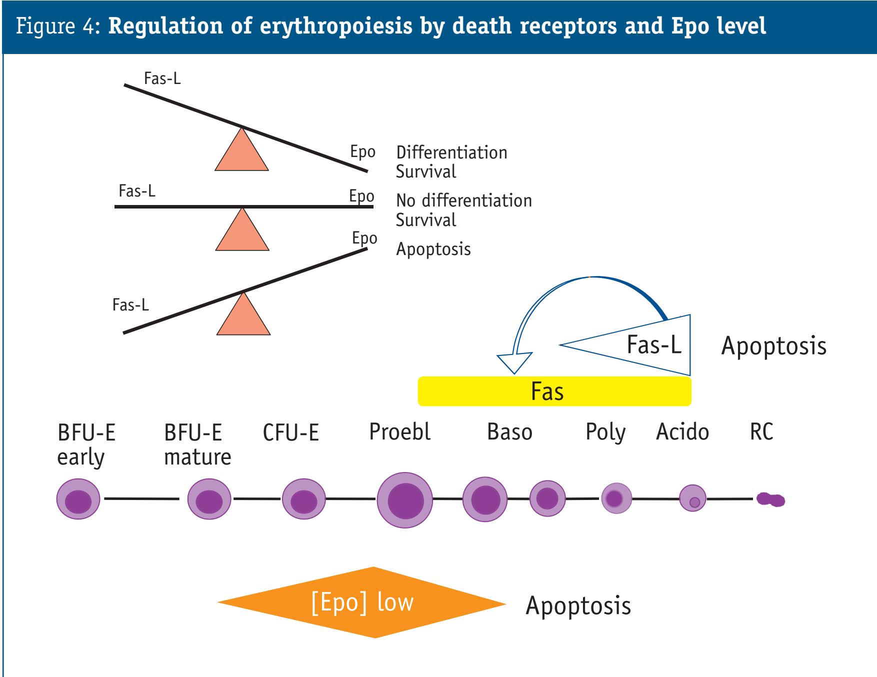
by renal oxygenation (see above and Chapter 1). Hence, a sufficient number of red cells and adequate oxygenation results in decrease of Epo production by the kidney, through degradation of HIF by the proteasome (see Chapter 1). As a consequence, the less Epo sensitive erythroid progenitors and precursors will die of apoptosis. In addition to the reduction of Epo production, a down-modulation of Epo action occurs after Epo/Epo-R interaction through the endocytosis and subsequent degradation of Epo and the Epo-R by the proteasome of erythroid progenitors (see below).

These multiple levels of down regulation contribute to the fine control of Epo concentration in the blood, preventing polycythaemia after recovery from bleeding or red cell haemolysis.

c. *Death receptors and their ligands*

Beside oxygenation and Epo production, erythropoiesis is also regulated in a paracrine fashion within the bone marrow erythroblastic islands. In this system, mature erythroblasts inhibit survival and differentiation of immature erythroblasts through Fas/Fas-L interactions, with death ligands being expressed on mature erythroid cells and the death receptor being present on the surface of immature erythroblasts (increased expression from BFU-E to CFU-E, to reach maximal level at the stage of pro-erythroblasts and basophilic erythroblasts) (75, 91). The pattern of expression of membrane bound Fas-L is different, Fas-L being expressed only in mature erythroblasts (orthochromatic erythroblasts). Sensitivity to Fas-L is increased by cytokines that induce cell cycling of immature erythroid progenitors, including SCF, IL-3 and TPO. In contrast, mature precursors are insensitive to Fas-L. Resting progenitors are protected by elevated levels of c-FLIP, an inhibitor of Fas activation. Furthermore, Fas triggering induces an arrest of erythroid maturation through the cleavage of TAL-1 and GATA-1 by caspases (91, 92). In addition to direct depolarisation of the mitochondrial membrane (see below), these mechanisms may account for the low, but significant, level of ineffective erythropoiesis associated with premature death of erythroid cells observed in normal subjects and also the maintenance of an available pool of erythroid progenitors. Interestingly, SCF or high dose of Epo are able to circumvent the apoptotic effect of Fas-L on erythroid precursors, allowing production of red cells during stress erythropoiesis. A similar system is observed with another death receptor (TRAIL-R) and its ligand (TRAIL) (93). Therefore, the control of the production of mature red cells may be summarised as follows (Figure 4). At low doses of Epo, cell die by apoptosis, at intermediate doses, cell are arrested in their maturation or enter a program of apoptosis, depending on the number of mature erythroblasts in the bone marrow, and at high doses of Epo, erythroid progenitors and precursors pursue their maturation

Figure 4: **Regulation of erythropoiesis by death receptors and Epo level**



independently of the number of mature erythroid precursors. This model allows the prevention of massive apoptosis of erythroid precursors during steady state erythropoiesis, while allowing the production of red cells during stress erythropoiesis, following haemolysis or haemorrhage.

This paracrine model is being investigated. In a mouse model, interactions between splenic early erythroblasts coexpressing Fas and Fas-L lead to their apoptosis, exerting a negative autoregulatory effect that limits basal erythropoietic rate in an autocrine fashion. With the induction of stress, high Epo suppresses Fas and Fas-L expression on erythroblasts, resulting in enhanced erythroblast survival and a consequent increase in the rate of erythropoiesis (94).

Furthermore, JAK2-mutated polycythaemia vera (PV) erythroblasts showed lower levels of Fas-induced caspase activation and incomplete caspase-mediated cleavage of GATA-1. JAK2 mutation was associated in PV erythroblasts with a deregulated expression of $cFLIP_{SHORT}$. This may contribute to death receptor resistance in PV erythroblasts and ultimately lead to a blunted response to death receptor-mediated regulation of erythropoiesis (95).

d. *Cytokines that negatively regulate erythropoiesis*

There are a number of pathological conditions where erythropoiesis is inhibited. The pathophysiologic mechanisms underlying this inhibition are reviewed in other chapters of this book. We will briefly review the mechanism of action of the inflammatory cytokines that are overexpressed in anaemia of chronic disease (67). In the inflammatory process various cytokines including tumour necrosis factor (TNF)-α, interferon (INF)-γ and transforming growth factor (TGF)-β and chemokines such as stromal derived factor (SDF)-1 are abnormally synthesised, and may impair erythropoiesis at several levels.

For example, inflammatory cytokines are responsible for a blunted erythropoietin response by the kidney. The level of Epo in the serum is therefore not adjusted according to tissue oxygenation. They also decrease the availability of iron to erythroid progenitors by inducing synthesis of hepcidin (for review see (96)). Interestingly, it has been suggested that hepcidin may also act directly on erythroid progenitors to decrease their threshold of sensitivity to Epo (97). Inflammatory cytokines can also directly act on erythroid progenitors.

› **d1.** *Tumour necrosis factor-α*

TNF-α induces a decrease in mature erythroblasts and an increased rate of apoptosis within the compartment of immature erythroblasts. It may act directly on the TNF receptor on immature erythroblasts and through the induction of ceramide synthesis. *In vivo*, treatment with a blocking anti-TNF-α monoclonal antibody results in improvement in anaemia and in the proportion of apoptotic erythroblasts (98). TNF-α could play a role in modulating NF-kB activity which positively regulates erythroid cell proliferation and survival and negatively regulates cell maturation.

› **d2.** *Interferon-γ*

INF-γ plays a complex role in the regulation of erythropoiesis. It has no apoptotic effect by itself and may even exert a protective effect on apoptosis at the mature stage of erythroblastic differentiation.

In contrast, by increasing expression of several death receptors and their ligands, including Fas-L/Fas and TRAIL as well as the recently characterised protein TWEAK and its receptor fibroblast growth factor-inducible 14 (Fn14), INF-γ contributes to indirectly inducing apoptosis of erythroid progenitors (99). Furthermore INF-γ induces the formation of ceramide and the blockade of ceramide synthesis abolishes apoptosis (100).

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› **d3.** *Transforming growth factor-β1*
TGF-β1 is a powerful inhibitor of erythropoiesis. However, its mechanism of action is different from other cytokines or death ligands. It has virtually no effect on apoptosis, but it markedly accelerates and increases erythroid differentiation to produce normal enucleated red cells even in the absence of macrophages. TGF-β1 inhibits cell proliferation by decreasing the cycle of immature erythroid cells and by accelerating maturation toward orthochromatic normoblasts that are not in cycle. Therefore, TGF-β1 is a paradoxical inhibitor of erythropoiesis that acts by blocking proliferation and accelerating differentiation of erythroid progenitors (101).

› **d4.** *Stromal derived factor-1*
Bone marrow stromal cells produce SDF-1, and haematopoietic cells express its receptor, CXC chemokine receptor 4. Low concentrations of SDF-1 promote haematopoietic cell growth, but high levels decrease erythroid progenitor growth through up regulation of Fas-ligand production and subsequent erythroid cell apoptosis via the Fas-ligand/Fas pathway (102). The factors that modulate this dual effect are not yet known.

4.4.2 Negative regulation of erythropoiesis at the molecular level

a. *Negative regulation of c-Kit phosphorylation*
The negative regulation of c-Kit signalling is mediated by various phosphatases including SHP-1, SHP-2, and SHIP. SHP-1 is known to interact with phosphorylated c-Kit at a specific tyrosine residue and dephosphorylate the receptor, and as such inactivates all pathways downstream of c-Kit signalling. SHP-2 interacts with c-Kit at another tyrosine residue and influences the activation of the MAP kinase pathway (52). C-Kit is also a target for the adaptor protein Lnk (see below), which may contribute to inactivation of c-Kit receptor (103).

b. *Negative regulation of the erythropoietin receptor at the molecular level*
Besides the control of Epo levels to avoid over stimulation, Epo-R activation is also finely regulated after its activation. Activation of the Epo-R after Epo binding is very transient because of the rapid activation of strong down-regulation mechanisms that quickly decrease Epo sensitivity of the cells. These down-regulation mechanisms include receptor internalisation, degradation and inhibition of tyrosine phosphorylation.

› **b1.** *Degradation of erythropoietin receptor*

The Epo receptor is rapidly ubiquitinated after ligand stimulation and the C-terminal part of the Epo receptor is degraded by the proteasome. Then, the Epo-Epo-R complexes are rapidly internalised and targeted to the lysosomes for degradation. Both ubiquitination and receptor degradation by the proteasomes occur at the cell surface, and require JAK2 activation (104).

› **b2.** *Inactivation of erythropoietin receptor by phosphatases*

In addition to its degradation, tyrosine phosphorylation in response to Epo is transient and returns to basal levels within 30 min of stimulation. Epo-R is rapidly dephosphorylated by several systems involving SOCS proteins and tyrosine phosphatases.

Several phosphatases have been involved in this process, but SHP-1 phosphatase appears to play an essential role. SHP-1 binds via its SH2-domain to a specific tyrosine on the phosphorylated Epo-R. Epo-R lacking this binding site is unable to bind SHP-1 and to dephosphorylate and inactivate JAK2 (105). The lack of expression of SHP-1, or impaired binding of SHP-1 to the Epo-R, leads to Epo hypersensitivity and erythrocytosis. These phenotypes have been described in humans as a consequence of the expression of truncated or frame-shift mutations of Epo-R that lack the C-terminal binding site for SHP-1 (106). Another manner of desensitisation involves the regulation of metabolic intermediates of inositol. Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) activates many crucial signalling events. Several enzymes dephosphorylate PtdIns (3,4,5) P3, including phosphatase and tensin homolog (which dephosphorylate the 3 phosphate) and SH2-inositol phosphatase, SHIP-1 and SHIP-2 (which dephosphorylate the 5 phosphate) (107). Their precise role in erythropoiesis remains to be determined.

› **b3.** *Inactivation of erythropoietin receptor by SOCS proteins*

In addition to phosphatases, the SOCS family of negative regulators of cytokine signalling is also involved in the control of Epo-R activation (108). Expression of SOCS genes is rapidly modulated in response to a variety of cytokines and the SOCS family acts via a negative feedback loop to suppress cytokine-induced signal transduction. Three SOCS genes are associated with erythropoiesis, including SOCS-1, CIS, and SOCS-3. Expression of CIS is elevated upon activation of JAK2 and STAT5. SOCS-1 inhibits the activation of JAK2. SOCS-3 is tyrosine phosphorylated in response to Epo stimulation and also suppresses Epo signalling by associating with the Epo receptor and JAK2. Recruitment of CIS to the Epo-R via the SH2 domain represses proliferation. SOCS genes are

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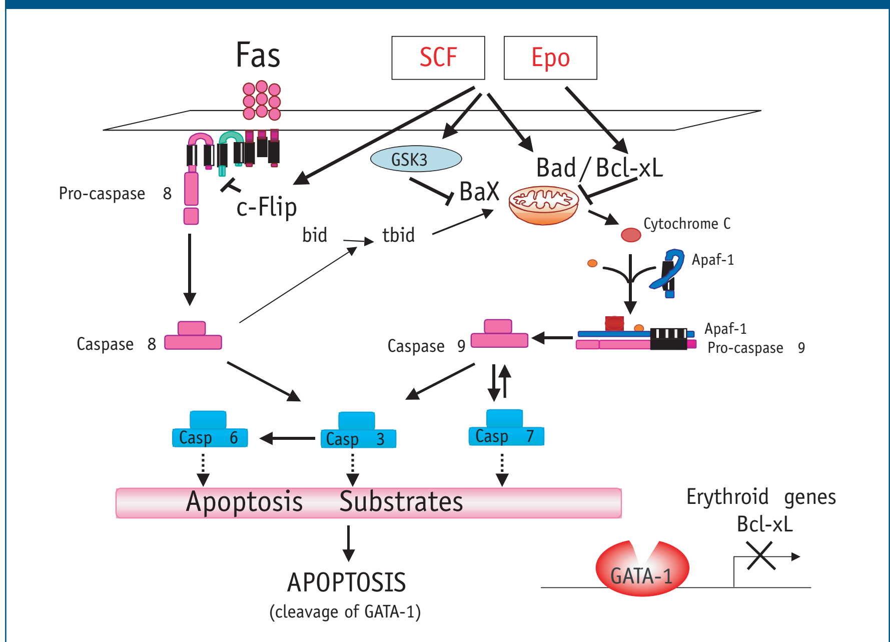
differentially regulated in normal and transformed erythroid cells depending upon the stage of maturation and may exert a complex regulation. Analysis of transgenic and knockout mice indicates that SOCS proteins are not vital to the regulation of erythropoiesis.

› **b4.** *Adaptor proteins, Lnk and modulation of erythropoietin receptor activation*
Cytokine receptor signalling networks rely heavily on adaptor proteins, and they may play a critical role in controlling receptor activation and threshold of sensitivity to Epo. Lnk, a member of a newly discovered adaptor protein family, is involved in erythroid regulation. Lnk does not possess a kinase domain but contains several protein-protein interaction domains including a proline-rich amino-terminus, a pleckstrin homology (PH) domain, a Src homology 2 (SH2) domain, and a conserved tyrosine near the carboxyl-terminus. Lnk becomes tyrosine-phosphorylated following Epo administration and inhibits Epo-induced Epo-R phosphorylation and JAK2 activation as well as downstream pathways including STAT5, AKT and MAPK. The Lnk Src homology 2 (SH2) domain is essential for its inhibitory function. However, its mechanism of action remains to be elucidated. Lnk may disrupt the binding of positive regulators of JAK2, recruit other JAK2 inhibitors, such as SOCS proteins or SHP-1. Finally, binding of Lnk to JAK2 may cause a conformational change that keeps JAK2 in a kinase-inactive state. Likewise, Lnk-deficient mice have an elevated number of erythroid progenitors, and exhibit superior recovery after erythropoietic stress. *In vitro*, CFU-E progenitors are hypersensitive to Epo, and Epo-R phosphorylation, JAK2 activation and Epo-induced signalling pathways, including STAT5, AKT, and MAPK are enhanced. Conversely, Lnk overexpression inhibits Epo-dependent erythroblast differentiation and induces apoptosis (103).
Taken together, the efficiency of these processes provides explanation for the short duration of intracellular signalling activated by Epo. In addition the negative regulator of Epo-R signalling may play a fundamental role in the control of the survival of erythroid progenitors and precursors. Indeed, as mentioned above, erythroid progenitor cells possess a vast heterogeneity in their sensitivity to Epo. This intrinsic difference of erythroblasts in their sensitivity to Epo might be tightly regulated through negative signalling molecules associated with the Epo-R, providing another checkpoint in addition to Epo level for the regulation of erythropoiesis.

c. *Molecular regulation of erythropoiesis by apoptosis*
As mentioned above, during erythropoiesis not all erythroid cells survive, because they are particularly sensitive to apoptosis. The rate of apoptotic cells might be

determined by the complex combination of Epo and Fas-L levels and the level of sensitivity of erythroid precursors and progenitors to these cytokines that can activate both extrinsic and intrinsic pathways of apoptosis. These two pathways may, however, converge in erythropoiesis (Figure 5). During erythroid differentiation, erythroid progenitors constitutively express pro-apoptotic proteins of the Bcl-2 family such as Bid and Bax (109). Both of these proteins cooperate in activating the intrinsic pathway of apoptosis through the formation of pores in the membrane of mitochondria. As a consequence, cytochrome c is released, and with Apaf-1 and caspase 9 they constitute the apoptosome, which in turn leads to activation of caspase 9 and then caspase 3. Caspase 3 cleaves GATA-1 and Tal-1 and proteins involved in cytoplasmic, nuclear, and DNA integrity. Bcl-xL through its antagonistic effect is probably the major protein involved in the protection of erythroid progenitors from apoptosis. Withdrawal of erythropoietin or stimulation of death receptors induces apoptosis by two different molecular mechanisms that may, however, converge in the formation of mitochondrial pores.
Epo deprivation induces Bcl-xL down-regulation through reduction of its transcription

Figure 5: **Regulation of apoptosis in erythropoiesis at the molecular level**



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as a consequence of decrease of STAT5 activation and GATA-1 cleavage. Furthermore, caspase 3 cleaves Bcl-xL. ERK1 and ERK2 activities may at this level control caspase activity and as such maintain expression of Bcl-xL.
GSK3 activity is suppressed by its phosphorylation induced by activation of the erythropoietin receptor and c-Kit in human erythroid progenitor cells. Therefore, increased GSK3 activity induced by growth factor withdrawal may result in a conformational change of Bax that increases its apoptotic activity (73).
The extrinsic pathway involves the death receptors (DR) at the plasma membrane level. In response to their specific ligand (DR-Ligand), these trimeric receptors recruit the adapter molecule FADD and either procaspase-8 or -10 in the death-inducing signalling complex (DISC). These enzymes, either directly through caspase 8 activation, or indirectly through cleavage and consequent activation of Bid, resulting in formation of pores in the mitochondria induce caspase 3 activation. Although not fully demonstrated, in erythroid cells the intrinsic pathway seems the most important since Epo and SCF are able to block apoptosis induced by Fas-L through the maintenance of mitochondrial membrane integrity.
Foxo3A alone can recapitulate all known elements of the apoptotic program normally induced by cytokine withdrawal through the activation of the intrinsic pathway, but also through activation of the extrinsic pathway. Foxo3A is expressed in erythroid cells, its expression decreases progressively during erythroid maturation. In immature erythroid precursors, Foxo3A is rapidly phosphorylated by Epo through a PI 3-K-dependent pathway and is exported outside the nucleus and therefore cannot induce the expression of the pro-apoptotic protein of the bcl2 family Bim and of Fas-L.
SCF and Epo may protect CFU-E from Fas-induced apoptosis, the protective effect of SCF being significantly more effective than the effect elicited by Epo alone. This protective effect of SCF involves the activation of Src-kinases, PI-3 kinase, and JAK/STAT5 activation, and MAP kinases. Activation of PKCε could also modulate the sensitivity of erythroid progenitors to death receptor by inducing anti-apoptotic proteins of the bcl2 family (100). The simultaneous addition of IFN-γ and Fas-L to CFU-E elicits a marked activation of caspase 8 and 3 with a consequent apoptotic response. This phenomenon is inhibited by SCF via a mechanism involving the up regulation of c-FLIP expression, an inhibitor of caspase 8 activation (111).

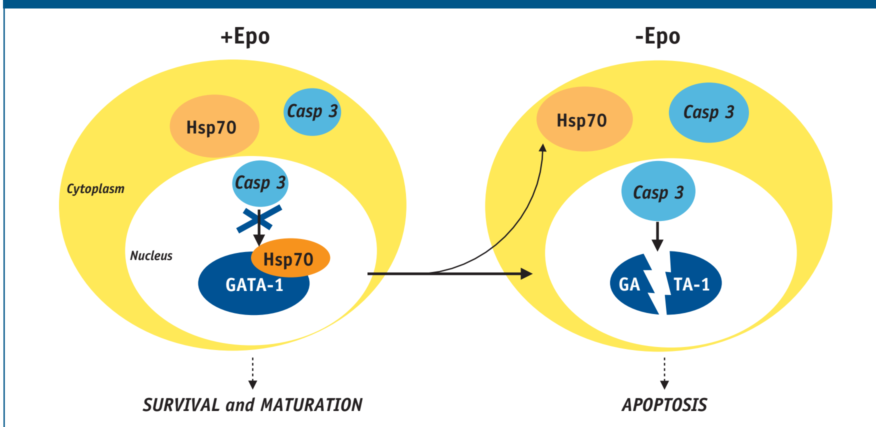
5. Role of caspases in maturation of erythroid cells

Taken together, in the regulation of erythropoiesis, caspases appear to play a critical role in the negative regulation of erythropoiesis. Both, low concentration of Epo or excess of mature erythroblasts (through activation of Fas-L and TRAIL)

induce caspase activation and, as a result, apoptosis of erythroid progenitors. However, as in other systems of differentiation with terminal enucleation, including keratinocytes and lens epithelial cells, or without enucleation such as megakaryocytes to proplatelets or monocytes to macrophages, caspases may also play a critical and positive role in erythroid precursor maturation (112).
The terminal differentiation of erythroid cells exhibits some similarities with apoptosis such as chromatin condensation and the degradation of nuclear components. A transient activation of several effector caspases is required for Epo-induced erythroid differentiation in both humans and mice. This transient caspase activation is not required for the process of enucleation itself, but occurs at earlier steps of red blood cell formation (113-115). The process of enucleation itself could involve p53 in erythroid cells (116), and as mentioned above DNase II from central macrophages in the erythroblastic island. However, caspase inhibition arrests the *in vitro* maturation of erythroid progenitors before nuclear and chromatin condensation at the transition between pro- and basophilic erythroblasts. Caspases are probably activated through a mitochondria-dependent pathway similar to that identified in various forms of apoptosis. Release of cytochrome c from the mitochondria and activation of caspase 9 and then caspases 3 and 6 are observed. Likewise, caspase activation in erythroid cells undergoing differentiation is negatively regulated by the Raf-1 kinase, which prevents premature differentiation of actively proliferating precursors (114). It has recently been demonstrated that a balance between Raf-1 and Fas expression permits erythroid differentiation. Indeed, Raf-1 by activating the MEK/ERK cascade induces progenitor proliferation. In contrast, differentiation is mediated by Fas via the activation of both ASK1/JNK/p38 pathway and caspases cascade. The point of convergence between the two cascades is activated ERK, which maintains Raf-1 expression and inhibits Fas expression. In turn, Fas, once expressed, exerts negative feedback on ERK activation and Raf-1 expression (117, 118). Emphasizing the role of mitochondria in erythroid differentiation, Nix$^{-/-}$ mice exhibit increased polychromatophils and abnormally shaped erythrocytes suggestive of a maturation defect. Nix deficiency caused anaemia, reticulocytosis and erythroid-myeloid hyperplasia (40). Those results show that pro-apoptotic proteins such as Fas or Nix are involved in erythroid differentiation. Further research is needed to determine by which mechanism caspase 3 is activated during terminal erythropoiesis. Caspase 3 induces cleavage of the protein acinus responsible for chromatin condensation without DNA degradation, and caspase 6 cleaves proteins involved in nuclear integrity such as lamin B (113), which may explain morphological changes in the nucleus occurring during erythroid differentiation. The identification of other targets cleaved during erythroid differentiation remains an important question to be elucidated. Interestingly, ICAD, a chaperone of CAD, is not cleaved by caspase

3 during erythroid differentiation. In another study on cell lines, ICAD is cleaved during erythroid differentiation but CAD is down-regulated at the mRNA and protein level. These changes provide a mechanism by which cells avoid DNA fragmentation with activated caspase 3 (118).
In addition, during erythroid differentiation, caspases are activated, but cells do not enter apoptosis as they do not cleave their DNA and do not expose phosphatidyl serine at the cell surface. Although GATA-1 is cleaved by caspases in erythroid cells undergoing apoptosis under Epo deprivation or death receptor stimulation, it remains uncleaved in erythroid cells undergoing terminal differentiation. An explanation for this apparent paradox came from the finding of a constitutive expression of HSP70 in erythroid cells. During differentiation but not during apoptosis, the chaperone protein Hsp70 protects GATA-1 from caspase 3-mediated proteolysis. At the onset of caspase activation, Hsp70 translocates and co-localises and interacts with GATA-1 in the nucleus of erythroid precursors undergoing terminal differentiation. In contrast, Epo starvation induces the nuclear export of Hsp70 and the cleavage of GATA-1 (Figure 6) (119). This model provides a potential explanation for the lack of cell death when caspases are activated in erythroid cells undergoing terminal differentiation, although the crucial question as to how Hsp70 translocates to the nucleus and remains nuclear localised under Epo is remains open. These results strongly indicate that Hsp70 is another key anti-apoptotic protein in erythroid cells

Figure 6: **Hsp70 protects GATA-1 from caspase 3-mediated proteolysis**



At the onset of caspase activation, Hsp70 co-localizes and interacts with GATA-1 in the nucleus of erythroid precursors undergoing terminal differentiation. In contrast, Epo starvation induces the nuclear export of Hsp70 and the cleavage of GATA-1.

by protecting GATA-1 from caspase 3-mediated cleavage, allowing Bcl-xL expression via GATA-1 protection.

Thus it appears that dysregulation of Hsp70 expression or localisation could account for some congenital or acquired erythroid disorders. The clarification of the mechanism responsible for Hsp70 nuclear localisation under Epo during terminal erythroid differentiation could shed light in the understanding of various acquired and congenital erythroid pathologies and may serve as a new therapeutic target in these disorders. Furthermore other chaperones including heat shock proteins HSP27 and 90 are also expressed during erythroid differentiation and they may also play an important role in the regulation of erythropoiesis that remains to be clarified.

6. Conclusions

Erythropoiesis is therefore a fascinating system of differentiation that is regulated at several levels. Differentiation is under the control of a complex set of transcriptional factors, and growth factors play a critical role in preventing apoptosis and in inducing proliferation. In this system caspases play an essential role in controlling the rate of red cell maturation and as such erythropoiesis is an example of a system in which regulation of apoptosis and differentiation share similar molecular pathways. Subtle differences in the set of cleaved proteins determine the fate of erythroid progenitors and precursors. It will be a great challenge to elucidate at the molecular level the mechanisms underlying this fine regulation as demonstrated with HSP70 in the protection of GATA-1. Understanding the complex role of stromal cells and the molecules that they produce will certainly help to elucidate the regulation of erythropoiesis within its natural bone marrow environment.

The understanding of all these processes will shed light on the pathophysiology of anaemia associated with various erythroid disorders. All these mechanisms may be translated beyond erythropoiesis and may be involved in neoplastic transformation of cells and in various degenerative disorders.

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Multiple Choice Questionnaire

To find the correct answer, go to http://www.esh.org/iron-handbook2009answers.htm

1. Which of the following combinations of growth factors promote the survival, proliferation and/or differentiation of erythroid progenitors?

a) Fas-L and TGF-β ☐
b) GM-CSF and IL-3 ☐
c) SCF and Epo ☐
d) TPO and IL-6 ☐

2. The transcription factor GATA-1 is essential during erythropoiesis because:

a) It induces cellular proliferation of non-committed and committed erythroid progenitors ☐

b) It cooperates with PU-1 ☐
c) It induces the expression of differentiation genes in cooperation with FOG-1 ☐
d) It reduces the expression of Bcl-xL ☐

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3) The major site of Epo production during adult erythropoiesis is the:
a) Bone marrow ☐
b) Kidney ☐
c) Liver ☐
d) Lungs ☐

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4) Which kinase is absolutely required for Epo-R activation?
a) Jak2 ☐
b) STAT5 ☐
c) Akt ☐
d) MAPK ☐

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5) Caspase activation is essential for which one of the following?
a) Positive regulation of erythroblasts proliferation ☐
b) Cleavage of Hsp70 ☐
c) Negative regulation of apoptosis ☐
d) Cleavage of protein implicated in erythroid differentiation ☐

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