THE ROLE OF ERYTHROPOIETIN AND ERYTHROPOIETIN RECEPTOR IN REGULATION OF HEMOPOIESIS

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ABSTRACT

To analyze the molecular mechanisms by which erythropoietin (Epo) can stimulate proliferation and differentiation of hemopoietic cells, I studied the effects of quantitative and qualitative alterations in the expression of erythropoietin receptors (EpoRs) in hemopoietic cell lines and in the primary bone marrow cells using retrovirus mediated gene transfer to engineer high level expresson of normal and mutant EpoRs in these cells. Interleukin-3 (IL-3)-dependent murine bone marrow derived Ba/F3 cells engineered to express normal EpoR increased their levels of β-globin mRNA in response to Epo, and this partial differentiation correlated with a marked Epo-induced growth delay, indicating that the transduced EpoR was capable of inducing a distinct set of intracellular events. The tyrosine kinase inhibitor genistein blocked both Epo-induced β-globin mRNA accumulation and proliferation in this model system. In contrast, inhibition of protein kinase C by Compound 3 suppressed only Epo-induced differentiation without affecting proliferation, indicating that the proliferative and differentiation functions of the EpoR can be uncoupled. Mutant EpoRs lacking all intracellular tyrosines were compromised only in proliferative signaling, implying that tyrosine phosphorylation of the EpoR itself is not required for its differentiation function. With IL-3 and Epo costimulation, IL-3 signaling appeared to be dominant, since no increase in β-globin mRNA occurred. Chimeric EpoRs comprising the extracellular domain of the EpoR and the transmembrane and cytoplasmic region of IL-3-R-β_{IL-3} were capable of inducing β-globin mRNA accumulation, suggesting the existence of a second EpoR subunit responsible for differentiation or that the α subunit of the IL-3 R prevents it. Arguing against the former, a truncated EpoR lacking an intracellular domain possessed no biological activity. Chimeric EpoRs comprising the

extracellular domain of the EpoR and the transmembrane and intracellular domains of the IL-3R α subunit were, however, capable of transmitting the Epo-induced mitogenic signal but failed to stimulate accumulation of β-globin mRNA. Moreover, coexpression of EpoR/IL-3Rα with EpoR/IL-3R β_{-IL-3} suppressed β-globin mRNA accumulation, which implicated an active role for the IL-3-Rα subunit in inhibiting EpoR-specific differentiating signals.

Epo also exhibited a marked effect on proliferation of EpoR-transduced primary mouse bone marrow cells. Epo alone supported proliferation of EpoR-transduced CFU-GM and CFU-GEMM in semi-solid and suspension cultures, indicating that Epo was capable of replacing other cytokines normally required for the in vitro proliferation of non-erythroid and multipotent clonogenic progenitors. No Epo-induced proliferation of control cells could be detected in cultures containing high numbers of irradiated EpoR-transduced cells, indicating that Epo stimulated proliferation directly, through activation of the transduced EpoR, and arguing against the possibility of Epo-induced secretion of growth factor(s) within the population of the EpoR-transduced cells.

To study effects of ectopic EpoR expression on proliferation of stem cells *in vivo*, EpoR- and neo-transduced bone marrow cells were transplanted into lethally irradiated mice. Recipients of the EpoR-transduced bone marrow developed within 6-14 weeks severe anemia, leukocytosis characterized by accumulation of undifferentiated blasts, and had significantly increased numbers of all clonogenic progenitor classes, consistent with development of myeloproliferative disease. Bone marrow and spleen cells recovered from the affected mice expressed high levels of surface EpoRs and proliferated in response to Epo, but not in the absence of growth factors, supporting a link

between the Epo-induced deregulation in proliferation of the EpoR transduced stem cells and development of neoplasia.

Together, the data presented in this thesis provide evidence that EpoRs may influence both proliferative and differentiative decisions of hemopoietic cells subject to their ability to interact with different signalling intermediates.

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ABBREVIATIONS

^{3}H-Tdr	tritiated thymidine
5-FU	5-flourouracil
aa	amino acid
bEpo	biotinylated erythropoietin
bp	base pair
BSA	bovine serum albumin
C3	aminoalkyl bisindolylmaleimide
cAMP	3'-5'cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CFU-E	CFU-erythroid
CFU-GEMM	CFU-granulocyte, erythroid, macrophage, megakaryocyte
CFU-GM	CFU-granulocyte-macrophage
CFU-Mk	CFU-megakaryocyte
CFU-S	colony forming unit-spleen
cGy	centi Gray
Ci	Curie
CSF	colony stimulating factor
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
Epo	erythropoietin
EpoR	erythropoietin receptor
ES	embryonic stem cells
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HMBA	hexamethylenebisacetamide
HXM	hypoxanthine-xanthine-mycophenolic acid
IBMX	isobutylmethylxanthine
IL	interleukin
IL-3 $R\beta_{IL-3}$	interleukin-3 -specific β subunit of IL-3 receptor complex
IL-3R α	α subunit of IL-3 receptor complex
LTR	long terminal repeat
MPSV	myeloproliferative sarcoma virus
MSCV	murine stem cell virus
NCS	newborn calf serum
pKC	protein kinase C
RNA	ribonucleic acid
SA-PE	streptavidin-phycoerythrin
SCF	stem cell factor

TPA	12-o-tetradecanoylphorbol-13-acetate
WT	wild type

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CHAPTER 1

INTRODUCTION

1.1. Overview

Mature blood cells have limited life spans and must be continuously replenished by the proliferation and progressive acquisition of highly specialized phenotypes from more primitive progenitors. This life-long process is maintained by a small population of primitive bone marrow cells - termed stem cells- that have the capacity to divide and give rise to virtually identical progeny cells (self-renew), or to differentiate to one of the lineages which constitute the hemopoietic system. Thus events occuring at the level of hemopoietic stem cells, such as recruitment of the mainly quiescent stem cells into active cell cycle, and decisions between self-renewal and commitment to one of hemopoietic lineages, are crucial to maintain normal hemopoiesis. Conversely, disruption in the control of these processes can have catastrophic consequences, ranging at one extreme to cessation of hemopoietic output leading to aplastic anemia, or to excessive production of poorly differentiated forms characterizing leukemias.

Although a variety of growth factors and their receptors with the capacity to support survival and proliferation of the primitive hemopoietic cells have been identified, little is known about the role growth factors play in the lineage commitment and differentiation of early cells. One possibility is that growth factors trigger events that enable execution of a predetermined differentiation program by preventing apoptosis and stimulating proliferation of committed progenitors. Alternatively, or in addition, expression of a specific cell surface

receptor may precede lineage commitment, and subsequent exposure to the respective growth factor may actually direct events leading to differentiation.

The goal of the research work described in this thesis was to examine further the roles growth factors and their receptors play in regulation of hemopoiesis. The capacity of the erythroid specific growth factor, erythropoietin, to induce erythroid differentiation was explored by examining the differentiation behavior of hemopoietic cells engineered by retroviral gene transfer to express high levels of erythropoietin receptor (EpoR). As an orientation to the issues studied, the following provides a review of the cellular components and organization of the hemopoietic system, the current concepts regarding hemopoietic growth factors, their receptors and molecular mechanisms of action, with a special emphasis on erythropoietin and the erythropoietin receptor.

1.2. Ontogeny of hemopoiesis

Precursors of hemopoietic cells are generated by differentiation of cells that form the ventral zone of embryonic mesoderm. In most vertebrate organisms, hemopoietic cells and vascular progenitors migrate to an extraembryonic position on the yolk sac to form blood islands. Blood island formation begins on day 7 of embryonic development of mouse (reviewed in (Zon, 1995)) and day 19 of gestation in humans (Kelemen et al., 1979). Yolk sac hemopoiesis yields predominantly nucleated erythrocytes synthesizing embryonic hemoglobins (Fantoni et al., 1981), although progenitors for most hemopoietic lineages have also been identified (Wong et al., 1986). Concurrently with yolk sac hemopoiesis an additional intra-embryonic population of hemopoietic cells, believed to represent ancestors of definitive hemopoiesis, arises in the dorsal mesentery (Godin et al., 1993; Medvinsky et

al., 1993). However, whether the primitive and fetal/adult hemopoietic stem cells develop from a common precursor (Moore and Metcalf, 1970) or represent two distinct ontogenetic lineages (Maeno et al., 1985) remains an open issue.

By day 12 of murine embryonic development and week 5-6 of human gestation the fetal liver become the major site of hemopoiesis. The spatial shift in blood cell production is associated with a change in the erythroid differentiation program, characterized by production of enucleated erythrocytes and a switch from embryonic to fetal hemoglobin synthesis (Gale et al., 1979; Lin et al., 1996). Concurrently, there is an increase in production of mature cells belonging to other myeloid lineages.

Hemopoietic activity characterized by the synthesis of adult hemoglobins and production of enucleated erythrocytes commences in the murine bone marrow and spleen by day 13-14, and by week 11 in humans (Kazazian and Woodhead, 1973). During subsequent fetal development liver hemopoiesis and production of fetal hemoglobins gradually decrease such that the bone marrow, and in mouse bone marrow and spleen, become the permanent sites of normal adult hemopoiesis.

1.3. Organization of the hemopoietic system

Blood contains at least 10 different mature cell types, each with a unique set of specialized properties and relatively short life span, ranging from approximately 48 hours for neutrophils to 100 days for erythrocytes. The turnover of cells in the hemopoietic system in adult man is estimated to be close to 10^{12} cells per day (Abkowitz et al., 1995). This impressive production is supported by a relatively small pool of pluripotent hemopoietic stem cells (PHSC) with the capacity to produce functionally indistinguishable daughter

cells, a process termed self-renewal, and the ability to generate progenitors committed to differentiate along the various myeloid and lymphoid lineages. The dividing and maturing progeny of progenitor cells committed to a particular lineage constitute the morphologically recognizable precursors that comprise the majority of cells seen in hemopoietic tissue. Unlike their differentiated progeny, primitive hemopoietic cells resemble lymphoid blasts and possess no distinguishing morphological characteristics when examined by light microscopy. Characterization of their functional properties became feasible only after development of assays for these cells based on their potential to give rise to morphologically recognizable progeny either in vivo or in vitro. These studies have shown that hemopoietic cells in each lineage can be stratified into three major sequential cell populations, each of progressively larger size and characterized by a gradual decrease in proliferation, differentiation and self-renewal. These populations can be operationally defined as stem cells, committed progenitor cells, and maturing cells.

1.3.1. Hemopoietic stem cells

Pluripotent hemopoietic stem cells (PHSC) with the capacity to generate all types of myeloid and lymphoid cells represent a small population of predominantly quiescent, cell-cycle specific drug resistant bone marrow cells (Harrison and Lerner, 1991; Hodgson and Bradley, 1979) that persist throughout life.

The most primitive hemopoietic stem cells (PHSC) can be detected by their capacity to provide long-term reconstitution of all blood cell lineages when transplanted into myeloablated animals. Studies to demonstrate the existence of donor-derived cells with lympho-myeloid reconstitution potential initially relied on detection of the donor-specific cytogenetic markers such as Y

chromosome (Lamar and Palmer, 1984) or chromosomal aberrations induced by prior sublethal irradiation of donor mice (Wu et al., 1967), detection of the donor specific Gpi-1^a/Gpi-1^b isotypes (VanZant et al., 1983), Hbb^d/Hbb^s hemoglobins (Harrison, 1980), and detection of cell surface Ly-5.1 and Ly-5.2 allo-antigens (Spangrude and Scollay, 1990). Retroviral infection to introduce clonal genetic markers has more recently been employed to identify and trace the growth of hemopoietic stem cells. The presence of identical proviral integration patterns in the bone marrow and thymus tissues of lethally irradiated recipients provided further evidence for the existence of cells with lympho-myeloid potential (Dick et al., 1985; Keller et al., 1985). Moreover, identical proviral integrations detected in hemopoietic tissues of the primary and several secondary recipients clearly demonstrated that individual marked PHSC were capable of self-replication (Jordan and Lemischka, 1990; Lemischka et al., 1986; Williams et al., 1984).

1.3.2. Progenitor cells

An approach to the quantitative detection of primitive hemopoietic cells was first provided in 1961 by Till and McCulloch (1961), who found that a fraction of bone marrow cells injected into lethally irradiated recipients lodged in the spleen and formed macroscopically visible colonies. Cells giving rise to spleen colonies were thus named colony-forming unit-spleen, or CFU-S. Later studies established that such spleen colonies comprise clonal cell populations (Becker et al., 1963), often contain mixed myeloid populations, and frequently also daughter CFU-S (Curry and Trentin, 1967; Lepault et al., 1993; Wu et al., 1967).

The multilineage potential and at least a degree of self-renewal capacity of CFU-S place these multipotent progenitors early in the hemopoietic hierarchy. Indeed, CFU-S were initially considered to represent PHSC, and studies focused on CFU-S behavior played a prominent role in developing concepts of the organization and regulation of mammalian hemopoiesis. Recent studies have shown, however, that the majority of CFU-S and cells with long-term in vivo repopulating potential can be physically separated (Mulder and Visser, 1987), indicating that these two types of primitive murine hemopoietic cells represent two distinct populations. The presence of CFU-S in multilineage mixed colonies that develop in vitro (Humphries et al., 1979) also indicates an overlap between populations of multipotent progenitors capable of forming colonies in vivo and in vitro. Together, these observations identify CFU-S as a transitional population, descending from PHSC and maturing into progenitors with restricted differentiation and proliferation potential.

Over the years great emphasis has been put into identification of conditions that would allow individual progenitor cells to express their growth potential in vitro. Initial attempts to grow bone marrow cells in semisolid medium revealed that formation of distinct granulocyte or granulocyte-macrophage colonies depended on the presence of feeder cells, normal or leukemic mouse sera or media conditioned by various tissues (Bradley and Metcalf, 1966; Ichikawa et al., 1966; Robinson et al., 1969). Later studies identified human urine as a source of macrophage, as well as erythroid colony stimulating activity (Robinson et al., 1969; Stephenson et al., 1971), and the pokeweed-stimulated lymphocyte conditioned medium was found to support development of multilineage-mixed colonies (Metcalf et al., 1979). A vast array of extracellular factors implicated in regulation of hemopoiesis has been identified and purified (Coze, 1994).

The mature cell content of the colonies generated in optimal conditions, the time required for appearance of maturing cells and the final size of the colonies that develop allow the discrimination of a hierarchy of progenitor cells with decreasing differentiation and proliferation potential. Multipotential progenitors capable of forming macroscopic colonies comprising all myeloid lineages (CFU-GEMM for colony-forming units-granulocyte, erythroid, megakaryocyte and macrophage), and frequently also identical daughter cells, represent primitive progenitor cells detectable in cultures of the total bone marrow (Humphries et al., 1981). The majority of clonogenic progenitors give rise to colonies comprising only one or at most two types of progeny such as maturing erythroblasts (CFU-E, and burst-forming unit, BFU-E), megakaryocytes (CFU-Mk and BFU-Mk), or granulocytes and macrophages (CFU-GM). The sequential decrease in proliferative potential of progenitors is particularly obvious in the erythroid lineage. Progeny of more differentiated erythroid progenitors (CFU-E) can undergo terminal maturation immediately and form erythroblast clusters of 8-64 cells in 2 days (mouse) or 8-49 cells in 7 days (human). The progeny of more primitive progenitors, BFU-E, appear to require several maturational divisions before any of the cells produced can initiate events leading to terminal maturation, and grouped clusters of greater than 500 erythroblasts develop in 8-10 days (mice) and 14-15 days (humans) (Gregory and Eaves, 1977; Gregory and Eaves, 1978). An analogous course of development was also demonstrated for granulocyte and granulocyte-macrophage progenitors (Bol and Williams, 1980; Metcalf and MacDonald, 1975).

More recently the methods and conditions for detecting early cells with myeloid and B lymphoid potential and their maturing B cell progeny have also been reported (Ball et al., 1995; Lemieux et al., 1995; Whitlock et al., 1985). As

mechanisms regulating production of lymphoid cells were not explored during research work underlying this thesis, they are not discussed in this review.

1.3.3. Maturing cell populations

Populations of dividing and terminally maturing cells represent the vast majority of cells present in the bone marrow. The final 3-5 amplifying divisions yield sufficient numbers of morphologically recognizable cells within each lineage to permit a variety of molecular and biochemical studies. Kinetic DNA measurements revealed, for example, that progression from proerythroblasts to basophylic erythroblasts involves two maturational divisions, followed by progression through an additional one or two cell cycles to polychromatic erythroblasts and then proceeding to nuclear condensation and extrusion to develop into orthochromatic erythroblasts (Starling and Rosse, 1976). Cell membrane remodeling commences during maturation of late erythroid progenitors (day 3 BFU-E), as detected by spectrin synthesis, to be followed first by ankyrin and band 4.1, and then by band 3 and glycophorin synthesis characterizing proerythroblasts (Ekblom, 1984; Wickrema et al., 1994). Hemoglobin synthesis, in contrast, can first be detected after the first proerythroblast division and peaks during enucleation and transition from orthochromatic erythroblasts to reticulocytes (Koury et al., 1987; Nijhof and Wierenga, 1984).

1.4. Regulation of hemopoiesis

Balanced blood cell production requires coordination of stem cell activation, lineage commitment, fidelity in initiation and completion of the differentiation program, as well as controlled release of mature cells and their appropriate functional activation. External factors regulating this complex

process include interactions between the various cells and extracellular matrices comprising the bone marrow environment, and numerous growth factors, with the capacity to modulate the hemopoietic cell behavior upon interaction with their specific cell surface receptors.

1.4.1. Bone marrow microenvironment

Ultrastructural studies of bone marrow revealed close associations between the maturing blood cells and fixed populations of endothelial, reticular and fibroblastoid cells, collectively referred to as stromal cells (Dorshkind, 1990; Lichtman, 1981). The luminal side of marrow sinuses is completely lined by endothelial cells, and reticular cells cover the abluminal face of the endothelial cell layer (reviewed in (Lichtman, 1984)). Newly generated blood cells must migrate through the stromal cell layer to enter the circulation. Reticular cells are able to decrease their volume and area of contact with endothelial cells in conditions demanding increased production of blood elements, such as hypoxia induced erythropoiesis, and thus facilitate the release of mature blood cells into the circulation (Chamberlain et al., 1975).

In addition to the physical regulation of blood cell circulation, stromal cells may also alter the local concentrations of growth factors and inhibitors by modulating the inflow of soluble mediators produced elsewhere (Shadduck et al., 1989). Moreover, stromal cells themselves produce a variety of growth factors, and can increase this production in response to appropriate stimuli (reviewed in (Coze, 1994)). Mice with mutations at the Steel (Sl) locus are probably the best documented case illustrating the importance of the marrow environment on hemopoiesis. Mutations at the Steel locus abolish or decrease production of stem cell factor (SCF) which appears to play a prominent role in supporting survival and proliferation of early hemopoietic cells in the adult

(Zsebo et al., 1990; de Vries et al., 1991; Li and Johnson, 1994). Sl/Sl embryos die in utero, and viable mice with the less severe Sl/Sl^{d} phenotype present with macrocytic anemia and mast cell deficiency. Anemia of these mice can be cured by bone implants, but not by marrow grafts; conversely, bone marrow from Sl mice can reconstitute the hemopoietic system of irradiated normal recipients, demonstrating that the Steel phenotype results from a defective hemopoietic environment (Bernstein et al., 1991; Russell, 1979).

Maturing and functional hemopoietic cells themselves may be considered constituents of the marrow microenvironment and have the potential to exert regulatory effects through cell-cell interactions and production of growth factors. Erythroblastic islands formed by maturing erythroid cells clustered around a central 'nurse' macrophage are, for example, a common feature of normal hemopoiesis. The nursing cell provides maturing erythroid cells with iron, and later ingests the expelled nuclei and effete erythrocytes (reviewed in Lichtman, 1984). Analogous clustering of maturing granulocytes around central macrophages was detected in granulocytic cobblestone areas within the adherent layer of long term bone marrow cultures (Dexter et al., 1990). Central macrophages in these clusters are believed to provide both positive and negative signals to the associated maturing cells.

The marrow stroma synthesizes a complex extracellular matrix (ECM), which provides structural support for hemopoietic cells, enables interactions between the stromal and hemopoietic cell populations and can by itself influence the behavior of developing blood cells (reviewed in Dorshkind, 1990). Constituents of ECM such as heparan sulphate were shown to sequester and concentrate IL-3 (Roberts et al., 1988), GM-CSF (Gordon et al., 1987), basic-fibroblast growth factor (b-FGF) (Gospodarowicz and Cheng, 1986) and

macrophage inflammatory protein (MIP)-1β (Tanaka et al., 1993). Through such mechanisms of local concentration of stimulators and inhibitors, as well as through the production of membrane bound growth factors, stromal cells may exert a pronounced effect on the behavior of hemopoietic cells in their immediate vicinity.

1.4.2. Growth factors and inhibitors

Nearly 100 years ago Carnot and Deflandre (1906) reported that serum obtained from rabbits made anemic by bleeding enhances erythropoiesis when injected into healthy recipients, and thus pointed to the existence of a soluble hemopoietic regulator in the anemic serum. Similar regulators were expected to exist for other blood lineages. However, no convincing evidence for such regulators was obtained until the development of tissue culture techniques that enabled screening of various cells, tissues extracts and tissue conditioned media for their ability to support proliferation of bone marrow cells (reviewed in Metcalf and Nicola, 1995).

The first hemopoietic growth factors to be characterized have been named according to the most obvious response they elicit (Table 1.1.), eg. erythropoietin for stimulation of erythropoiesis and colony-stimulating factors for enabling an in vitro development of single or mixed lineage colonies. In addition to colony-stimulating factors, numerous hemopoietic regulators produced predominantly by various subpopulations of leukocytes (hence the name interleukins was coined) have since been characterized.

Table 1.1. The major hemopoietic growth factors implicated in regulation of erythro- and myelopoiesis.

Name	Major myeloid target cells and responses
Erythropoietin (Epo)	Stimulation of CFU-E, BFU-E, erythroblast survival
Thrombopoietin (TPO or c-mpl ligand)	Stimulation of CFU-Mk, megakaryocytes
Macrophage colony-stimulating factor (M-CSF or CSF-1)	Stimulation of CFU-M, activation and survival of monocytes/macrophages
Granulocyte colony-stimulating factor (G-CSF)	Stimulation of CFU-G
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Stimulation of CFU-GM, BFU-E
Interleukin-1 (IL-1)	Activation of macrophages
Interleukin-3 (IL-3 or multi-CSF)	Stimulation of single and multilineage myeloid progenitors
Interleukin-5 (IL-5)	Stimulation of CFU-eosinophil
Interleukin-6 (IL-6)	Stimulation of CFU-Mk
Interleukin-8 (IL-8)	Activation of neutrophils
Interleukin-9 (IL-9)	Proliferation of erythroid and mast cells
Interleukin-11 (IL-11)	Stimulation of CFU-MK and primitive hemopoietic cells
Interleukin-12 (IL-12)	Stimulation of NK cells
Steel factor (SF, mast cell growth factor or c-kit ligand)	Stimulation of mast cells, BFU-E, CFU-GM, CFU-Mk
Flk-2/Flt-3 ligand	Stimulation of multilineage progenitors
Transforming growth factor-β (TGF-β)	Inhibition of primitive myeloid progenitors stimulation of mature myeloid cells
Macrophage inflammatory protein-1α (MIP 1-α)	Inhibition of primitive myeloid progenitors stimulation of mature myeloid cells

None of the molecularly cloned cytokines is restricted in its action to a single lineage or developmental stage. These factors can be, however, roughly grouped into three categories based on their potential to stimulate early as opposed to later stages of hemopoietic cell differentiation. Late-acting factors such as Epo, M-SCF and IL-5, stimulate predominantly the lineage-committed cells; intermediate-acting factors such as IL-3 and GM-CSF act on a wide variety of cells; and early-acting factors such as IL-6, IL-11 and G-CSF were proposed to be involved in activation of the quiescent pluripotent progenitors (Ogawa, 1993).

1.4.2.1. Biological actions of hemopoietic regulators

Assays for specific progenitor types, recognition of specific growth factors and improved methods for isolation of early hemopoietic cell populations highly enriched for cells with the desired phenotype opened the way to detailed characterization of the biological effects of hemopoietic factors.

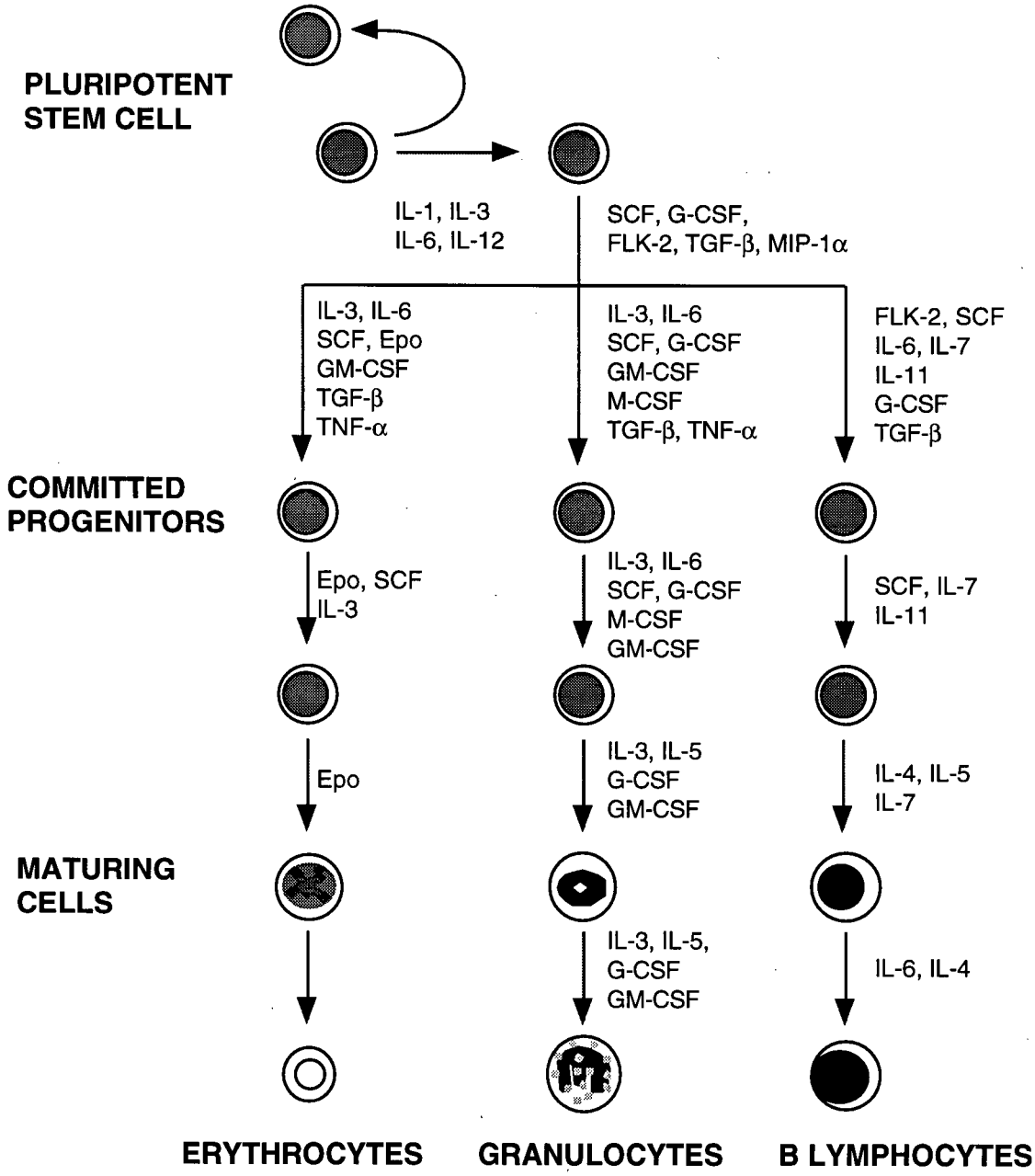

Figure 1.1. Schematic diagram showing some regulators controlling hemopoietic cell proliferation.
Numerous hemopoietic growth factors exhibit an overlap in their target cell ranges, and individual regulators can act on cells in different lineages at various stages of hemopoietic hierarchy.

Most of these humoral regulators can also act on various cells outside the hemopoietic system. SCF, for example, maintains viability and in combination with other growth factors promotes proliferation of early myeloid

and lymphoid progenitors (de Vries et al., 1991; Hirayama et al., 1994), and also promotes development of primordial germ cells and melanocytes during embryogenesis (Russell, 1979). As schematically depicted in Figure 1.1, virtually all hemopoietic regulators act on cells within different lineages and at different stages of hemopoietic differentiation. Epo, although frequently reported as erythroid lineage specific growth factor, cooperates with other growth factors in stimulating proliferation of megakaryocyte progenitors (Clark and Dessypris, 1986) and increased platelet counts were observed after Epo administration to rats (Berridge et al., 1988). Endothelial cells were also reported to proliferate and secrete endothelin ET-1 in response to Epo (Carlini et al., 1993). There appears to be a considerable functional overlap between different regulators: IL-3, IL-5, GM-CSF, G-CSF and SCF all support formation of neutrophil colonies (Metcalf, 1993). The quantitative importance of a particular cytokine in regulating cell production in a lineage may vary with the stage of hemopoietic cell differentiation. Epo is probably the best candidate for a sequentially acting cytokine. Within the erythroid lineage its action is limited to relatively late erythroid cells (CFU-E), whereas earlier erythroid committed cells (BFU-E) depend for their proliferation on growth factors such as IL-3, GM-CSF, SCF and IL-11, but not Epo (Emerson, 1988; Emerson 1985; Wu, 1995; Lin, 1996; Kieran, 1996). Whether or not a cytokine appears to be active on cells within a particular lineage may depend on which other regulators are acting on these cells. SCF alone appears to support proliferation for only a minor subset of BFU-E, but synergizes with Epo to induce more than an additive increase in the output of their maturing progeny (Kieran et al., 1996). Between the different lineages the magnitude of response to a given factor may vary significantly. When acting on committed progenitors, G-CSF acts predominantly on populations of neutrophils and to a lesser extent on macrophage and

granulocyte-macrophage progenitors, while M-CSF predominantly stimulates formation of macrophage and some granulocyte and granulocyte-macrophage colonies (Metcalf and Nicola, 1995). Combined action of these two factors results in an enhanced production of all three mature cell types, which can accelerate an optimal response to emergency situations (Metcalf, 1993). Whether a true redundancy exists among hemopoietic regulators, or they rather represent multiple subtle means for regulation of hemopoiesis remains an open question. If the regulator under examination was genuinely redundant then in its absence the hemopoietic cell differentiation should proceed normally.

Table 1.2. Examples of hemopoietic aberrations occurring in mice deficient in production of various hemopoietic growth factors.

Regulator	Consequences
SCF (Steel mutants)	Severe macrocytic anemia, mast cell deficiency (Bernstein et al., 1991)
M-CSF (op mutants)	Osteopetrosis, macrophage deficiency (Yoshida et al., 1990)
Epo	Absence of definitive erythropoiesis (Wu et al., 1995)
G-CSF	Neutrophil deficiency (Metcalf and Nicola, 1995)
GM-CSF	Defective resistance to lung infections (Stanley et al., 1994)

Aberrations in hemopoiesis observed in mice naturally deficient in the production of hemopoietic regulators or in which the gene encoding the regulator has been inactivated by targeted gene disruption (Table 1.2) suggest, however, that each factor under examination exerts at least some unique, nonredundant actions. Mice carrying an inactivating mutation in the Epo gene exhibit reduced primitive erythropoiesis and die around embryonic day 13 due to the failure of the definitive fetal liver erythropoiesis. In semisolid medium supplemented with Epo, however, the $Epo^{-/-}$ fetal liver cells do give rise to hemoglobinized colonies comprising the adult type erythroid cells,

demonstrating that Epo is crucial for proliferation of CFU-E and functional maturation of their progeny (Wu et al., 1995). Studies using purified populations of human and mouse erythroid colony forming cells showed that during terminal erythroid differentiation either Epo or SCF prevent apoptosis, as detected by the absence of DNA fragmentation (Muta et al., 1994). In several such studies Epo appeared to be indispensable for induction of cell membrane remodeling and hemoglobinization (Koury et al., 1987; Muta and Krantz, 1995; Muta et al., 1994; Papayannopoulou et al., 1993). Surprisingly however, $EpoR^{-/-}$ fetal liver cells gave rise to fully hemoglobinized erythroid colonies in response to Tpo and SCF, suggesting an entirely permissive role for Epo in regulation of erythroid cell maturation (Kieran et al., 1996). As mentioned above the formation of large erythroid bursts by BFU-E depends on Epo and IL-3, GM-CSF or SCF (Krantz, 1991; Sawada et al., 1990; Sawada et al., 1991). Analyses of the specific ^{125}I-Epo binding by the populations enriched for human BFU-E showed that the numbers of cell surface erythropoietin receptors (EpoR) increase as cells progress from BFU-E to CFU-E, suggesting that the magnitude and the nature of the cellular response to Epo may be regulated through the numbers of available cell surface EpoRs (Sawada et al., 1990).

1.4.2.1.1. Hemopoietic growth factors and regulation of differentiation

Stem cell commitment or lineage determination represents the first step in the qualitative change during which PHCS lose their pluripotentiality and their progeny acquire the potential to proliferate and differentiate in response to an appropriate stimulation. In contrast to the well documented effects various cytokines exert on regulation of progenitor cell proliferation, the role extrinsic

regulators may play in events leading to lineage commitment has not yet been resolved.

Early studies focusing on CFU-S have played key roles in developing current concepts of the regulation of mammalian hemopoiesis. Based on the variability in the self-renewal and differentiation capacity determined for populations of CFU-S by clonogenic progenitor assays Till and co-workers (1964) developed the stochastic model of stem cell commitment (Hemopoiesis Engendered Randomly, HER). In this model, the fate of individual cells within a population is not tightly regulated, and events leading to either self-renewal or lineage commitment occur randomly. Cells exposed to identical environmental conditions were predicted to generate dissimilar progeny due to the stochastic nature of the intrinsic events which establish the probability of cycling, self renewal and differentiation. An alternative model termed Hemopoietic Inductive Microenvironment (HIM) evolved from histological analyses of the mature cell content and the localization of spleen colonies (Curry and Trentin, 1967). The major assumption in this model is that the fate of a multipotential cell is determined by interactions with its local microenvironment, whose constituents direct or instruct commitment to a particular lineage. Cells exposed to a microenvironment directing erythroid differentiation would thus all undergo commitment to erythroid lineage to yield progeny competent to proliferate and differentiate in response to Epo. This model, however, does not discriminate between the ability of the microenvironment to influence the commitment of multipotential cells and its capacity to provide lineage-specific support to their committed progenitors.

The in vitro colony-forming assays in which multipotential progenitors are exposed to a controlled environment allowed for more rigorous analysis of

events occuring early during hemopoietic cell differentiation. Replating of individual macroscopic multilineage mixed colonies demonstrated, for example, that the frequency of the multipotential daughter cells in individual colonies varied markedly from one clone to another (Humphries et al., 1981), consistent with a stochastic process influencing the outcome of stem cell divisions. More detailed studies of mechanisms regulating stem cell commitment, however, became feasible only after in vitro model systems involving pure populations of multipotential progenitors in which the fate of individual cell could be studied had been developed. These studies showed that paired progenitors (two daughter cells derived from a single multipotential cell) frequently generated colonies containing different types and numbers of various lineages, suggesting that stochastic processes influence commitment at each division of the multipotential cell (reviewed in Ogawa, 1993).

A concept that parallels the stochastic model of differentiation is that growth factors support survival and proliferation, but do not necessarily direct differentiation of multipotential progenitors. If Epo were essential for commitment of multipotent cells to the erythroid lineage, then in its absence no erythroid precursors should develop. Using clonogenic progenitor assays Wu and co-workers (1995) demonstrated that fetal liver cells of $Epo^{-/-}$ mice contain erythroid progenitors, indicating that in the absence of Epo erythroid committed progenitors (BFU-E and CFU-E) can develop, but cannot mature into functional erythrocytes. The apparent induction of differentiation by a growth factor can thus be interpreted as a consequence of proliferation and functional maturation of the lineage committed, growth factor responsive progenitors.

Some studies indicate that lineage commitment of multipotential progenitors may not be exclusively stochastic and can be modulated by growth

factors. Metcalf (1991), for example, compared the progenitor cell content of blast colonies co-stimulated with SCF and G-CSF or GM-CSF or IL-3 and found that GM-CSF significantly increased the proportion of granulocyte progenitors, and IL-3 specifically augmented production of eosinophils.

Whether a particular regulator can induce differentiation may depend on whether or not the cells examined express cell surface receptors for that regulator. Early pro-B cells engineered by the retrovirus mediated gene transfer to express M-CSF receptor acquired morphologic and functional characteristics of macrophages upon exposure to M-CSF (Borzillo et al., 1990). While these examples are few they do indicate that cytokines can, to a certain degree, influence commitment of oligopotential progenitors and thus bias the subsequent formation of their maturing progeny.

1.4.3. Hemopoietic growth factor receptors

Cellular responses to various growth factors are initiated by the interaction between the growth factor and its specific cell surface receptor. The receptors for most hemopoietic growth factors have been cloned, and elucidation of their structure and the intracellular signaling pathways that they utilize have begun to provide some explanations for the apparent functional redundancy, as well as for the unique functions assigned to some cytokines.

Responsiveness of hemopoietic cells to a given growth factor always correlates with expression of the corresponding cell surface receptor. Before cloning of the EpoR cDNA, investigators used radiolabelled Epo to demonstrate specific binding sites on the surface of BFU-E and CFU-E, as well as yolk sac derived erythroid cells, various mouse and human erythroleukemia-derived cell lines and megakaryocytes (reviewed in D'Andrea and Zon, 1990). Low level

expression detectable at mRNA levels by RT PCR has been reported for populations of early hemopoietic progenitors (Nakamura et al., 1992; Orlic et al., 1995), and nondifferentiated ES cells (Schmitt et al., 1991). Outside the hemopoietic system, surface EpoRs were detected on endothelial cells, placenta and in distinct areas of mouse brain (Anagnostou et al., 1990; Digicaylioglu et al., 1995; Sawyer et al., 1989). The functional relevance of the latter is unclear, given that disruption of the Epo and/or EpoR affected only the erythroid lineage (Lin et al., 1996; Wu et al., 1995).

Expression of various receptors is regulated in a cell-type specific and temporal manner. Multipotent progenitors, for example, express mRNA encoding receptors for Epo and IL-3 (Orlic et al., 1995). With progressive differentiation along granulocyte-macrophage pathways, cells retain and enhance expression of IL-3 receptors, and acquire receptors for IL-5 and M-CSF, which are not expressed by early cells (Byrne et al., 1981; Rolink et al., 1989). Erythroid development is, in contrast, characterized by progressive increase in the EpoR levels and cessation of IL-3 receptor expression (Nicola and Metcalf, 1986; Sawyer et al., 1989). Hemopoietic cell differentiation thus appears to correlate with changes in expression of cytokine receptors.

1.4.3.1. The cytokine receptor superfamily

Structural characteristics of hemopoietic growth factor receptors, deduced from nucleotide sequences of their cDNAs, identify them as members of two major receptor superfamilies. Receptors for SCF (c-kit), M-CSF (c-fms) and Flt-3 (Flk-2) are structurally related to the platelet-derived growth factor receptor. Their cytoplasmic region contains a domain encoding a protein tyrosine kinase, and their extracellular regions comprise five immunoglobulin-like loops (Rosnet and Birnbaum, 1993). Receptors for the majority of

hemopoietic growth factors, including EpoR, are members of a cytokine receptor superfamily characterized by the absence within their intracellular regions of any recognizable catalytic domain and by conserved elements in their extracellular regions. The following section presents features that are common to a number of cytokine receptors and focuses on the structural and functional characteristics of the EpoR.

The murine EpoR cDNA was cloned by transfecting pools of recombinant plasmids from an MEL cDNA library into COS cells, which do not express endogenous EpoR, and then screening for the binding of ^{125}I-Epo by the transfected cells (D'Andrea et al., 1989). As inferred from the sequence of the cDNA, the cloned murine EpoR encodes a polypeptide with a single membrane spanning domain. Cleavage of the 24-amino acid leader sequence leaves a 223 amino acid extracellular region encompassing the Epo-binding domain, a 24-amino acid transmembrane domain and a 236-amino acid cytoplasmic region. On the basis of the predicted amino acid sequence, the EpoR was initially found to share a conserved motif within the extracellular domain, and some limited amino acid homology within the cytoplasmic region with the IL-2 receptor β chain (IL-2 Rβ). As amino acid sequences of both receptors showed an absence of tyrosine kinase catalytic domain, this conserved region suggested that EpoR and IL-2Rβ shared similar intracellular signal transduction mechanisms (D'Andrea et al., 1989).

By sequence and structural pattern matching techniques Bazan (Bazan, 1989) identified EpoR as a member of the cytokine receptor superfamily, which in addition to EpoR has now been found to include receptors for IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, G-CSF, leukemia inhibitory factor (LIF), growth hormone (GH), prolactin, neurociliary trophic factor (CNTF), and thrombopoietin

(Bazan, 1989; Bazan, 1990; Gearing et al., 1991; Vigon et al., 1992). Members of the cytokine receptor superfamily are characterized by the absence of an intrinsic tyrosine kinase domain and share two distinctive features in their extracellular regions: a conserved position of four cysteine residues and a presence of a conserved motif, Trp-Ser-X-Trp-Ser (WSXWS), where X can represent any amino acid. The region encompassing the cysteines and WSXWS motif is subdivided into two domains, each of them forming a compact structure containing two antiparallel β sheets of four and three strands, where the four conserved cysteines stabilize the structure of the membrane-distal subdomain. Crystallographic studies also revealed that the WSXWS motif represents an integral part of the membrane-proximal subdomain and does not directly participate in the ligand binding (de Vos et al., 1992).

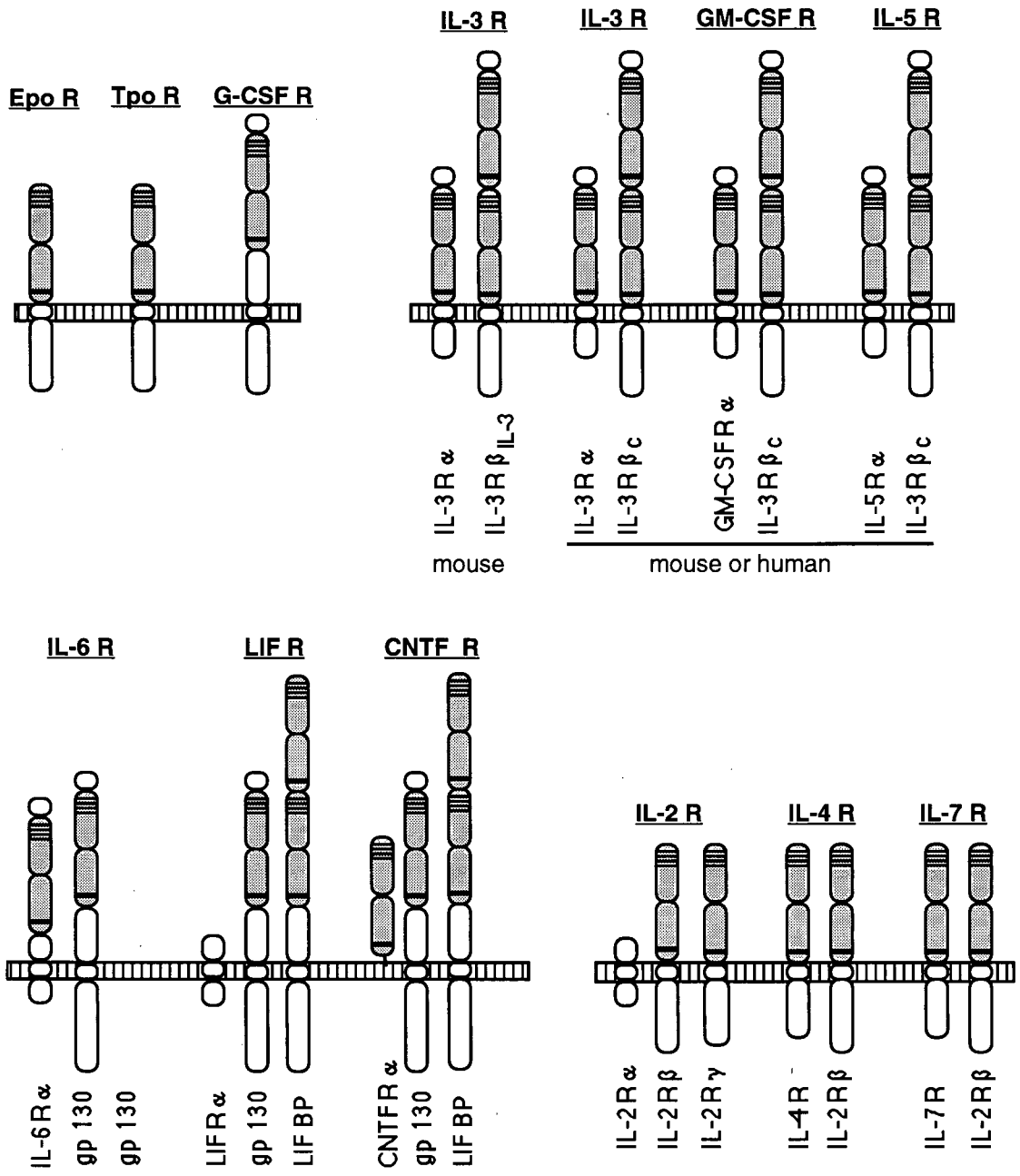

Figure 1.2. The cytokine receptor superfamily.

As the numbers of the cloned cytokine receptors increased, it became apparent that high affinity binding of many cytokines required oligomerization of two or more dissimilar receptor subunits. Receptors for human IL-3, IL-5 and GM-CSF have distinct α subunits, which bind their respective ligand with low affinity and associate with a common β subunit (β_C) to form high affinity binding sites. The β_C subunit is unable to bind any of the growth factors and is believed to represent the major signaling component of the receptor complex. In mouse, in addition to β_C there is an IL-3 specific β chain (β_{IL-3}), that unlike βc has a low binding affinity for IL-3 (reviewed in Miyajima et al., 1993). The high affinity IL-2 receptor is composed of three subunits, α, β and γ. The α and the β subunit form a high affinity IL-2 binding complex (Leonard et al., 1984), although the α (TAC) chain possesses no cytokine receptor specific features (Asao et al., 1993; Takeshita et al., 1992). The γ subunit, which does not contribute to the ligand binding, also associates with the IL-4, IL-7 and IL-9 specific chains to form biologically active receptors for their respective ligands (Kawahara et al., 1994; Renauld et al., 1995). The IL-6, IL-11 and LIF specific chains have high affinity for the ligand binding, and share a common signal transducing component, the gp130 (Hibi et al., 1990; Hilton et al., 1994). The ciliary neurotrophic factor (CTNF) receptor complex comprises in addition to the CTNF binding component and gp130 also the LIF specific chain (Ip et al., 1992).

EpoR, TpoR and G-CSF R are the only known single chain representatives of the cytokine receptor superfamily. Based on the structural similarities between the EpoR and other cytokine receptors, as well as on the results of the chemical cross-linking studies (to be presented in Section 1.4.3.2.), a multichain structure for the EpoR has likewise been proposed (D'Andrea and Zon, 1990; Krantz, 1991). A second subunit of the EpoR

complex, however, has not yet been cloned, and evidence for its existence remains inconclusive.

Elucidation of the structure of cytokine receptors raised several questions regarding their mechanism of action. For example, how they activate intracellular signaling pathways in the absence of a tyrosine kinase domain? The majority of cytokines induce proliferative responses, and several also can act as inducers of terminal differentiation. Are these responses ligand/receptor specific, and therefore controlled, at least in part, through regulation of receptor expression? Do dissimilar receptor subunits, or perhaps specific subdomains within their structure, play distinct roles in regulation of hemopoietic cell proliferation and differentiation?

1.4.3.2. Expression and structure of the EpoR

Before cloning of the EpoR cDNA, investigators used radiolabelled Epo to demonstrate specific binding sites on the surface of normal human and mouse erythroid progenitors, virally transformed spleen cells (i.e. Friend cell), various mouse and human erythroleukemia derived cell lines, as well as megakaryocytes and yolk sac and fetal liver derived cells (reviewed in D'Andrea and Zon, 1990). Scatchard analyses of specific Epo binding revealed that some Epo nonresponsive erythroleukemia cell lines, such as MEL cells, express only low affinity EpoR, whereas low- and high-affinity binding sites were demonstrated on CFU-E, fetal liver cells and some Epo responsive cell lines. This suggested a correlation between Epo responsiveness and expression of high-affinity binding sites. However, COS cells engineered to express EpoR cDNA exhibit high- and low-affinity receptors but do not proliferate in response to Epo, it appears that expression of high-affinity Epo-binding sites alone is not sufficient to confer Epo responsiveness (D'Andrea et

al., 1989). In cross-linking studies with radiolabelled Epo two proteins of approximately 85-95 and 100-115 Kd have consistently been observed, regardless of the erythroid cell type examined or the number of affinity classes reported (reviewed in D'Andrea and Zon, 1990). The relationship between the Epo-cross-linked protein species and the presence of high- and low-affinity receptors has not yet been resolved. The affinity cross-linking studies also showed that the transfected COS cells express two Epo-binding cell surface proteins of 65 and 100 Kd, but only the 65 Kd membrane bound protein was recognized by the EpoR antibody (D'Andrea et al., 1989; Wognum et al., 1990). These observations suggested that the EpoR cDNA encodes one subunit of the EpoR complex, and that the Epo-cross-linked 100 Kd protein represents an accessory protein required for the formation of the high affinity Epo binding complex. In hemopoietic cell lines engineered to express the EpoR cDNA three major Epo-binding proteins were identified by the cross-linking studies: a 63-66 Kd polypeptide that is recognized by the anti EpoR antibodies, and 85-95 and 105 Kd proteins that are immunologically unrelated to the cloned EpoR (Damen et al., 1992; Miura and Ihle, 1993; Quelle et al., 1992). Although the nature and the function of these proteins in Epo binding has not yet been elucidated, their hemopoietic cell restricted expression favors the model predicting that the EpoR complex comprises at least two subunits.

1.4.3.2.1. Experimental models for studying the EpoR action

Given the overall functional similarities among members of the cytokine receptor superfamily, any insight into the mechanism of action by one member may be applicable to others. As the EpoR was one of the first receptors to be cloned, studies into the mechanisms of its action have been particularly extensive. The major difficulties encountered in studies of EpoR action have

been the small number of cell surface receptors on normal erythroid progenitors and the lack of cell lines that depend exclusively on Epo for their proliferation and differentiation. The latter problem has been partly overcome by engineering ectopic expression of wild type or mutant EpoR in hemopoietic cell lines such as Ba/F3, DA-3, FDC-P1 and CTLL-2, that normally depend for their proliferation on IL-3 or IL-2, and not on Epo (Li et al., 1990; Miura et al., 1991; Quelle and Wojchowski, 1991; Showers et al., 1992; Yamamura et al., 1992). These model systems enabled an in depth comparison of the intracellular events associated with the Epo, IL-3 or IL-2 R induced proliferative responses. Moreover, EpoR expressing Ba/F3 cells not only proliferate, but also accumulate β-globin mRNA in response to Epo (Carroll et al., 1994; Liboi et al., 1993) and thus represent a valuable experimental system for dissecting the Epo specific proliferative and differentiation pathways. The following sections will briefly present the current knowledge about the Epo induced activation of the EpoR, the intracellular signaling pathways engaged in transmission of the Epo-induced proliferative signal, and the functional domains within the EpoR implicated in its proliferative and differentiation signaling.

1.4.3.3. Activation of cell surface receptors

The initial ligand-induced event is assembly of the functional receptor by homo- or oligomerization of the receptor subunits.

Most available evidence indicates that Epo triggered homodimerization of the EpoR chains is sufficient for its activation. In an effort to identify possible "gain-of-function" mutations arising within the EpoR, Yoshimura et al (1990) infected IL-3 dependent Ba/F3 cells with recombinant spleen focus forming virus encoding EpoR (SFFV-EpoR) and selected transduced cells for their capacity to proliferate in the absence of Epo and IL-3. A mutant EpoR that

enabled autonomous proliferation of Ba/F3 cells contained a cysteine for an arginine substitution at codon 129 in the extracellular domain, which led to formation of disulfide-linked EpoR oligomers and constitutive activity of receptor in the absence of Epo. Based on the crystal structure of the ligand bound, homodimeric growth hormone receptor (GH-R) and sequence alignments between the GH-R and EpoR, residue 129 and four other amino acids were predicted to form the EpoR dimer interface region, and two additional constitutively active, disulfide-linked homodimeric forms of the EpoR were engineered by substituting these residues with cysteine (Watowich et al., 1992).

In addition to Epo, the EpoR can also be activated by interaction with the envelope gene product of a murine erythroleukemia virus. Friend virus induces rapid development of erythroleukemia in infected mice (Ben-David and Bernstein, 1991). This virus complex consists of the replication-competent murine leukemia virus (F-MuLV) and replication defective spleen focus-forming virus (SFFV), which harbors a mutant envelope gene derived from recombination of ecotropic F-MuLV *env* with xenotropic-like sequences endogenous to mouse genome (Clark and Mak, 1983). The resulting gp55 fusion protein interacts with the extracellular and transmembrane region of the EpoR (Yoshimura et al., 1990; Zon et al., 1992), and induces constitutive activity of the EpoR and Epo independent proliferation of infected erythroid cells (Li et al., 1990; Ruscetti et al., 1990).

Analogous models for the ligand induced oligomerization of the receptor subunits were proposed for receptors comprising dissimilar constituents. IL-3, IL-5 and GM-CSF are believed to induce first dimerization of their the ligand specific α subunits, and then oligomerization of the α-α dimers and shared βc chains (Miyajima et al., 1993). IL-2 induces assembly of β, γ and α chains of the

IL-2 receptor complex, in which interaction between the β and γ chains appears to be required for receptor activation, and the α subunit was proposed to stabilize the complex (Takeshita et al., 1992). Binding of IL-6 to its ligand-specific α subunit induces formation of disulfide-linked gp130 homodimers. In contrast to the IL-3 and IL-2 receptor subfamily, activation of the IL-6 receptor complex depends solely on interactions between the extracellular domains of the ligand-binding chain and gp130 (Ip et al., 1992).

1.4.3.4. Transmembrane signaling by the EpoR

Early studies of the Epo-induced intracellular events showed that Epo Induces influx of extracellular Ca^{2+} and mobilizes its intra-cellular pool, and calcium-specific ionophores enhanced the effect of Epo on marrow CFU-E (reviewed in Barber and D'andrea, 1992). Intracellular cyclic adenosine monophosphate (cAMP) levels were reported to increase in response to Epo, and cAMP-elevating agents potentiated the effect of Epo on erythroid maturation of SKT6 cells (Kuramochi et al., 1990). Epo was also reported to activate protein kinase C in murine erythroleukemia cells (Spangler et al., 1991), and to increase levels of the active $p21^{ras}GTP$ complex in human erythroleukemia cells (Torti et al., 1992). Together, these observations indicated that EpoR signaling is mediated by more than one intracellular pathway.

Many growth factors, such as platelet-derived growth factor, epidermal growth factor and insulin, as well as M-CSF and SCF, bind to receptors with tyrosine kinase activity. The ligand dimerization of these receptors is followed by rapid phosphorylation of several cellular proteins, as well as phosphorylation of tyrosine residues within the receptor subunits (reviewed in (Heldin, 1995). The capacity of the tyrosine kinase inhibitors to suppress proliferation and promote differentiation of the Epo-dependent erythroleukemia

cells suggested that, although the EpoR lacks an intrinsic tyrosine kinase activity, tyrosine phosphorylations also play an important role in the EpoR-mediated signaling (Noguchi et al., 1988; Watanabe et al., 1989).

The growth factor dependent hemopoietic cell lines engineered to express high levels of normal or mutant EpoRs enabled more detailed studies of the early Epo-induced intracellular events. Several such studies showed that the EpoR and a number of other cellular proteins become transiently phosphorylated on tyrosine residues within minutes of Epo binding, suggesting a physical association between the EpoR and cytoplasmic tyrosine kinase(s) (Carroll et al., 1991; Damen et al., 1992; Dusanter-Fourt et al., 1992; Miura et al., 1991; Quelle and Wojchowski, 1991). Miura et al (1991) further demonstrated that a mutant EpoR, which lacked 20 amino acids within the region of similarity between the EpoR and IL-2 Rβ cytoplasmic domains, no longer induced tyrosine phosphorylations and was not capable of supporting proliferation.

To identify the known and potential novel kinases in hemopoietic cells Mano and co-workers used a PCR approach and Northern blot analysis and found that the IL-3 dependent DA-3 cells express lyn, c-fes, tec, Jak-1 and Jak-2 mRNA (Mano et al., 1993). In the EpoR expressing DA-3 cells, however, of these only Jak-2 was phosphorylated and activated in response to Epo, as detected by the in vitro kinase activity assays (Witthuhn et al., 1993). DA-3 cells expressing the mitogenically inactive EpoR described above (Miura et al., 1991) failed to activate Jak-2, further supporting the role for Jak-2 kinase activity in Epo-induced mitogenesis (Witthuhn et al., 1993). IL-3, GM-CSF, G-CSF, growth hormone and interferon-γ, however, also activate the Jak-2 kinase (Argetsinger et al., 1993; Witthuhn et al., 1993). The specificity of the cytokine induced

response was therefore proposed to depend on the lineage specific expression of the substrates available to Jak-2 and other tyrosine, as well as serine/threonine-specific protein kinases.

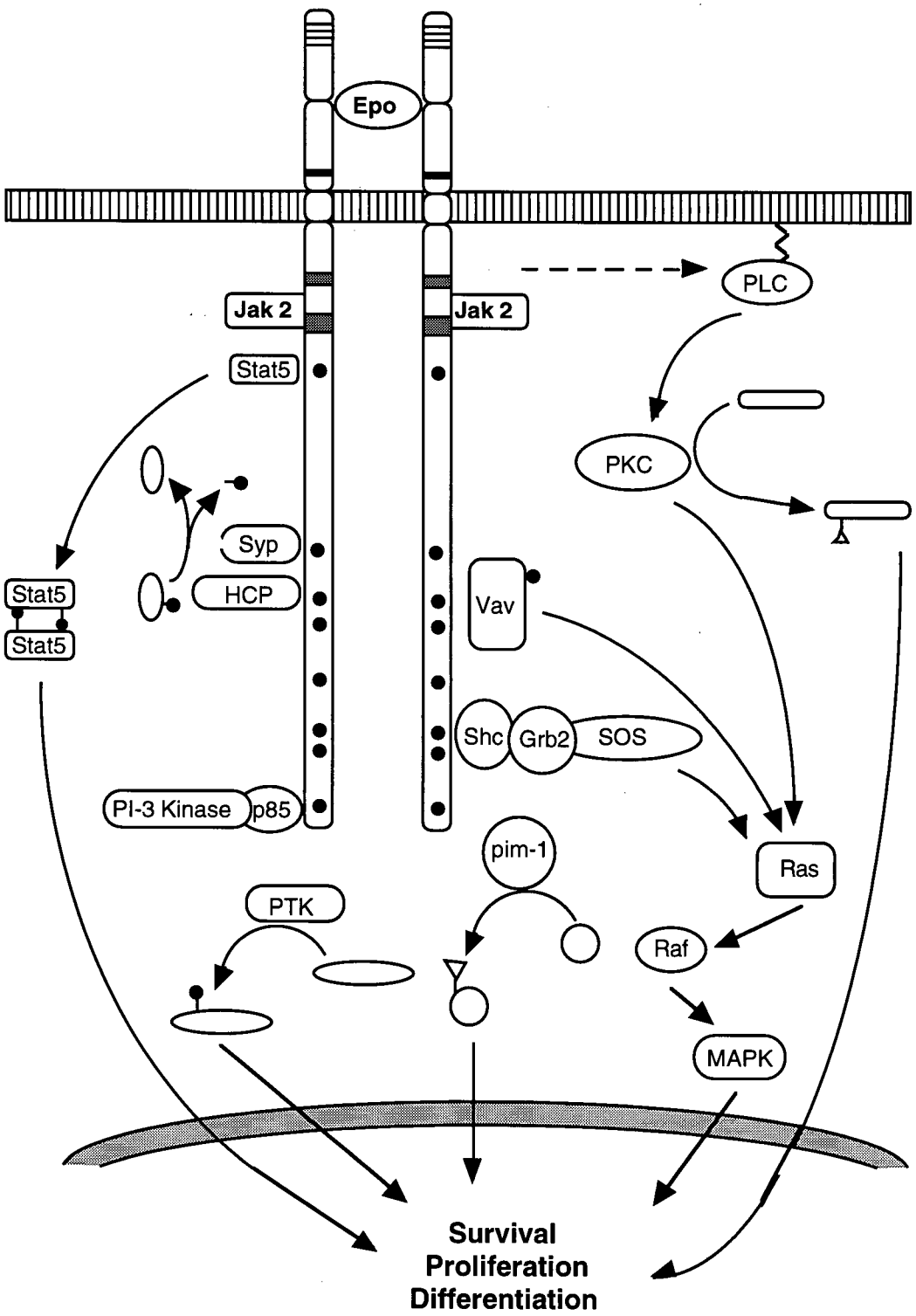

Figure 1.3. A model of signaling pathways activated by the erythropoietin receptor.

A schematic representation of interactions between the activated EpoR and the intracellular signal transducing proteins. PTK, protein tyrosine kinase; black dots, phosphorylation/dephosphorylation of the tyrosine residues; triangles, serine/threonine phosphorylations.

In the current model of EpoR activation, Epo induced oligomerization enables intermolecular phosphorylation and activation of the receptor-associated tyrosine kinase Jak-2, which then phosphorylates the tyrosine residues within the EpoR. These phosphotyrosines enable association between the EpoR and various proteins possessing the Src homology-2 domain (SH-2) domain. SH-2 domains were first described as regions mediating interaction between the cytosolic kinases of the Src family and the phosphorylated tyrosine residues within their target proteins (Pawson and Gish, 1992). SH-2 domains were later identified as an integral part of numerous proteins involved in intracellular signaling. The SH-2 containing proteins that interact with the activated EpoR include the signal transducer and transcription activating factor 5 (STAT5) (Damen et al., 1995), Grb2 and Shc (Cutler et al., 1993; Damen et al., 1993), the regulatory subunit of phosphatidylinositol-3 kinase (Damen et al., 1993; He et al., 1993) and phosphatases SH-PTP1 and Syp (Klingmuller et al., 1995; Tauchi et al., 1995). Interactions with the EpoR chains bring these proteins into close proximity of Jak-2, which enables their phosphorylation and activation.

The Epo induced tyrosine phosphorylation is transient, and dephosphorylations are believed to be carried out, at least in part, by the EpoR-associated phosphatases SH-PTP1 and Syp (Klingmuller et al., 1995; Wakao et al., 1995). The deleted EpoR lacking the phosphatase binding region was implicated in an increased Epo sensitivity of erythroid progenitors detected in some patients with benign erythrocytosis and primary polycythemia (de la Chapelle et al., 1993; Sokol et al., 1995). Similarly, the absence or reduction of SH-PTP1 activity in the mutant motheaten (me) mice results in a variety of hematological abnormalities, including increased sensitivity to Epo and accumulation of activated macrophages (Tsui et al., 1993; Van Zant and Shultz,

1989). Transient dephosphorylation and deactivation of the EpoR, as well as other cytokine receptors, is therefore likely crucial for normal regulation of hemopoiesis.

The EpoR-activated signaling proteins were, however, also implicated in transduction of mitogenic and survival signals activated by other cytokine receptors. The Jak-2/STAT5 pathway is, for example, activated in response to Epo, prolactin, IL-2, IL-3, IL-5 and GM-CSF (Damen et al., 1995; Gouilleux et al., 1995; Mui et al., 1995; Wakao et al., 1995), and activation of the Ras pathway was in addition to Epo also documented in response to IL-2, IL-3, IL-5 and GM-CSF (Duronio et al., 1992; Satoh et al., 1992; Torti et al., 1992). The cytokine induced mitogenic signaling thus appears to converge into common intracellular signaling pathways, which likely represents the molecular basis for the apparent interchangability of different cytokines in supporting hemopoietic cell proliferation.

1.4.3.5. Functional domains of the erythropoietin receptor

In addition to promoting proliferation and survival, Epo also appears to play a role in regulating terminal differentiation of the erythroid committed cells. In an effort to identify the regions within the EpoR that are associated with its proliferation and differentiation functions, several groups have engineered high level expression of normal and mutated EpoRs in growth factor dependent hemopoietic cell lines, which do not express endogenous EpoR, and examined the capacity of the mutated EpoRs to support proliferation and induce expression of the erythroid specific genes.

1.4.3.5.1. Proliferation domain of EpoR

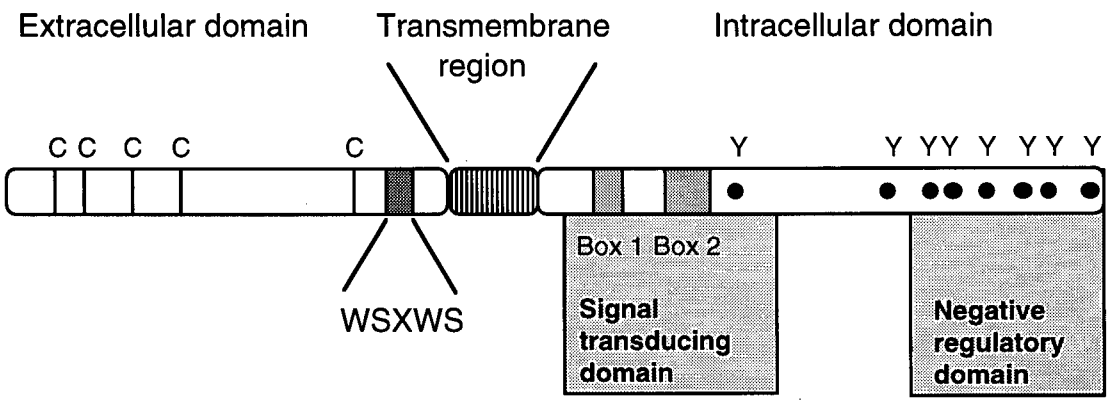

Figure 1.4. Functional domains of the erythropoietin receptor.
The EpoR comprises a 223 amino acid extracellular domain, 24 amino acid transmembrane region and 236 amino acid cytoplasmic region. In the intracellular domain, the shaded boxes denote the conserved box 1/box 2 regions of homology, and dots show positions of the tyrosine residues. The membrane proximal region is indispensable for the proliferative function of the EpoR, and the membrane distal region comprising the majority of tyrosine residues was associated with downregulation of the EpoR activity.

In an attempt to generate high EpoR expressing cells D'Andrea et al (1991) infected the IL-3 dependent Ba/F3 cells with recombinant retroviruses encoding EpoR, and selected clones of the infected cells in medium containing only 1 pM/L of Epo, or 1/10 of the previously determined concentration required for optimal proliferation of these cells (Yoshimura et al., 1990). One of the selected clones was found to express a truncated EpoR that lacked the carboxy terminal 40 amino acids, and Ba/F3 cells engineered to express this -40 EpoR deletion mutant exhibited hypersensitivity to Epo in proliferation assays, comparable to that determined for the original selected cell line. A similar increase in Epo sensitivity was seen in Ba/F3 cells expressing an EpoR lacking the terminal 91 amino acids of the cytoplasmic domain, suggesting that the carboxy-terminal region of the cytoplasmic domain suppresses the mitogenic activity of the EpoR. A comparable effect was, however, not evident when the Epo-induced proliferative responses of FDC-P1 and DA-3 cells engineered to

express these or very similar EpoR deletion mutants were examined (Damen et al., 1995; Miura et al., 1991; Quelle and Wojchowski, 1991). The negative regulatory action of the EpoR carboxy terminus may thus involve activation of intracellular signaling proteins expressed by some, but not all cell lines.

A number of additional EpoR carboxy terminal deletions have been generated using a PCR approach, and examined for their capacity to promote proliferation (D'Andrea et al., 1991; Damen et al., 1995; Miura et al., 1991; Nakamura et al., 1992; Quelle and Wojchowski, 1991). Results of these studies have been remarkably consistent in demonstrating that receptors retaining more than 125 amino acids of the cytoplasmic domain are mitogenically active and induce tyrosine phosphorylation, and that receptors lacking more than 100 amino acids of their cytoplasmic region are inactive.

In the membrane proximal region indispensable for the mitogenic function of the EpoR there are some sequence homologies, termed box 1 and box 2, with the IL-2 receptor β chain and the IL-6 signal transducing protein gp130 (Murakami et al., 1991). These regions were proposed to link cytokine receptors to a common mitogenic signal transducing pathway. The effects of deletions and point mutations within the membrane proximal region were therefore examined to define more precisely the domains that are required for mitogenic activity of the EpoR. A deletion spanning residues 33 to 54 of the cytoplasmic domain, including a part of the conserved region, inactivated the capacity of the EpoR to induce tyrosine phosphorylations and promote proliferation (Miura et al., 1991). Finally, Witthun et al (1993) demonstrated that Jak-2 physically associates with the conserved membrane proximal region in the cytoplasmic domain, that this interaction is required for the Jak-2 activation,

and that capacity of the EpoR to activate Jak-2 correlates with its ability to promote proliferation.

1.4.3.5.2. The differentiation-active domain of the EpoR

The observation that EpoR-expressing Ba/F3 cells respond to Epo with proliferation and accumulation of β-globin mRNA (Chiba et al., 1993; Liboi et al., 1993) indicated that the EpoR activates both a proliferative and specific differentiation signal. To determine if the cytoplasmic region of the EpoR was sufficient for transduction of the putative differentiation signal, Jubinsky et al (1993) constructed a chimeric receptor consisting of the extracellular and transmembrane region of the granulocyte-macrophage colony stimulating receptor α2 chain and the intracellular domain of the EpoR (GM-CSFα_2/EpoR) and showed, that GM-CSF induces expression of glycophorin in GM-CSFα_2/EpoR expressing Ba/F3 cells. Moreover, a chimeric receptor consisting of the epidermal growth factor receptor (EGF-R) derived extracellular and transmembrane domain, and only the membrane proximal 127 amino acids of the EpoR intracellular domain was capable of inducing globin synthesis in the murine erythroleukemia derived TSA8 cells (Maruyama et al., 1994). Together, these studies suggested that the membrane proximal region of the cytoplasmic domain of the EpoR is sufficient for activation of the Epo-specific differentiation signal. A surprisingly contrasting set of results was obtained when tyrosine phosphorylation events induced by various chimeric EpoRs were analyzed (Chiba et al., 1993). A chimeric receptor consisting of the extracellular domain of the IL-2 receptor β chain (IL-2 Rβ) and the transmembrane and intracellular domain of the EpoR was found to induce an IL-2-specific pattern of tyrosine phosphorylations. The Epo-specific patterns of tyrosine phosphorylated proteins were, in contrast, detected in cells expressing chimeric receptors consisting of

the extracellular domain of the EpoR, and the intracellular region of either IL-2 Rβ (EpoR/ IL-2 rβ) or IL-3 Rβ (EpoR/IL-3R β) (Chiba et al., 1993), consistent with Epo-specific signaling occurs through the extracellular domain of the EpoR. Moreover, accumulation of β-globin mRNA was detected in Epo-stimulated Ba/F3 cells expressing chimeric receptors comprising the extracellular domain of the EpoR, but not in the IL-2 stimulated cells expressing the hybrid receptor consisting of the extracellular domain of the IL-2 R β, and the intracellular region of the EpoR (Chiba et al., 1993). It was proposed, therefore, that the Epo-induced differentiation signal involves interactions between the extracellular domain of the EpoR, and the putative second, so far unidentified second subunit of the EpoR complex.

Epo, however, failed to promote survival, proliferation and β-globin accumulation in Ba/F3 cells expressing a truncated EpoR lacking the cytoplasmic domain (Chapter 4 and Carroll et al., 1994). This observations suggested an overlap between the proliferation and putative differentiation domain of the EpoR and implied that Epo may induce terminal maturation of the erythroid committed cells through the EpoR-specific regulation of survival and proliferation. Results presented in Chapter 4. suggest, moreover, that in EpoR-expressing Ba/F3 cells, the α subunit of IL-3R can actively inhibit the Epo-induced accumulation of β-globin mRNA. An EpoR-mediated survival signal may thus enable terminal erythroid differentiation only in the absence of the IL-3R activity

1.4.3.5.3. Alterations of the EpoR expression and modulation of hemopoietic cell behavior

During hemopoietic cell differentiation, Epo responsiveness appears to correlate with high levels of cell surface EpoR expressed by late erythroid

progenitors, indicating that the action of Epo is restricted by the developmental stage-specific expression of the EpoR. The transduction of the Epo-specific signal may, alternatively, or additionally, involve some specific intracellular signaling proteins that are expressed by the late erythroid committed cells, but not by the multipotential progenitors.

To explore the capacity of the EpoR-mediated signaling to alter the proliferation and differentiation behavior of early hemopoietic progenitors, several groups have utilized retrovirus mediated gene transfer to engineer high levels of EpoR expression by primary hemopoietic cells. Fetal liver cells infected with a retrovirus encoding a mutated EpoR, which signals in the absence of Epo (Section 1.4.3.2.2.), gave rise to CFU-E derived colonies in the absence of added growth factors, whereas early erythroid and lineage non-committed progenitors still depended for their proliferation on IL-3, IL-6 and SCF (Pharr et al., 1993). Epo alone, however, supported development of multilineage-mixed colonies in cultures of bone marrow cells infected with a retrovirus harboring a normal EpoR (Dubart et al., 1994). The mixed colonies generated by the EpoR-transduced multilineage progenitors were composed of cells from all hemopoietic lineages, suggesting that lineage commitment of clonogenic progenitor cells was not affected by the EpoR mediated signaling (Dubart et al., 1994; Pharr et al., 1994).

An alternative approach to studying the role Epo and its receptor play in regulation of hemopoietic cell behavior was a creation of mouse strains carrying an inactivating mutation in the Epo and EpoR gene (Kieran et al., 1996; Lin et al., 1996; Wu et al., 1995). Both, the $Epo^{-/-}$ and $EpoR^{-/-}$ mice die *in utero* at embryonic day 12-15 with severe anemia due to the failure of the definitive erythroid committed progenitors to undergo terminal differentiation. These

observations are remarkably consistent with the results obtained by the over-expression of the EpoR in the multipotential bone marrow derived cells in that they demonstrate that neither Epo nor EpoR are required for commitment of stem cells to erythroid lineage, and that the EpoR activity is essential for proliferation and functional maturation of late erythroid progenitors.

1.5. Thesis objectives

As presented in the previous sections, several lines of evidence support a major role for Epo in regulating mammalian erythropoiesis. These include the well documented elevation in erythrocyte production in response to hypoxia-induced increase in Epo levels; the absolute Epo dependency of the late erythroid clonogenic progenitors; and the absence of mature erythroid cells in Epo and EpoR deficient mice. Observations in both *in vitro* and *in vivo* experimental models suggested that Epo promotes the proliferation and differentiation of erythroid committed cells. However, at the time this project was initiated there was no direct evidence for the capacity of the EpoR to induce the onset of terminal erythroid differentiation. Two major questions were thus addressed during the course of this research.

1- Does Epo induce a distinct set of events in EpoR expressing cells

2- Is the absence of EpoR expression by non-erythroid and multipotential cells the only determinant preventing their Epo responsiveness

To address these questions studies were carried out towards specific aims as presented in Chapters 3 to 5.

1- To search for the potential qualitative differences between the IL-3 and Epo induced cellular responses using the IL-3 dependent hemopoietic

cell line Ba/F3 engineered to express EpoR as a model system (Chapter 3).

2- To identify domains within the EpoR that are specifically involved in induction of the Epo-specific events by exploring the capacity of various mutant and chimeric EpoRs to induce β-globin mRNA accumulation in Ba/F3 cells (Chapter 4).

3- To investigate the effect of Epo on the proliferation and differentiation behavior of early progenitor cells engineered by the retrovirus mediated gene transfer to constitutively express high levels of EpoR (Chapter 5).

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Generation of EpoR mutant and chimeric cDNAs

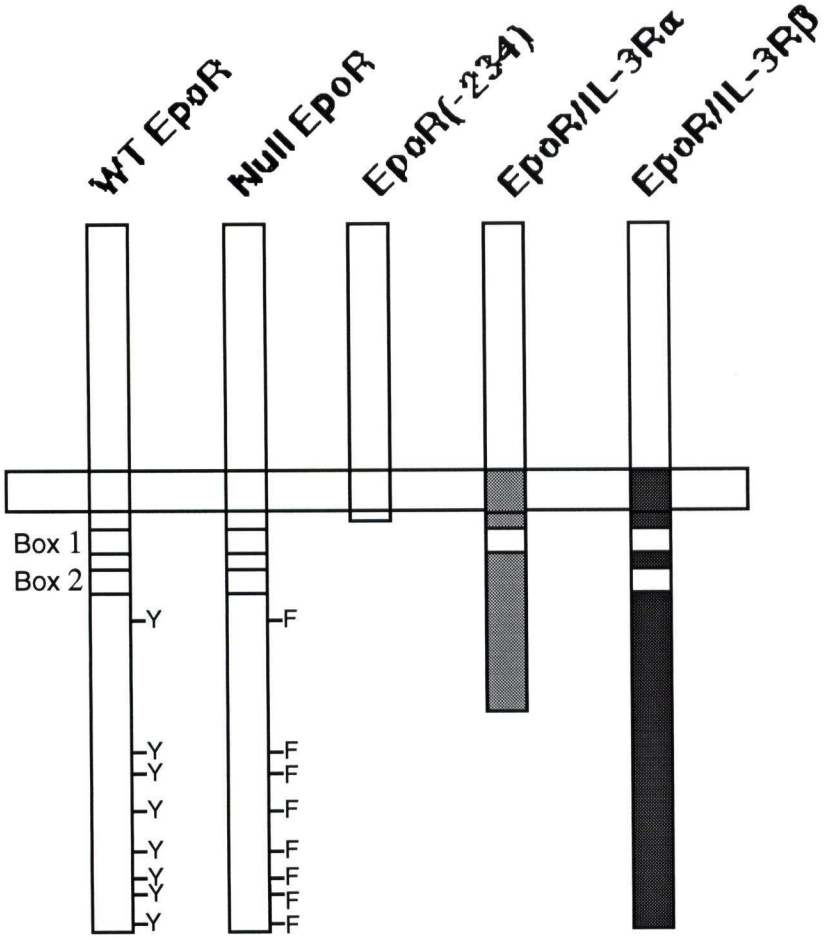

Figure 2.1. Schematic representation of the normal (WT) and modified EpoRs used in this study.
The null EpoR cDNA encodes a full-length 483-amino acid EpoR polypeptide in which all eight cytoplasmic tyrosines (Y) are substituted with phenylalanines (F). All depicted chimeric and truncated EpoRs possess the extracellular domain encoded by the EpoR cDNA. The EpoR/IL-3Rβ and EpoR/IL-3Rα chimera comprise the transmembrane and cytoplasmic domains of the IL-3Rβ_{IL-3} subunit or the transmembrane and cytoplasmic domains of the IL-3Rα subunit, respectively. The cytoplasmic domain of the C-terminal truncated EpoR$_{(-230)}$ possesses only two primer-derived arginine residues.

The Null EpoR mutant in which all cytoplasmic tyrosines were replaced with phenylalanines was constructed using site-directed mutagenesis as

described by Damen et al (1995) To generate a hybrid gene encoding for the EpoR/IL-3Rβ_{IL-3} chimera, *Kpn I-BamH I* fragment of pXM EpoR(190) (A.D'Andrea, Harvard Medical School, Boston, MA) encompassing cDNA encoding for the extracellular, transmembrane and a portion of intracellular domain of the EpoR, and *BamH I-Not I* fragment of pAIC2 26 (A.Miyajima, DNAX, Palo Alto, CA) encoding for a truncated extracellular domain along with the transmembrane and cytoplasmic regions of the IL-3Rβ_{IL-3} subunit were subcloned into *Kpn I-Not I* digested pBS (KS+) (Stratagene, LaJolla, CA). The resulting intermediate termed pBS-EpoR/IL-3Rβ encoded for both EpoR and IL-Rβ_{IL-3} derived transmembrane domains which could be removed by virtue of the *Nhe I* site located upstream of the EpoR transmembrane domain and the *Nde I* site downstream of the IL-3R β_{IL-3} specific transmembrane region. The transmembrane region of IL-3R β_{IL-3} was amplified by polymerase chain reaction (PCR) using pAIC2-26 as a template and primers 5'-TGG GCT AGC GAC TGG GTG ATG CCC AC-3' (nt 1493-1525) and 5'-CAA ATG TTC ATA TGA CAC CC-3' (nt 1749-1769). An *Nhe I* site was introduced into the sense primer, and the antisense primer contained endogenous *Nde I* restriction site. The *Nhe I-Nde I* digested 265 bp PCR product encoding for the transmembrane region of IL-3Rβ_{IL-3} subunit was then sucloned into *Nhe I -Nde I* linearized pBS-EpoR/IL-3Rβ to create a hybrid cDNA encoding for the extracellular domain of the EpoR and the transmembrane and intracellular domain of the IL-3Rβ_{IL-3} subunit. Ligation of the PCR product with *Kpn I-Nhe I* fragment of EpoR cDNA generated substitutions T437A and T438S at positions -4 and -3 relative to the predicted boundary of the transmembrane region of IL-3Rβ_{IL-3} subunit.

To create a cDNA encoding a chimera consisting of the extracellular domain of the EpoR and the transmembrane and intracellular domain of the

IL-3Rα subunit, the transmembrane and intracellular domains of IL-3Rα cDNA were amplified by PCR using pSUT-1 (A.Miyayima, DNAX, Palo Alto, CA) as a template. Primers used for amplification were 5'-CCC CCA GAG GTG CTA GCG TGA AG-3' (nt 1110-1133) and 5'-ATG CCC CAG GGC GGC CGC AGT TCT CAG GCG GTC-3' (nt 1332-1365). An *Nhe I* restriction site was engineered into the sense primer, and the antisense primer complementary to the nontranslated region of IL-3R α cDNA contained a *Not I* restriction site. The amplified 255 bp fragment was digested with *Nhe I* and *Not I* and subcloned into *Nhe I -Not I* linearized pBS-EpoR/IL-3Rβ downstream of the EpoR cDNA to yield a hybrid gene encoding for the EpoR/IL-3Rα chimera. Ligation of the PCR amplified sequences of IL-3Rα subunit and EpoR cDNA created substitutions M328A and P329S at positions -4 and -3 relative to the transmembrane region of the IL-3α subunit.

To generate the EpoR deletion mutant $EpoR_{(-230)}$ in which 230 COOH-terminal aa had been removed leaving only 2 aa of the cytoplasmic domain, the extracellular and transmembrane domains of the EpoR cDNA were amplified by PCR using pBS(KS+) encoding for the EpoR cDNA as a template. The sense primer used for amplification was complementary to vector. Antisense primer 5'-CGG TTA CGG CGG CGG CGG TGG GAC AGC AGG-3' was complementary to nucleotides 835-853 of the EpoR cDNA and introduced R^{252} and R^{253} as the only two amino acids of the intracellular domain. The PCR product was blunted and then digested with *Nhe I* to isolate sequences encoding for the transmembrane domain of the EpoR. The region of cDNA encoding for the transmembrane and intracellular domain of the EpoR was excised from pBS-EpoR with *Nhe I* and *Sma I* and was then replaced with PCR product to create the EpoR deletion mutant. All amplification reactions were carried out for 20 cycles using Taq polymerase and following recommendations

of manufacturer (Canadian Life Technologies, Burlington, Ontario). The products of PCR amplifications were sequenced to verify the fidelity of their respective coding sequences.

2.2. Retroviral vectors

The JZen EpoR TKneo vector carrying a murine cDNA encoding the EpoR and a thymidine kinase promoter neo^r gene cassette (Tkneo) was constructed as reported previously (Damen et al., 1992). Briefly, a 1596-bp *Kpn-I -Mse I* fragment encompassing the coding region of the murine EpoR (D'Andrea et al., 1989) was isolated from pXMEpoR(190) and inserted by blunt end ligation into the *Xho I* site of JZenTkneo upstream of the neo^r gene such that the expression of the EpoR cDNA is under the control of the myeloproliferative sarcoma virus long terminal repeat.

To generate the JZenEpoR/IL-3Rβ_{IL-3} retroviral vector, a 2279-bp *Kpn-I -Not I* fragment encompassing the coding region of the chimeric receptor was isolated from pBS-EpoR/IL-3Rβ_{IL-3} and inserted by blunt end ligation into the *Xho -I* site of JZen TKneo upstream of the neo^r gene. Expression of the hybrid gene was thus under control of the myeloproliferative sarcoma virus long terminal repeat.

To create MSCV-EpoR/IL-3Rα, a 1010-bp *Kpn-I -Not I* fragment encompassing the hybrid EpoR/IL-3Rα gene was isolated from the pBS-EpoR/IL-3Rα and inserted by blunt end ligation into the *Hpa I* site of the MSCV PGKpac^r vector upstream of the PGKpac^r cassette.

A MSCV-EpoR$_{(-230)}$ PGK retroviral vector was generated by subcloning a 843-bp *Kpn I -BamH I* fragment of pBS-EpoR$_{(-230)}$, encoding the truncated

EpoR, into the *Hpa I* site of the MSCV PGKneo vector upstream of the PGKneo^r cassette (Hawley et al., 1994).

The JZen null EpoR TKneo vector was constructed as reported previously (Damen et al., 1995).

2.3. Cell lines

The ecotropic GP+E-86 retrovirus packaging cell line (Markowitz et al., 1988) was obtained from Dr. A. Banks (Columbia University, New York, NY) and was maintained in Dulbecco's modified Eagle medium (DMEM) with 4500 mg/L glucose and 10% heat inactivated newborn calf serum, supplemented with 15 µg/mL hypoxanthine, 250 µg/mL xanthine, 25 µg/mL mycophenolic acid (HXM selective medium). The amphotropic packaging cell line GP+envAM12 (Markowitz et al., 1988) was maintained in HXM medium containing 200 µg/mL of hygromycin (Boehringer, Mannheim, FRG). GP+E-86 and GP+envAM12 subclones transfected with recombinant retroviral vectors were maintained in HXM medium containing 1 mg/mL of G-418 (Canadian Life technologies, Burlington, Ontario), or 2 µg/mL of puromycin (Sigma, St.Louis, MO), as appropriate for selection of virus encoded selectable marker.

The murine bone marrow derived IL-3 dependent cell lines Ba/F3 and DA-3 (Palacios and Steinmetz, 1985) were kindly provided by Dr. A. Miyajima (DNAX Research Institute, Palo Alto, CA) and Dr. J. Ihle (St. Judes Children's Hospital, Memphis, TN), respectively. Both cell lines were maintained in RPMI medium with 10% heat inactivated fetal calf serum (FCS) and 3 nmol/L COS cell derived mIL-3. The retrovirally infected cells were maintained in the same medium, supplemented with 1.8 mg/mL of G-418 and/or 2 µg/mL of puromycin.

2.4. Viral production

The plasmid DNA was transfected by calcium phosphate precipitation into GP+E-86 cells. After 18-hr incubation with the DNA precipitate, cells were washed, incubated for additional 24 hr in HXM medium, and were then selected and maintained in HXM medium containing 1 mg/mL G-418 (Canadian Life Technologies, Burlington, Ontario, Canada) or 2 μg/mL puromycin (Sigma), as appropriate for selection of the virus-encoded selectable marker.

In an effort to generate high retrovirus-producing cell lines supernatants obtained with clones of GP-E-86 Jzen EpoR TKneo and GP-E-86 MSCVneo cells were used to infect GP+envAM12 cells, and the supernatants obtained with polyclonal G-418^r populations of GP+envAM12 cells used to superinfect the initial clones of ecotropic EpoR and neo virus producers. The EpoR and neo virus producers were then subcloned to identify clones producing >1x10^7 cfu/mL as assessed by transfer of G-418 resistance to NIH 3T3 fibroblasts. Absence of replication competent helper viruses was verified by failure to serially transfer virus conferring G-418 resistance to NIH 3T3 cells.

2.5. Infection of Ba/F3 and DA-3 cells

Ba/F3 or DA-3 cells were cocultured with irradiated (1500 cGy, X-ray source) retroviral producer cells for 24 hours in the presence of 3 nmol/L COS cell derived mIL-3 and 4 μg/mL polybrene. Non-adherent cells were recovered and cultured for 1 week in growth medium containing 3 nmol/L COS cell-derived mIL-3 and 1 mg/mL G-418. Polyclonal G418-resistant cells were then plated in standard methylcellulose (Humphries et al., 1979) supplemented

with mIL-3 and 1 mg/mL G418 and the resulting colonies were clonally expanded for further studies.

The C5 clone of Ba/F3 cells expressing high levels of the murine EpoR following infection with the EpoR virus has been described previously (Damen et al., 1992). In contrast to the lines described here, C5 were initially selected and continuously grown in Epo supplemented medium.

2.6. Infection of primary bone marrow cells

BM cells from adult B6C3F1 mice injected 4 days previously with 5-fluorouracil (150 mg/kg of body weight) were flushed from femoral shafts with α medium containing 2% FCS. The recovered cells were cultured for 2 days on petri dishes in DMEM containing 15% FCS (Stem Cell Technologies Inc., Vancouver, BC), 100 ng/mL of murine stem cell factor (SCF), 6 ng/mL of murine IL-3 and 10 ng/mL of human IL-6, and were then co-cultured with confluent monolayers of irradiated (1500 cGy, X-ray source) EpoR or neo virus producing cells for 2 days in the same medium supplemented with 6 μg/mL of polybrene. Bone marrow cells recovered from the cocultures were washed with DMEM containing 2% FCS, resuspended in fresh growth medium with SCF, IL-3 and IL-6, and cultured on petri dishes for a further 24 hr to allow for expression of the transduced EpoR and neo^r genes. All growth factors were used as diluted supernatants from cultures of COS cells transfected with appropriate growth factor expression vectors.

2.7. Proliferation assays

2.7.1. Proliferation of Ba/F and DA-3 cells in liquid culture

To assess growth rates of EpoR Ba/F3 and EpoR DA-3 cells in liquid culture, growth factor deprived cells (see below) were resuspended in RPMI with 10% FCS and containing either 3 nmol/L COS cell-derived mIL-3, 1.5 U/ml recombinant human Epo or a combination of both growth factors. One mL aliquots, containing 6000 cells/mL, were dispensed into 24-well plates and incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. Duplicate wells were harvested at different times and cell numbers determined using a hemocytometer.

2.7.2. ^{3}H-Tdr incorporation assays

Cells were grown for 2-4 days in the absence of G-418 and/or puromycin, washed and then deprived of IL-3 and FCS for 8 hr in 0.1% BSA in RPMI. Growth factor deprived cells were then washed, resuspended in RPMI 1640 containing 0.1% BSA, and aliquoted into 96-well U-shaped microtiter plates at 2.5 x 10^4 cells/well, and growth factors were added to a final volume of 0.1 mL/well. Following 22 hr of incubation at 37°C in a humidified atmosphere containing 5% CO_2, cells were pulsed with 1 μCi of [^{3}H]thymidine (3H-Tdr, 2 Ci/mmol, DuPont NEN) for 2 hr. The cells were then harvested onto filter mats using an LKB 1295-001 -Skatron cell harvester, and ^{3}H-Tdr incorporation was determined in an LKB 1205 Betaplate liquid scintillation counter (LKB Wallac, Turku, Finland).

2.7.3. Proliferation of neo^r- and EpoR-transduced bone marrow cells in serum free suspension cultures

Bone marrow cells recovered 24 hr after co-culture with the neo^r or WT EpoR virus producers were washed twice with DMEM containing 2% BSA and then resuspended in serum-free medium (Lansdorp and Dragowska, 1992) consisting of Iscove's modification of DMEM supplemented with 2% BSA, 10 μg/mL of insulin, 200 μg/mL of transferrin, 10^{-5} mol/L of β-mercaptoethanol, 40 μg/mL of low-density lipoprotein (Sigma, St.Louis, MO) and 1.6 mg/mL of G-418. Cell suspensions were then aliquoted into 96-well U shaped microtiter plates at $2x10^4$ cells/well, and medium containing growth factors (Epo, or IL-3, or SCF; Epo and IL-3; and Epo, IL-3 and SCF) was added to a final volume of 0.2 mL per well. Concentrations of cytokines used were: Epo, 1 Unit /mL; IL-3, 10 ng/mL; and SCF, 50 ng/mL. After 2-day incubation at 37°C in a humidified atmosphere containing 5% CO_2 duplicate wells were harvested, cells washed with serum free medium and numbers of viable cells (excluding Eosin Y dye) were determined using a hemocytometer. Cells were then transferred into new wells ($2x10^4$/well) containing fresh growth medium, and were counted and transferred on day 4 and day 6 after initiation of the serum-free culture as described. At the indicated time points aliquots of washed cells were plated in methylcellulose supplemented with 1 Unit/mL of Epo, 10 ng/mL of IL-3 and 25 ng/mL of SCF and the numbers of multilineage mixed colonies were determined on day 14.

2.8. In vitro clonogenic progenitor assays

Bone marrow cells were washed twice with DMEM supplemented with 2% FCS and were plated in 35 mm petri dishes in 1 mL culture mixture

containing 0.8% methylcellulose in α medium supplemented with 30% FCS, 1% bovine serum albumin (BSA) and $1x10^{-4}$ mol/L of β-mercaptoethanol. Semisolid cultures contained 2% spleen cell conditioned medium (SCCM) and 3 Units of Epo with and without 1 mg/mL of G-418, or 3 Units of Epo alone, or no added growth factors. In two experiments varying proportions of the neo^r- and WT EpoR-infected cells were plated in the presence and in the absence of $1x10^5$ irradiated WT EpoR-infected cells (1500 cGy, X-ray source) in methylcellulose supplemented with 3 Units of Epo/mL. Bone marrow cells recovered 24 hr after co-cultivation infection were plated at $2x10^3$ cells/dish. Bone marrow cells isolated from recipients of the WT EpoR or neo^r- infected bone marrow were plated at $3x10^4$ cells per dish, and spleen cells at $5x10^4$ and $5x10^5$ cells/dish for recipients of the WT EpoR- and neo^r-transduced bone marrow, respectively. Colonies were scored after 2 to 14 days of incubation according to standard criteria (Humphries et al., 1979).

2.9. Cell cycle analysis

Growth factor deprived EpoR Ba/F3 cells were stimulated with 3 nmol/L IL-3 or 1.5 U/ml Epo in RPMI, containing 0.2% BSA. The progression of cells through the cell cycle was then analyzed as described by Sato et al (1993). At the indicated time points, $1x10^6$ cells were washed twice with PBS, resuspended in 1 ml of staining solution containing 4 mmol/L trisodium citrate, 0.1% Triton-X100, 0.1 mg/mL RNase A and 0.05 mg/mL propidium iodide (PI) and incubated for 30 min at room temperature. Stained cells (10,000/-sample) were analyzed using a FACSort (Beckton Dickinson, San Diego, CA) and the percentage of cells in different phases of the cell cycle were determined using the CellFIT program (Beckton Dickinson).

2.10. Flow cytometric analysis of biotinylated Epo binding.

Specific binding of biotin-Epo (b-Epo) by the neo^r and EpoR-transduced cells was performed as described by Wognum et al (1990). Briefly, cells were washed twice with Hank's balanced salt solution containing 2% FCS and 0.05% of sodium azide (HFN buffer) and were then incubated in HFN buffer containing 1 nmol/L of b-Epo or, for controls, 1 nmol/L of b-Epo and 100 nmol/L of unlabelled Epo, for 30 min at 37°C. Cells were then washed twice with ice-cold HFN and incubated with a streptavidin-R-phycoerythrin (RPE) conjugate (Molecular Probes, Eugene, OR) for 30 min on ice. After incubation, cells were washed twice with HFN and resuspended in HFN containing 1 μg/mL of 7-amino-actinomycin (7 AAD, Sigma, St.Louis, MO) to distinguish dead cells before analysis by flow cytometry using FACScan cell analyzer (Beckton Dickinson, San Jose, CA).

2.11. Scatchard analysis of the specific ^{125}I-Epo binding

^{125}I human Epo (Amersham, Oakville, Ontario) was first diluted with binding buffer (Hank's balanced salt solution, 2% FCS, 0.02% NaN_2) to an initial concentration of 1.3 $x10^{-9}$ mol/L. Twofold serial dilutions were then performed in 0.5-mL microfuge tubes, with and without a 100-fold excess of unlabeled recombinant Epo to correct for nonspecific binding. Cells were then added in binding buffer to give $1x10^6$ cells in a total volume of 100 μL. After 4 hours of gentle rocking at room temperature cell suspensions were transferred to 0.5 mL microfuge tubes containing 250 μL of dibutylphtalate/dioctylphalate oil mixture (3:2 ratio) and centrifuged for 3 min at 12, 000 rpm to separate cells from unbound radioactivity. The tubes were then snap-frozen at -70°C and the tips, containing the cell pellets, cut off, transferred to γ counter tubes, and

counted in a Beckman gamma 5500 γ-counter. The concentration of ^{125}I-Epo specifically bound at each dilution was calculated as the difference between the nonspecific binding detected in the presence of unlabeled Epo, and the total binding determined in the absence of competitor. The numbers of Epo-binding sites (n) and the dissociation constants (K_d) for the Epo-EpoR complexes were then calculated using Scatchard analysis.

2.12. Mice

Mice used in these experiments were 12- to 16-week-old (C57Bl/6J -x-C3H/HeJ)-F1-(B6C3 F1) male and female mice bred and maintained in the animal facility of the British Columbia Cancer Research Center from parental strain breeders originally obtained from the Jackson Laboratories (Bar Harbor, ME). All animals were housed in sterilized microisolator cages and provided with sterilized food and acidified water.

2.13. Transplantation of the retrovirally transduced bone marrow

Lethally irradiated 14- to 16-week-old (B6C3)F1 mice (950 cGy, 110 cGy/min, ^{137}Cs source) were injected intravenously with 0.2-1x10^6 bone marrow cells recovered 24 hours after co-culture with the neo^r and WT EpoR retrovirus producing cells. For secondary transplantations, four affected primary recipients of the WT EpoR-infected bone marrow were sacrificed and their bone marrow (2x10^6 cells, 3 recipients for each donor) and spleen cells (5x10^6, 3 to 4 recipients for each donor) transplanted together with 1x10^5 of normal bone marrow cells into lethally irradiated secondary recipients. Mice were observed daily and were sacrificed when showing any visible signs of disease such as lethargy, ruffled fur or hunched posture. At the time of sacrifice, hematocrit values and the total and the differential peripheral blood cell counts were

determined. Hematocrit values were obtained by sedimentation in heparinized capillaries, and the total peripheral blood cell counts were determined using hemocytometer. Blood smears were examined after Wright staining and the differential counts for 200 hundred cells were determined.

2.14. RNA isolation and Northern blot analysis

Exponentially growing parental Ba/F3 or EpoR-transduced Ba/F3 cells were cultured in the absence of IL-3 for 12 hr as described for proliferation assays, washed with phosphate buffered saline (PBS) and resuspended in fresh growth medium supplemented with either 1.5 U/ml Epo or 3 nmol/L COS cell-derived mIL-3, or a combination of both growth factors. After incubating for various times, cells were lysed in RNAzol (Canadian Life Technologies, Burlington, Ontario) and the total cellular RNA isolated as recommended by the manufacturer. For Northern blot analyses, 10 μg aliquots of total RNA were separated on 1% agarose, 5% formaldehyde gels and transferred by blotting to (BioRad, Mississauga, ONT). These membranes were then prehybridized in 50% formaldehyde, 0.5 M NaH_2PO_4, 2.5 mM EDTA, 5% SDS and 1 mg/ml BSA at 42°C, and subsequently hybridized under the same conditions with cDNA probes ^{32}P-labeled by the random primer method (Feinberg and Vogelstein, 1984). Probes used for hybridization were: a 295 bp *Sau3A-I -Acc-I* fragment encompassing the first exon and intron of murine ß-major globin gene (provided by Dr. P. LeBoulch, MIT, Boston, MA); a 1.8 kb fragment of GATA-1 cDNA isolated from the pXM-GATA-1 expression vector (provided by Dr. S. Orkin, Howard Hughes Medical Institute, Boston, MA); a 595 bp *BamH-I -Mse I* fragment of the murine EpoR from pXM EpoR(190); a 1.6 kb *Pst-I* fragment of chicken ß-actin cDNA; and a 1.3 kb fragment of rat glyceraldehyde-3-phosphate-dehydrogenase cDNA (GADPH, provided by

Dr.P.Jeanteur, Centre Paul Lamarque, Montpellier, France). After overnight hybridization, membranes were washed twice at 55°C, first with 2 x SSPE, 0.3% SDS (2xSSPE: 0.3 mol/L NaCl, 20 mmol/L NaH_2PO_4, 2 mmol/L EDTA, pH 7), then with 1 x SSPE, 0.5% SDS and finally with 0.3 x SSPE, 1% SDS. The membranes were then exposed to Kodak X-Omat AR film for autoradiography.

2.15. DNA isolation and Southern blot analysis

Bone marrow, spleen and thymic cells were lysed in DNAzol (Canadian Life Technologies, Burlington, Ontario) and genomic DNA isolated as recommended by manufacturer. For Southern blot analyses, DNA was digested with *EcoR I* , which cuts once within the integrated provirus. Fragments were separated on 0.8% agarose gels, denatured by incubation of gels in solution containing 0.4 mol/L NaOH and 1.5 mol/L NaCl and transferred onto Zetaprobe nylon membranes using 20 x SSC (3 mo/L NaCl, 0.3 mol/L Na_3-citrate). These membranes were then prehybridized in 3 x SSC, 5% deionized formamide, 0.5% SDS, 1 nmol/L EDTA, 20 mg/mL skim milk, 50 mg/mL dextran sulfate, 250 µg/mL of denatured salmon sperm DNA at 65°C, and subsequently hybridized under the same conditions with radiolabelled EpoR cDNA. After overnight hybridization, membranes were washed 3 times at 65°C with 0.3 x SSC, 0.1% SDS, 1 mg/mL sodium pyrophosphate.

CHAPTER 3

ERYTHROPOIETIN (EPO) AND INTERLEUKIN-3 INDUCE DISTINCT EVENTS IN ERYTHROPOIETIN RECEPTOR EXPRESSING BA/F3 CELLS[1]

3.1. Abstract

To compare the signal transduction pathways utilized by Epo and IL-3, the cDNA for the murine erythropoietin receptor (EpoR) was introduced into the IL-3 responsive cell lines, Ba/F3 and DA-3, using retrovirally mediated gene transfer. After selection in G-418 and IL-3, clones expressing comparable levels of cell surface EpoR were identified using biotinylated Epo and flow cytometry. A comparison of the effects of Epo and IL-3 on these cells revealed that most EpoR-expressing Ba/F3 clones, when first exposed to Epo, dramatically increased their levels of ß-globin mRNA. The kinetics of appearance of this message, following exposure to Epo varied considerably from clone to clone, with some clones showing a marked increase in ß-globin mRNA within 1 hour while others required several days before an increase was observed. Interestingly, not only was this increase not seen with IL-3 but IL-3 prevented the Epo-induced appearance of ß-globin message. Furthermore, none of the EpoR-expressing DA-3 cell clones tested increased their levels of ß-globin mRNA in response to Epo. While the EpoR DA-3 clones showed identical proliferative responses to IL-3 and Epo, most EpoR Ba/F3 clones displayed a

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[1] The data presented in this chapter have been published in:

Krosl, J., Damen, J. E., Krystal, G., and Humphries, R. K. (1995). Erythropoietin and interleukin-3 induce distinct events in erythropoietin receptor-expressing BA/F3 cells. Blood *85*, 50-56.

marked, al beit transient, proliferative lag when first exposed to Epo. This was manifested as both an increased doubling time in liquid culture and a decreased colony size in methylcellulose. Plating efficiencies of EpoR Ba/F3 cells in methylcellulose, however, were identical in response to IL-3 and Epo, suggesting that the Epo induced lag in proliferation reflected a growth delay of the entire population of cells to Epo rather than a selection of an Epo responsive subpopulation. FACS analysis established that this growth delay was due to a lengthening of the first G1 period following exposure to Epo. Interestingly, this Epo induced delay in entry into the S phase was not detected in cells stimulated with both Epo and IL-3 nor in EpoR Ba/F3 cell clones that did not show an increase in ß-globin mRNA in response to Epo. Thymidine induced growth arrest however, revealed that this alone was not sufficient to stimulate ß-globin mRNA in the absence of Epo. Further studies established that the Epo induced increase in ß-globin mRNA could be inhibited by the tyrosine kinase inhibitor, genistein, and the protein kinase C inhibitor, Compound 3. Taken together, these results confirm that Epo and IL-3 can trigger qualitatively different responses in EpoR-expressing Ba/F3 cells, that the Epo induced increase in ß-globin transcription correlates with a lengthening of the first G1 period following exposure to Epo and that protein phosphorylation events play a critical role in this Epo-induced partial differentiation.

3.2. Introduction

Studies to examine the signal transduction pathways utilized by the activated EpoR have been impeded by low receptor numbers on normal erythroid progenitors and difficulties in obtaining pure Epo responsive erythroid cell populations. However, with the recent isolation of murine and human EpoR cDNAs (D'Andrea et al., 1989; Jones et al., 1990) cells displaying high levels of

both normal and mutant forms of the EpoR have been generated. Since these cells have been primarily IL-3 dependent cell lines, this has made possible both an in depth analysis of the signaling pathways utilized by the EpoR and a comparison with the pathways used by IL-3. In several such studies Epo appeared to be equivalent to IL-3 in supporting cell proliferation (D'Andrea et al., 1991; Damen et al., 1992; Miura et al., 1991; Quelle and Wojchowski, 1991). Both growth factors appeared to induce identical patterns of protein tyrosine phosphorylations suggesting that, at least in these experimental systems, receptors for IL-3 and Epo can utilize the same signal transducing intermediates (Damen et al., 1992; Miura et al., 1991).

Although these studies have suggested considerable overlap in the signaling pathways used by Epo and IL-3, other data point to significant qualitative differences in receptor function. For example, Epo stimulation of EpoR expressing Ba/F3 cells has recently been shown to induce accumulation of ß-globin mRNA (Liboi et al., 1993) and expression of cell surface glycophorins (Jubinsky et al., 1993), suggesting a correlation between EpoR activation and Epo-associated differentiation and thus favoring an instructive model for Epo-mediated differentiation. However, whether the proliferation and differentiation functions of the EpoR can be separated and which of the major signaling pathways is specifically involved in induction of erythroid differentiation remains an open issue.

To further investigate the erythroid differentiation inducing potential of the activated EpoR we used retroviral gene transfer to engineer high level expression of the EpoR in two IL-3 responsive cell lines, Ba/F3 and DA-3, and compared the early effects of Epo and IL-3 on the proliferation and gene expression of these cells. Our results reveal that Epo stimulation of the EpoR-

expressing Ba/F3 cells, but not the EpoR-expressing DA-3 cells, induces a transient growth delay and can stimulate a very rapid onset of ß-globin mRNA accumulation. The EpoR specific differentiation signal appears to be mediated through tyrosine and serine/threonine specific phosphorylation events. Moreover, differentiation signals can be effective under conditions in which proliferation is inhibited; conversely, conditions permissive for proliferation but resulting in suppression of ß-globin mRNA accumulation were identified. Taken together, these results not only provide additional evidence that the EpoR initiates both proliferative and erythroid specific differentiation signals but also suggest, that the differentiation and proliferation signals of the EpoR can be uncoupled.

3.3. Results

3.3.1 Generation of EpoR expressing Ba/F3 and DA-3 cells

Ba/F3 and DA-3 cells were retrovirally infected with a JZenTKneo vector carrying the murine EpoR cDNA and various EpoR expressing clones were selected and maintained in G-418 supplemented medium containing IL-3. A number of Ba/F3 and DA-3 cell clones expressing comparable levels of cell surface EpoR were then identified by flow cytometry using biotin-labeled Epo and these were designated EpoR Ba/F3 and EpoR DA-3 cells, respectively. Importantly, these cell clones were never exposed to Epo during the selection or maintenance phases of this study.

3.3.2. Effect of Epo on erythroid differentiation

To determine if Epo stimulation of our EpoR Ba/F3 and DA-3 cells was associated with induction of erythroid specific genes, several independent clones were examined for ß-globin and GATA-1 mRNA expression by Northern blot analysis of total cellular RNA. The EpoR DA-3 cells expressed low levels of GATA-1, but no ß-globin mRNA under any experimental conditions (data not shown). In contrast, low but detectable levels of ß-globin mRNA were found in all EpoR Ba/F3 clones grown in IL-3 (Fig. 3.1), and most of these clones responded to Epo stimulation with a sharp increase in the levels of this message.

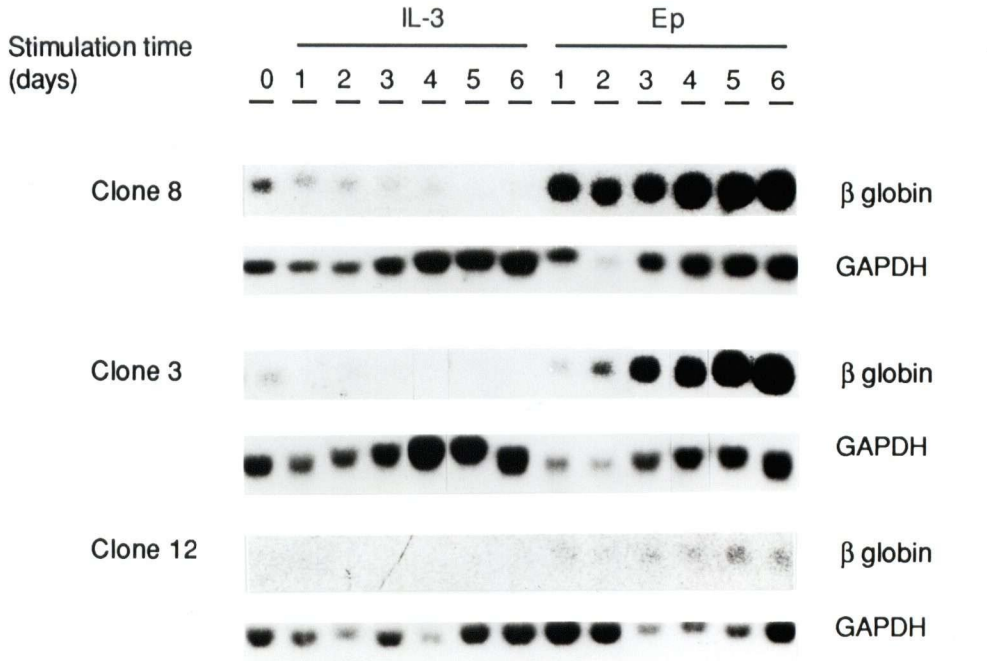

Figure 3.1. Northern blot analysis of β-globin mRNA levels after exposure of various EpoR-expressing Ba/F3 clones to Epo or IL-3.
Growth factor deprived cells were stimulated with Epo (1.5 Units/mL) or IL-3 (3 nmol/L) in RPMI containing 10% FCS for the indicated periods of time. Shown are representative results from clones responding within 1 day (clone 8) or 3-4 days (clone 3) or unresponsive after up to 6 days' exposure (clone 12). Rows 1, 3 and 5, hybridization to β-globin probe; rows 2, 4 and 6, hybridization to GAPDH probe.

The time of appearance of this increase varied substantially from one clone to another. For example, high levels of ß-globin message were first detected after 3 to 4 days continuous growth in Epo for some clones (e.g. clone 3, Fig 3.1), analogous to the delayed response in clones described by Liboi et al (Liboi et al., 1993). Clones with a much more rapid response were however also observed, with high levels of ß-globin message detected within only 1 day of Epo stimulation (e.g. clone 8, Fig 3.1), and in early passages of another clone (clone 3, Fig 3.1) a marked increase in ß-globin mRNA could be detected within 1 hr of Epo stimulation. In 2 of 9 clones analyzed, no significant increase in ß-globin mRNA levels was observed during up to 6 days of Epo supported proliferation (e.g. clone 12, Fig. 3.1). With continuous passages in IL-3, all clones inducible for ß-globin mRNA showed a gradual increase in basal levels of this message. Consequently, all our further studies were performed using only early passages of EpoR-expressing Ba/F3 cells.

Since no induction of ß-globin mRNA was detected in any of the EpoR-Ba/F3 cell clones stimulated with IL-3, we examined ß-globin mRNA levels following costimulation with IL-3 and Epo and found that the combination of these two growth factors suppressed the Epo induced increase in ß-globin mRNA (Fig. 3.2, representative result). This inhibitory effect of IL-3 on Epo induced accumulation of ß-globin mRNA was observed with all the Epo inducible clones and no increase in ß-globin mRNA levels was ever detected during the standard 6 days of costimulation (data not presented).

Epo stimulation of EpoR Ba/F3 cells, following the standard 12 hr of starvation, was also associated with a rapid and persistent elevation in GATA-1 mRNA levels (Fig. 3.2). This rapid increase was suppressed when cells were stimulated with both IL-3 and Epo. However, it is important to note that GATA-1

mRNA levels were high when these cells were exponentially growing in IL-3, prior to growth factor deprivation, and by 96 hr of continuous growth factor stimulated proliferation, the levels of this message returned to this pre-stimulation level, regardless of whether they were grown in IL-3, Epo or IL-3 plus Epo.

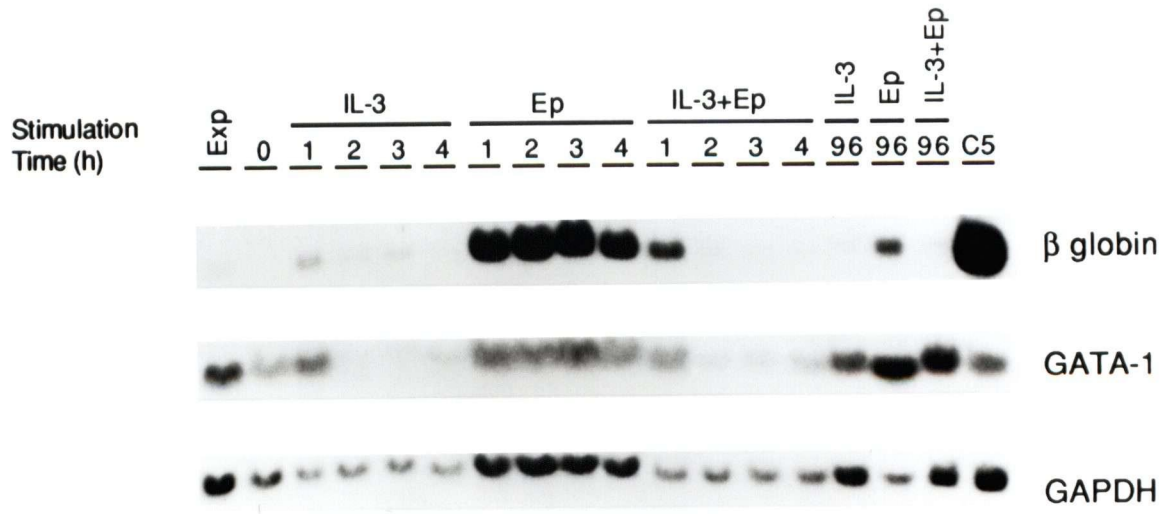

Figure 3.2. Northern blot analysis of β-globin mRNA levels in EpoR-expressing Ba/F3 cells (clone-1, early passage) stimulated with IL-3, Epo or IL-3 plus Epo. Growth factor deprived cells were stimulated with 1.5 Units of Epo/mL, or 3 nmol/L of IL-3 of combination of both growth factors for the indicated periods of time. The sample in the last lane was obtained from our previously described EpoR+ Ba/F3 clone, C5, grown continuously in Epo (0.5 Units/mL) since its isolation. Hybridization probes are listed on the right.

3.3.3. Epo vs IL-3 induced proliferative responses of EpoR expressing Ba/F3 and DA-3 cells

To determine whether the Epo induced increases in ß-globin and GATA-1 mRNA were correlated with Epo induced proliferation, Epo stimulated EpoR Ba/F3 and EpoR DA-3 were counted over a 6 day incubation period and growth kinetics compared to the same cells grown in IL-3 or in the presence of a combination of IL-3 plus Epo (Fig 3.3). For EpoR DA-3 cells, the growth curves were identical under all conditions (Fig 3.3.A). In contrast, EpoR Ba/F3 clones that responded to Epo stimulation with a rapid increase in ß-globin mRNA, e.g. clone 8, grew more slowly in Epo than in IL-3 or in IL-3 plus Epo during the first 24 hours of culture (Fig 3.3.B). As a consequence, by 48 hr, with Epo stimulation alone, cell numbers were increased only 4.4 ± 0.3 fold over starting cell

numbers compared to 8.8 ± 0.3 fold observed with IL-3 stimulation (Fig. 3.3 C, mean ± SE, five separate experiments; difference significant by Student t-test , $p<0.005$). This reduction in growth rate was transient and, by 72 hr, cell doubling times in response to Epo were equivalent to those for IL-3 or IL-3 plus Epo. In contrast, EpoR Ba/F3 clones that did not show an increase in ß-globin mRNA in response to Epo or that increased the level of this message only after a delay of three to four days displayed no or only a modest growth delay respectively (Fig. 3C, clone 3).

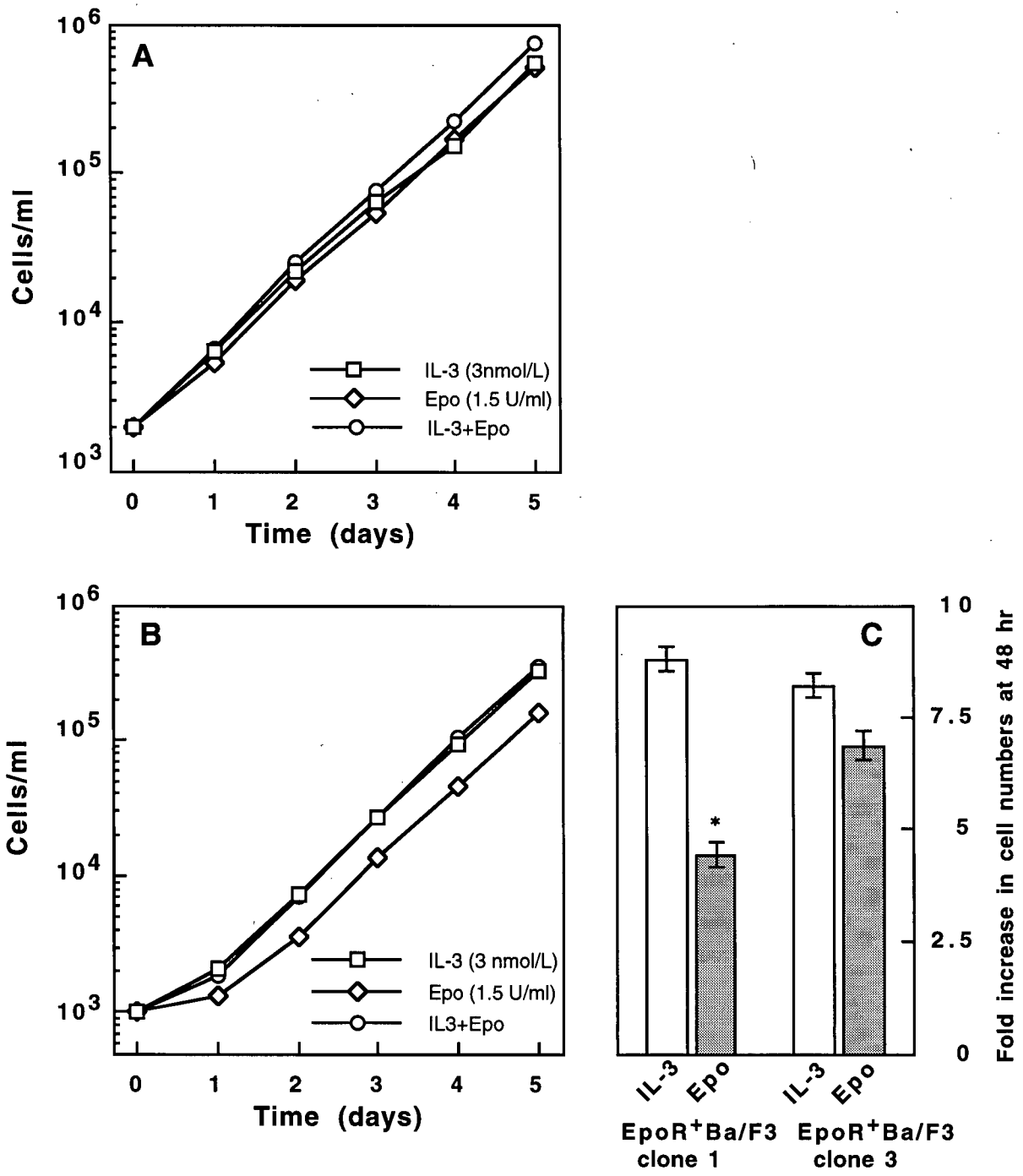

Figure 3.3. Proliferative responses of EpoR DA-3 and EpoR Ba/F3 cells to IL-3 and Epo.
Growth factor deprived cells were restimulated with either 1.5 U/ml Epo, 3 nmol/L IL-3 or a combination of both growth factors and cell numbers were determined daily. Shown are mean values of two separate experiments for each time point. A) Growth curves of EpoR DA-3 cells; B) Growth curves of EpoR Ba/F3 cells, clone 1; C) Fold increase over input of EpoR Ba/F3 cells, clone 1 and clone 3, after 2 days of stimulation with IL-3 or Epo. Shown are mean values ± SE of five independent experiments.

To determine if the Epo induced lag in proliferation of EpoR Ba/F3 cells reflected a decrease in the number of proliferating cells, due to selection of an Epo responsive subpopulation, or to a growth delay of the entire cell population, EpoR Ba/F3 cells were plated in methylcellulose with Epo or IL-3. The number of colonies which developed in response to Epo and IL-3 were found to be similar, with an average plating efficiency of 43 ± 3% for IL-3 and 41 ± 3% for Epo. However, the colonies grown in Epo contained 30 to 40% fewer cells than those formed in the presence of IL-3 suggesting that the Epo induced growth delay of EpoR Ba/F3 cells resulted from a lag in proliferation of all the cells present in the population.

To further characterize the apparent Epo-induced growth delay we examined the progression of growth factor deprived EpoR Ba/F3 cells through the cell cycle upon stimulation with IL-3 or Epo (Fig. 3.4). The results indicate that exposure to Epo leads to a marked delay in G_1 to S transition of the cell cycle. The onset of DNA synthesis was detected within 6-7 hr for the IL-3 stimulated cells (Fig. 3.4 A), whereas no increase in the proportion of cells in S phase could be detected until 13 hrs of Epo stimulation (Fig. 3.4 B). Moreover, by 16 hr of incubation with Epo, 60% of the cells were in G_1 while only 30% of those grown in IL-3 were in this stage, suggesting that growth delay observed with EpoR Ba/F3 upon first exposure to Epo is due to a slower progression through G1. These observations were confirmed by ^{3}H-Tdr incorporation assays. Cells exposed to Epo for up to 16 hr incorporated 50% less ^{3}H-Tdr than those incubated with IL-3. Within 26-28 hr, however, the Epo stimulated cells resumed their cycling activity and the levels of Epo stimulated ^{3}H-Tdr incorporation approached those determined in response to IL-3 (data not shown).

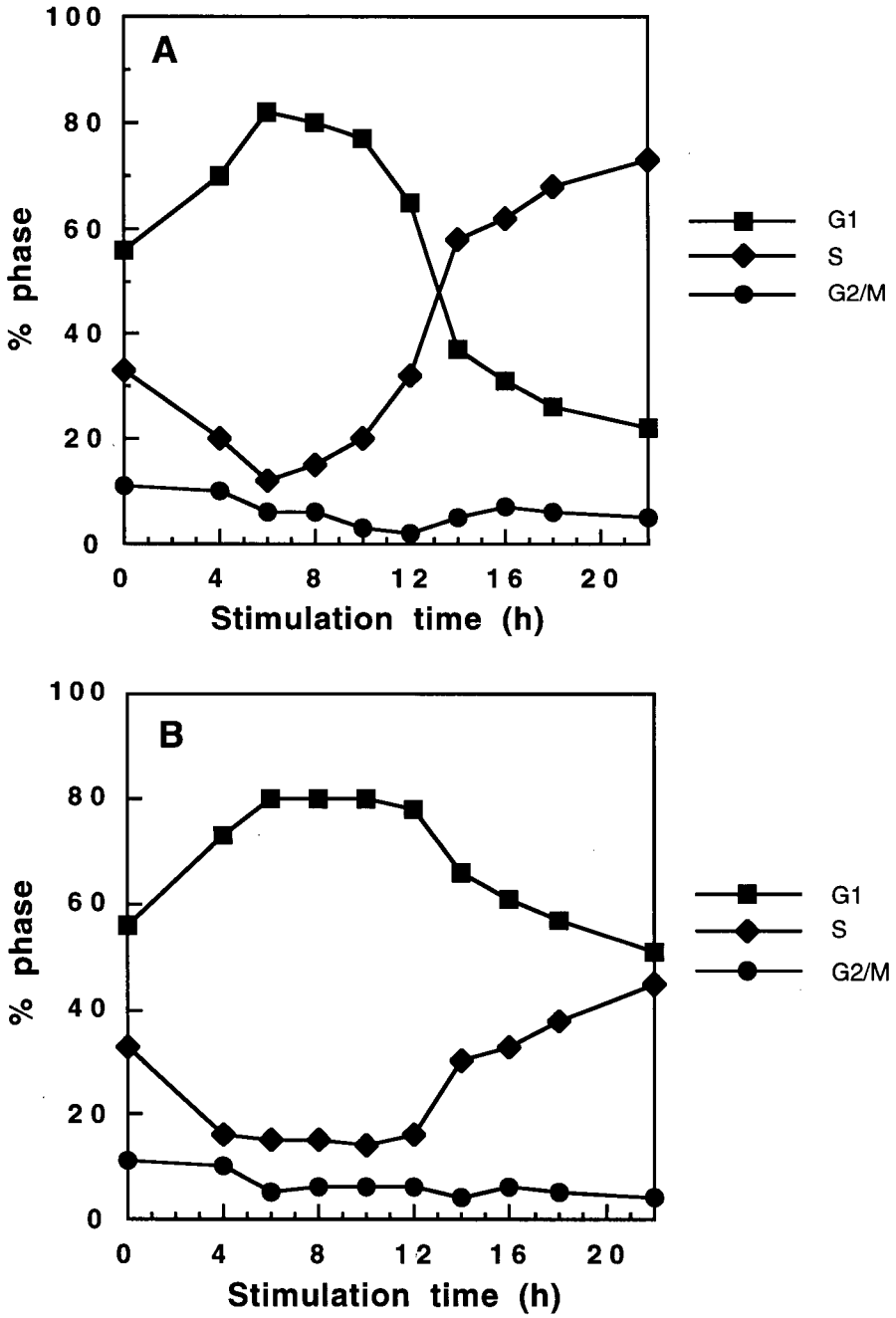

Figure 3.4. Cell cycle analysis of IL-3- and Epo- stimulated EpoR Ba/F3 cells (clone 8).
Logarithmically growing cells were deprived of IL-3 for 12 hours in RPMI containing 0.2% BSA and then stimulated with 3 nmol/L IL-3 (A) or 1.5 U Epo/ml (B). At the indicated times after stimulation, cells were stained with propidium iodide and analyzed by flow cytometry.

3.3.4. Effect of signal pathway modulators on Epo induced accumulation of ß-globin m RNA

To gain some insight into the intracellular mechanisms involved in Epo induced accumulation of ß-globin mRNA, we examined the effects of various agents on IL-3 and Epo stimulated EpoR Ba/F3 cells. These studies were carried out with a clone of these cells (clone 8) that showed a rapid response to Epo stimulation with ß-globin mRNA accumulation reaching maximal levels within 1 day, thus allowing short exposure times to the various agents and minimizing their possible toxic effects. When these cells, which had never been exposed to Epo, were simply starved in RPMI plus 10% FCS for 24 hours, only approximately 30% remained viable based on eosin red exclusion (Table 3.1) and were predominantly in the G1 phase of cell cycle (data not shown). However, recovered cells expressed high levels of ß-globin mRNA (Fig 3.5 and Table 3.1). This is consistent with a growing body of data suggesting that certain cells can be induced to differentiate if they are delayed in their progression through G1 (Fairbairn et al., 1993; Johnson et al., 1993; Kiyokawa et al., 1993). As can be seen in Table 3.1, thymidine effectively blocked both IL-3 and Epo induced proliferation to the same extent with minimal or no reduction in cell viability. However, prevention of entry into S is obviously not sufficient to induce the partial differentiation since no increase in ß-globin mRNA was observed when cells were exposed to thymidine in the presence of IL-3. However, a marked increase in levels of this message was seen in the presence of Epo.

We also tested the tyrosine kinase inhibitor, genistein (Akiyama et al., 1987) since growth factor induced entry in S phase in general and Epo induced proliferation in particular (Miura et al., 1991; Spivak et al., 1992) has been shown to be dependent upon tyrosine phosphorylation events. Interestingly, this

tyrosine kinase inhibitor not only inhibited proliferation, as expected, but markedly reduced Epo induced ß-globin mRNA accumulation as well. Thus, even though genistein delayed entry into S and thus might be expected to enhance ß-globin levels, reduced levels were observed, suggesting that tyrosine phosphorylation events are critical for this differentiation step (Fig 3.5). Related to this, orthovanadate, a known inhibitor of tyrosine phosphatases, was added to either Epo or IL-3 stimulated cells to see if enhancing tyrosine phosphorylation levels in these cells might have an effect on ß-globin induction. However, ß-globin mRNA was neither induced by this agent in the presence of IL-3 nor further increased in the presence of Epo.

Table 3.1. Modulation of Epo-induced accumulation of β-globin mRNA in EpoR Ba/F3 cells.

Growth factor	Modifier	Viability	Proliferation	β-globin mRNA
IL-3	None	91± 5	100	±
	Genistein	74 ± 3	5 ± 2	±
	Thymidine	84 ± 2	7 ± 3	±
	Orthovanadate	83 ± 2	67 ± 5	±
	Compound 3	84 ± 3	90 ± 3	±
	TPA	83 ± 2	65 ± 3	±
	IBMX	85 ± 5	46 ± 3	±
	DMSO	89 ± 4	42 ± 5	±
Epo	None	89 ± 3	100	++++
	Genistein	69 ± 2	6 ± 3	±
	Thymidine	79 ± 1	7 ± 2	++++
	Orthovanadate	87 ± 2	65 ± 4	++++
	Compound 3	82 ± 2	85 ± 4	+
	TPA	77 ± 5	57 ± 5	+
	IBMX	80 ± 4	52 ± 1	++++
	DMSO	86 ± 2	52 ± 1	±
None	None	29 ± 7	3 ± 1	+++++

Data are from early passage of EpoR Ba/F3 cells clone 8. Growth factor-deprived cells were incubated for 20 minutes at 37°C in complete growth medium containing the agents indicated and were then stimulated with IL-3 (3 nmol/L) or Epo (1 U/mL) for 20 hours. Control cultures were incubated for the same period of time in the absence of growth factors. Viability was determined by eosin red exclusion and proliferation by ^{3}H-Tdr incorporation. Levels of β-globin mRNA were assessed by Northern blot analysis. All values represent mean ± SE of three experiments. Abbreviations: ±, basal level; ++++, approximately 8- to 10-fold higher levels, as determined by densitometric analysis.

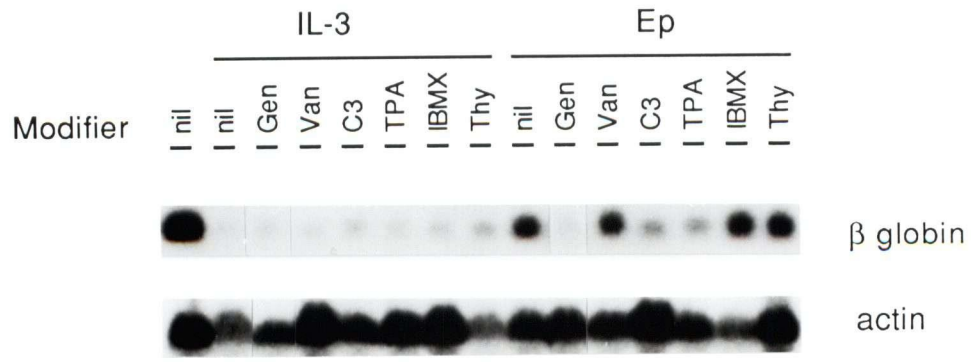

Figure 3.5. Northern blot analyses of β-globin mRNA levels in EpoR Ba/F3 cells (clone 8), stimulated with IL-3 or Epo in the presence of modifiers.
Growth factor deprived cells were incubated for 20 minutes at 37^0C in complete growth medium containing the agent indicated and then stimulated with 3 nmol/L IL-3 or 1 unit of Epo/mL for 20 hours. The lanes are labeled with reactant conditions: nil, cells deprived of growth factors for 24 hours; nil, IL-3, no modifier; nil, Epo, no modifier; Gen, Genistein (5 μg/mL); Van, orthovanadate (20 μmol/L); C3, compound 3 (0.3 μmol/L); TPA (5 ng/mL); IBMX (0.5 mmol/L); Thy, thymidine (100 μmol/L).

The highly specific inhibitor of protein kinase C, Compound 3 (Davis et al., 1989; Toullec et al., 1991) was also tested and found to have no effect on Epo-induced proliferation but, like genistein, strongly suppressed ß-globin mRNA induction thus implicating serine/threonine specific phosphorylations as additionally important in this Epo-induced differentiation event. Consistent with this finding, the phorbol ester, TPA, which exhausts protein kinase C when added to cells for the time period used here, also inhibited Epo induced ß-globin mRNA accumulation.

Interestingly, dimethylsulfoxide (DMSO), an inducer of erythroid differentiation for MEL cells (Terada et al., 1977) and human MB-07 cells (Morgan et al., 1991) inhibited both Epo-induced proliferation and ß-globin

mRNA accumulation, whereas increasing levels of cAMP by IBMX suppressed only proliferation without significantly affecting ß-globin mRNA levels.

3.4. Discussion

In the present study we compared the effects of Epo and IL-3 on the proliferation and differentiation of two hemopoietic cell lines, Ba/F3 and DA-3, that we retrovirally infected with the murine EpoR cDNA. Although we found a number of EpoR-expressing Ba/F3 cell clones that responded quite differently to these two cytokines, none of the EpoR-expressing DA-3 cell clones studied showed any qualitative difference in response to Epo and IL-3 and proliferated equally well in response to these two growth factors, which is consistent with observations reported by Miura et al (1991). Interestingly, although Ba/F3 cells were originally described as pro-B cells, based on their low level of B220 expression (Palacios and Steinmetz, 1985) it is likely that they are actually more erythroid or have evolved into a more erythroid cell type, since they express GATA-1 and NF-E2 (Liboi et al., 1993).

Our characterization of several EpoR Ba/F3 cell clones that increased their level of ß-globin mRNA as rapidly as within 1 hr in response to Epo, but not to IL-3, confirm and extend findings of Liboi et al (1993) and Jubinsky et al (Jubinsky et al., 1993) who observed EpoR mediated induction of ß-globin mRNA (Liboi et al., 1993) or glycophorin (Jubinsky et al., 1993) after relatively prolonged exposure of engineered cell lines to Epo. More recently, Maruyama et al (1994) electroporated a chimeric receptor containing the extracellular region of the EGF receptor and the cytoplasmic domain of the EpoR into both the Epo-responsive MEL cell line, TSA8 and Ba/F3 cells and found that EGF stimulated globin synthesis in the former but not the latter. This is not necessarily inconsistent with our results nor with those of Liboi et al since

neither of us have observed any hemoglobin synthesis (as judged by benzidine staining) in any of our EpoR Ba/F3 cell clones (data not shown and Liboi et al., 1993).

One of our most intriguing findings is the correlation between rapid Epo induced differentiation, as assessed by ß-globin mRNA levels, and the delay in Epo stimulated progression through the G1 phase of the cell cycle. Evidence that this lengthening of the first G1 period, following exposure to Epo, is important in inducing ß-globin message comes from our finding that incubation of the same cell clones with IL-3 plus Epo eliminates both the delay through G1 and the increase in globin mRNA (Fig. 3B). This prolongation of G1, which could occur mechanistically through a delay in the formation of active CDK/cyclin complexes (Ewen et al., 1993; Geng and Weinberg, 1993; Koff et al., 1993) may simply be triggering a predetermined erythroid differentiation program, as seems to be the case for DMSO (Terada et al., 1977) or hexamethylenebisacetamide (Kiyokawa et al., 1993) induced differentiation of MEL cells, as well as in MEL cells engineered to express high levels of p53 (Johnson et al., 1993). However, this is unlikely in our experimental system since only EpoR-expressing and not parental Ba/F3 cells increased their ß-globin mRNA levels in response to starvation induced growth arrest. It is possible that under these conditions the EpoR cells were triggered by low levels of Epo present in the FCS used for our studies or that the high level of EpoR expression on these cells (approximately 3,000/cell based on Scatchard analysis) led to a low level of spontaneous dimerization. The fact that EpoR cells stimulated with IL-3 do not accumulate ß-globin mRNA and our finding that growth arrest alone, as induced by thymidine addition, was not sufficient in the absence of Epo to stimulate this accumulation further point to an active role of EpoR mediated stimulation in triggering erythroid differentiation events.

Together these data add to evidence that the EpoR plays an instructive rather than just a permissive role in Epo-stimulated differentiation. A similar conclusion was reached by Liboi et al (1993), Jubinsky et al (1993) and Maruyama et al (1994) and work by the latter two groups suggests that only the cytoplasmic portion of the EpoR is required to transmit this differentiation signal. Indeed, studies by Maruyama et al (1994), using a truncated EGF/EpoR chimera, suggest that only the membrane proximal 127 amino acids are required for differentiation. Our results with genistein, Compound 3 and TPA indicate that protein phosphorylation plays an essential role in this phenomenon. These results indicate that the EpoR specific signaling events which lead to the accumulation of ß-globin mRNA in permissive cells involves both tyrosine and serine/threonine specific phosphorylation events and suggest that the differentiation function of the EpoR can be uncoupled from its proliferative function.

The dominant negative effect of IL-3 on Epo induced accumulation of ß-globin mRNA that we observe is reminiscent of the work of Fukunaga et al (1993) in which they demonstrated that the G-CSF induced expression of myeloperoxidase in FDC-P1 cells expressing the G-CSF receptor cDNA was inhibited when these cells were costimulated with IL-3 and G-CSF. It is conceivable that in both these FDCP-1 cells and in our EpoR Ba/F3 cells, IL-3 dominates over these more lineage specific cytokines because the activated IL-3R has a higher affinity for certain common signal transduction intermediates, as has been suggested by previous studies in our laboratory (Damen et al., 1992). Alternatively, IL-3R activated intermediates (e.g., kinases) involved in mediating entry into S phase may override (e.g., inactivate) the Epo or G-CSF activated intermediates involved in G1 prolongation and turning on of

differentiation related genes via post translational modification (e.g., phosphorylation).

Although there is a steadily growing body of data concerning the Epo induced signaling pathways involved in promoting cell proliferation, little is known about the EpoR generated signals involved in inducing erythroid specific differentiation. Our data are consistent with an instructive role for the EpoR in erythroid differentiation and suggest that this differentiation process is contingent upon a prolongation of the G1 phase of the cell cycle and both tyrosine and serine/threonine specific phosphorylation events.

CHAPTER 4

INTERLEUKIN-3 (IL-3) INHIBITS ERYTHROPOIETIN-INDUCED DIFFERENTIATION IN Ba/F3 CELLS VIA THE IL-3 RECEPTOR α SUBUNIT[1]

4.1. Abstract

Introduction of erythropoietin receptors into the interleukin-3 (IL-3)-dependent murine hemopoietic cell line, Ba/F3, enables these cells to not only proliferate, after an initial lag in G1, but also to increase β-globin mRNA levels in response to Epo. With IL-3 and Epo costimulation, IL-3-induced signaling appears to be dominant since no increase in globin mRNA occurs. Differentiation and proliferation signals may be uncoupled since EpoRs lacking all eight intracellular tyrosines were compromised in proliferative signaling but retained erythroid differentiation ability. Intriguingly, a chimeric receptor of the extracellular domain of the EpoR and the transmembrane and intracellular domains of IL-3Rβ IL-3 chain (EpoR/IL-3Rβ IL-3) was capable of Epo-induced proliferative and differentiating signaling, suggesting either the existence of a second EpoR subunit responsible for differentiation or that the α subunit of the IL-3 receptor (IL-3R) prevents it. Arguing against the former, a truncated EpoR

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[1] The data presented in this chapter have been published in:

Krosl, J., Damen, J. E., Krystal, G., and Humphries, R. K. (1996). Interleukin-3 (IL-3) inhibits erythropoietin-induced differentiation in Ba/F3 cells via the IL-3 receptor α subunit. J. Biol. Chem. *271*, 27432-27437.

Damen, J. E., Wakao, H., Miyajima, A., Krosl, J., Humphries, R. K., Cutler, R. L., and Krystal, G. (1995). Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and stat5 activation. EMBO J *14*, 5557-5568.

lacking an intracellular domain was incapable of promoting proliferation or differentiation. An EpoR/IL-3Rα chimera, in contrast, was capable of transmitting a weak Epo-induced proliferative signal but failed to stimulate accumulation of β globin mRNA. Most significantly, coexpression of the EpoR/IL-3Rα chimera with either EpoR/IL-3Rβ or wild-type EpoRs suppressed Epo-induced β globin mRNA accumulation. Taken together, these results suggest an active role for the IL-3Rα subunit in inhibiting EpoR-specific differentiating signals.

4.2. Introduction

EpoR belongs to the hemopoietin receptor superfamily and does not possess intrinsic tyrosine kinase activity (Bazan, 1990). Nonetheless, within minutes of binding Epo, the EpoR and several intracellular proteins become tyrosine phosphorylated (Damen et al., 1992; Dusanter-Fourt et al., 1992; Quelle and Wojchowski, 1991) through the action of an EpoR-associated tyrosine kinase, Jak2 (Witthun et al., 1993). These Epo-induced tyrosine phosphorylations have been shown to correlate with both the expression of immediate-early response genes, such as c-jun and c-fos, and with mitogenesis (Miura et al., 1993). Moreover, tyrosine phosphorylation of the EpoR itself appears to be critical for activation of Stat5 and initiation of Epo-induced proliferation at physiological concentrations of Epo (Damen et al., 1995).

In addition to its role in stimulating proliferation, Epo may have roles in preventing apoptosis (Koury and Bondurant, 1990; Wickrema et al., 1992) and in stimulating erythroid differentiation (Bondurant et al., 1985; Koury et al., 1986; Minegishi et al., 1994). As described in Chapter 3 and reported by other groups (Carroll et al., 1994; Carroll et al., 1995; Krosl et al., 1995; Liboi et al., 1993) Ba/F3 cells engineered to express the EpoR rapidly accumulate β-globin mRNA upon exposure to Epo. Interestingly, the tyrosine kinase inhibitor genistein

blocks both Epo-induced proliferation and β–globin mRNA accumulation in this model system, whereas inhibition of protein kinase C by Compound 3 suppresses only Epo-induced differentiation without affecting proliferation (Chapter 2.). These observations suggest that protein phosphorylation events play a critical role in Epo-induced differentiation and that the proliferative and differentiating functions of the EpoR can be uncoupled.

As pointed out in Chapter 1., both the extra- and intracellular domains of the EpoR have been implicated in Epo-induced differentiation. To examine in more detail the regions within the EpoR that might be responsible for eliciting differentiation-specific signals, we have monitored the effects of various mutant and chimeric EpoRs on the induction of β-globin mRNA accumulation in Ba/F3 cells.

4.3. Results

To examine the regions within the EpoR that might be inducing β-globin mRNA, we engineered retroviral vectors carrying coding regions for various mutant and chimeric EpoRs that could be compared with the WT EpoR. The forms of the various receptors studied are illustrated in Fig. 2.1. These included a full-length EpoR in which all eight intracellular tyrosines were exchanged for phenylalanines (null EpoR) (Damen et al., 1995), two chimeric EpoRs containing the extracellular domain of the EpoR and the transmembrane and cytoplasmic domains of the IL-3Rβ or IL-3Rα subunit (termed EpoR/IL-3Rβ or EpoR/IL-3Rα, respectively), and a C-terminal truncated EpoR (EpoR($_{-230}$)) in which the entire cytoplasmic domain was replaced with two primer-derived arginine residues. Following retroviral infection, IL-3-responsive Ba/F3 cellsexpressing the various EpoR forms were then selected for assessment of Epo-stimulated proliferation and differentiation.

4.3.1. EpoR tyrosine phosphorylation is not required for EpoR-mediated β-globin gene induction in Ba/F3 cells

To determine the importance of EpoR tyrosine phosphorylation to Epo-induced β-globin gene expression, we tested the mutant EpoR in which all eight cytoplasmic tyrosines were substituted with phenylalanines (null EpoR). Several independent Ba/F3 clones were obtained following retroviral mediated gene transfer and assessed for expression of cell surface null EpoRs by both flow cytometry using biotin-labeled Epo and by Scatchard analysis using ^{125}I-labeled Epo. Following selection of clones expressing similar numbers of cell surface WT or null EpoRs (approximately 3000/cell, as determined by Scatchard analysis), the Epo-induced proliferation and induction of β-globin message were compared. Null EpoR-expressing Ba/F3 cells proliferated in response to Epo as determined by 3H-Tdr incorporation assays but required approximately 5-fold higher concentrations of Epo to achieve levels of 3H-Tdr incorporation comparable with those obtained with WT EpoR-expressing cells (Fig. 4.1.A). The null and WT EpoR-expressing cells accumulated comparable levels of β-globin mRNA upon stimulation with Epo, and for both, no induction of β-globin mRNA could be detected in response to IL-3 or to IL-3 plus Epo (Fig. 4.1.B, representative Northern blot analysis). Interestingly, this Epo-induced differentiation response could be detected in both cell types at concentrations of Epo that stimulated proliferation of WT EpoR cells but were markedly less effective in promoting proliferation of null EpoR cells. Moreover, cell cycle analyses revealed that exposure of null EpoR cells to Epo leads to a marked delay in the transition of G1 phase of cell cycle compared that determined for the WT EpoR cells (Fig 4.1.C), which is consistent with recently published observations that Epo-induced differentiation of Ba/F3 cells can occur in the

absence of proliferation (Carroll et al., 1995; Krosl et al., 1995). Our results further suggest that tyrosine phosphorylation of the EpoR itself is not required for induction of β-globin mRNA.

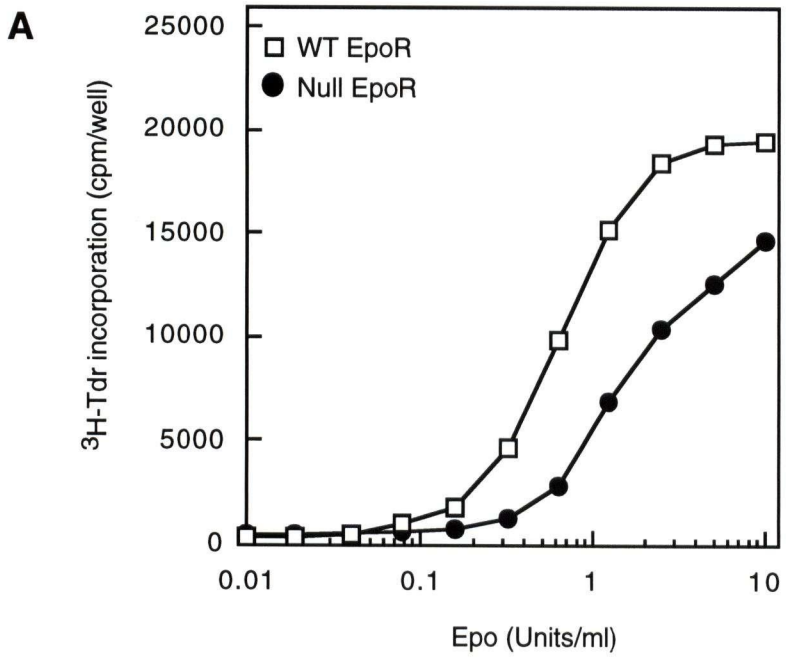

Figure 4.1.A., Epo-induced proliferative responses of WT and null EpoR-expressing Ba/F3 cells.
Growth factor-deprived cells were resuspended in RPMI 1640 supplemented with 0.1% BSA and various concentrations of Epo. 3H-Tdr incorporation assays were performed as described under ``Materials and Methods.'' The graph shows results representative of five independent experiments.

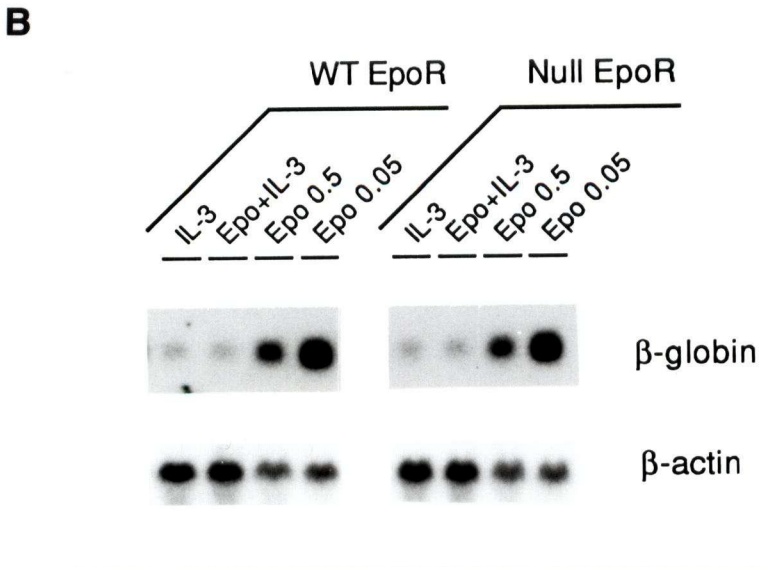

Figure 4.1.B., Northern blot analysis of β-globin mRNA levels in WT and null EpoR-expressing cells.
Growth factor-deprived cells were incubated for 20 h in RPMI 1640 supplemented with 0.1% BSA in the presence of 3 nmol/liter IL-3 or IL-3 and 0.5 unit of Epo/ml or Epo alone (0.5 or 0.05 unit/ml). Lanes contained approximately 10 μg of total cellular RNA. Hybridization probes are listed on the right.

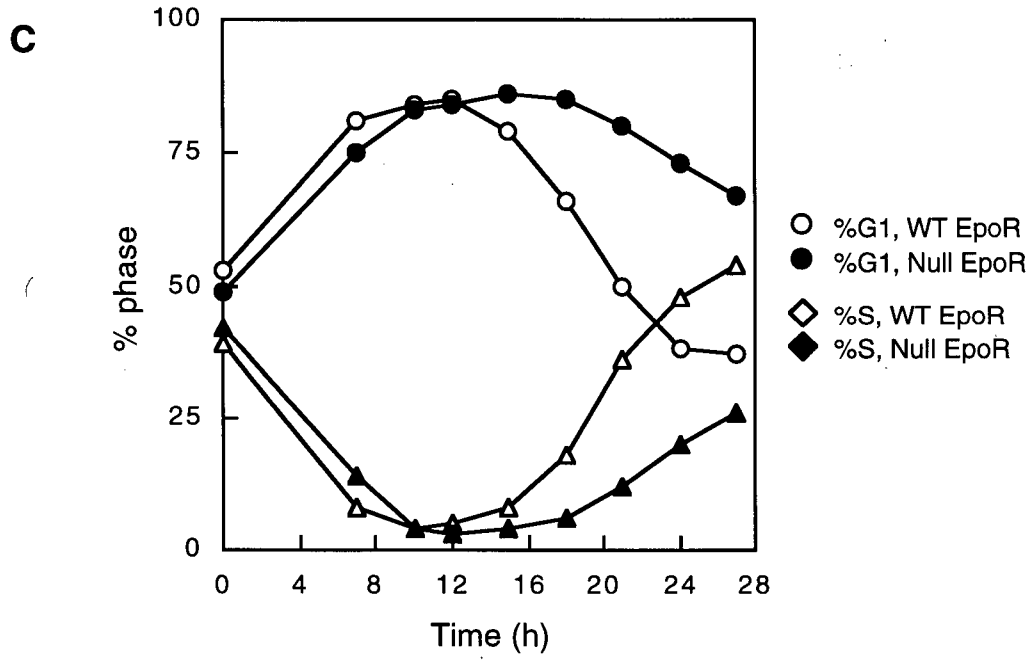

Figure 4.1.C. Cell cycle analyses of Epo stimulated- null EpoR and WT EpoR Ba/F3 cells.
Cells were deprived of IL-3 for 12 hours in RPMI containing 0.2% BSA and then stimulated with 0.5 Units of Epo/mL. At indicated times after stimulation, cells were stained with propidium iodide and analyzed by flow cytometry.

4.3.2 The intracellular domain of the IL-3Rβ$_{IL-3}$ subunit induces β-globin gene expression

IL-3 was previously reported to inhibit the Epo-induced increase in β globin mRNA seen in Ba/F3 cells engineered to express the normal, wild-type EpoR (Chapter 3. and Carroll et al., 1995). To identify domains within the EpoR and/or the IL-3R that might be involved in regulating β-globin mRNA induction in this model system, we first examined a chimeric receptor (EpoR/IL-3Rβ), consisting of the extracellular domain of the EpoR and the transmembrane and cytoplasmic domains of the major signal transducing component of the IL-3R complex, the IL-3Rβ$_{IL-3}$ subunit. Ba/F3 cells engineered to express this chimeric receptor proliferated as well as cells expressing similar numbers of WT EpoRs

(3000/cell as determined by Scatchard analysis) in response to Epo (Fig. 4.2.A); moreover, the β-globin message was induced in these cells in response to Epo (Fig. 4.2.B) but not in response to IL-3 or IL-3 plus Epo, consistent with results obtained by other groups (Carroll et al., 1995; Chiba et al., 1993).

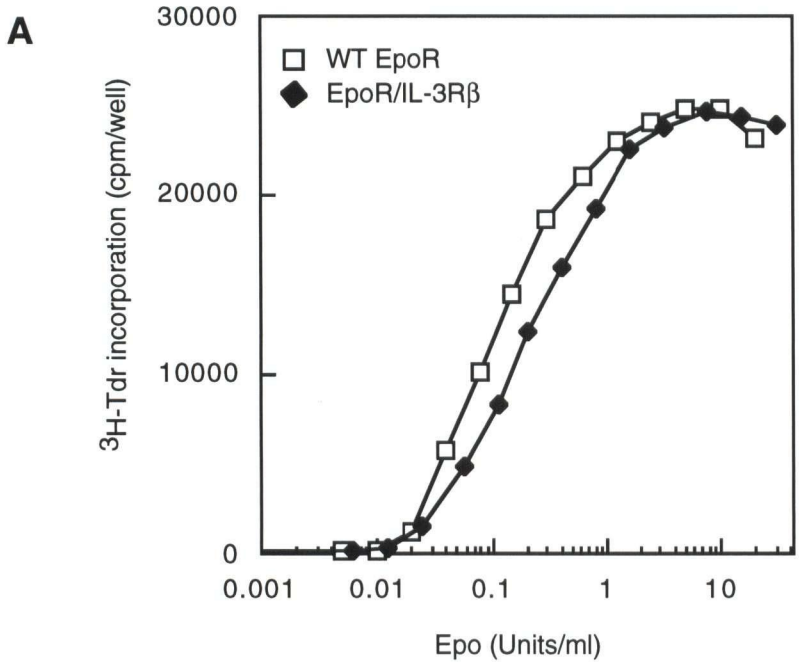

Figure 4.2.A. Epo-induced proliferative responses of EpoR/IL-3Rβ_{IL-3} expressing Ba/F3 cells.
Levels of Epo-stimulated 3H-Tdr incorporation were determined as described for Fig. 2A.

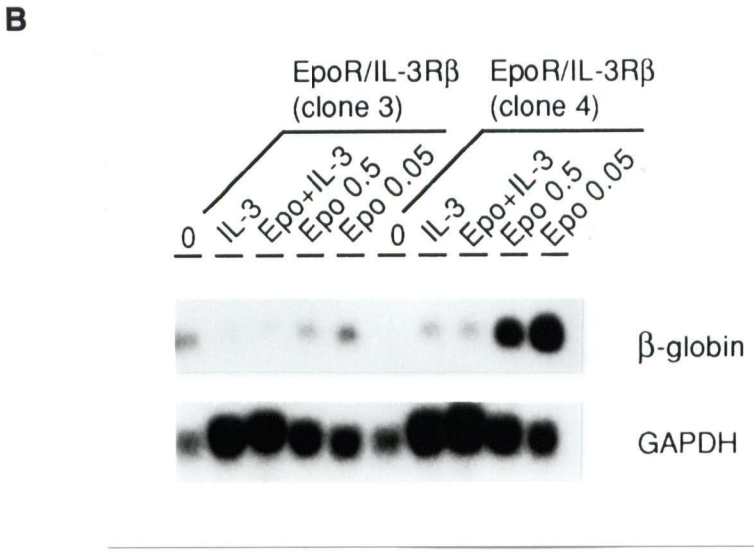

Figure 4.2.B. The Epo-induced accumulation of β-globin mRNA by cells of two representative Ba/F3 clones expressing EpoR/IL-3Rβ_{IL-3} chimera.
Growth factor-deprived cells were incubated for 20 h in RPMI 1640 supplemented with 0.1% BSA in the absence of growth factors (lane 0) or in the presence of 3 nmol/liter of IL-3 or IL-3 and 0.5 unit of Epo/ml or Epo alone (0.5 or 0.05 unit/ml). Lanes contained approximately 10 μg of total cellular RNA. Hybridization probes are listed on the right. Clone 4 was subsequently used to assess the effect of coexpressing the EpoR/IL-3Rα (see Fig. 6). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

One possibility suggested by these observations is that a second as yet unidentified subunit associates with the extracellular domain of the EpoR and provides the differentiating signal. Another intriguing possibility is that the cytoplasmic domain of the EpoR/IL-3Rβ_{IL-3} subunit on its own is permissive for differentiation, but signaling through the intact IL-3R suppresses differentiation, thus pointing to a specific inhibitory role for the α subunit of the IL-3R. To discriminate between these two possibilities, we tested a C-terminal truncated EpoR (EpoR$_{(-230)}$) possessing only 2 amino acids within the cytoplasmic domain (Fig. 2.1). The differentiating and proliferative capacity of this truncated EpoR was examined in several independent EpoR$_{(-230)}$-transduced Ba/F3 clones, expressing between 8000 and 12,000 cell surface EpoRs as determined Scatchard analyses. Viability of these cells decreased in Epo-supplemented medium within 24 hr to approximately 25-30%, and no viable cells could be detected by 48 hr (Fig. 4.3.A). Moreover, no accumulation of β - globin mRNA by the EpoR$_{(-230)}$-transduced cells could be detected in response to Epo or Epo plus IL-3 (Fig. 4.3.B). This indicates that an EpoR lacking the intracellular domain is not capable of promoting the survival and differentiation of Ba/F3 cells in response to Epo and argues against differentiating signaling being activated through molecules associated with the extracellular domain of the EpoR, at least not in the absence of a functional cytoplasmic domain.

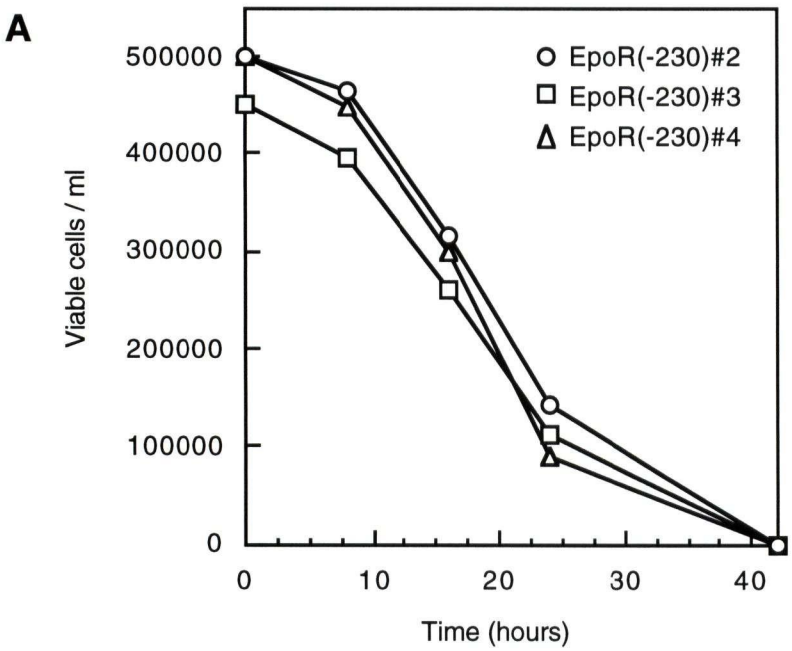

Figure 4.3.A. viability of EpoR(-230)-expressing Ba/F3 cells in Epo-supplemented medium.
Growth factor-deprived cells were incubated in RPMI 1640 containing 0.1% BSA and 0.5 unit of Epo/ml. Viability of cells at the indicated time points was determined by Eosin Y exclusion. Each data point represents a mean value of two experiments.

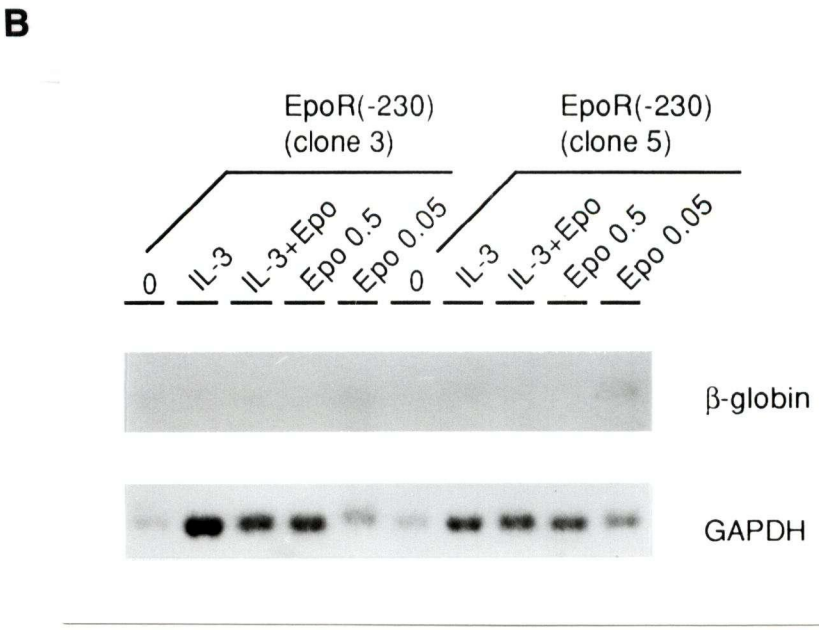

Figure 4.3.B. β-globin mRNA levels in Epo-stimulated EpoR(-230) cells.
Hybridization probes are listed on the right. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

4.3.3. The α subunit of IL-3R inhibits Epo-induced β-globin gene expression

To test the hypothesis that the IL-3Rα subunit can suppress Epo-induced differentiation, we examined the differentiating and proliferative capacities of a chimeric receptor composed of the extracellular domain of the EpoR and the transmembrane and cytoplasmic domains of the IL-3Rα subunit (EpoR/IL-3Rα). Ba/F3 cells expressing this EpoR/IL-3Rα chimera proliferated in Epo-supplemented medium but required approximately 5-fold higher concentrations of Epo to achieve 3H-Tdr incorporation levels comparable with those obtained by WT EpoR-expressing cells (Fig. 4.4.A). β-Globin mRNA levels were then examined in several independent EpoR/IL-3Rα clones, and no accumulation of β-globin mRNA could be detected (Fig. 4.4.B), suggesting that this EpoR/IL-3Rα chimeric receptor was not capable of promoting differentiation.

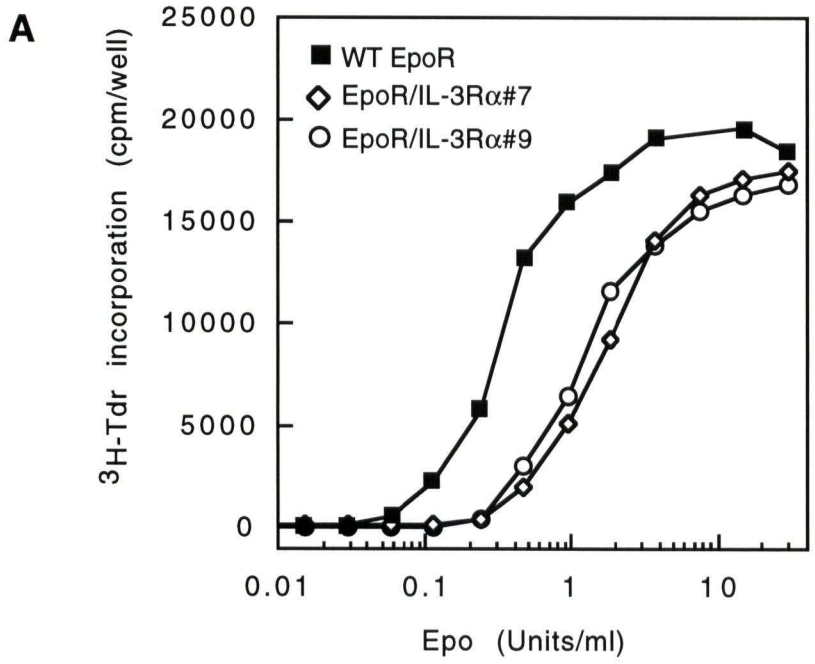

Figure 4.4.A Epo-induced proliferative responses of Ba/F3 cells expressing EpoR/IL-3Rα chimera.
Conditions for determining the Epo-induced incorporation of 3H-Tdr by WT and EpoR/IL-3Rα cells were as described for Fig. 2A. Results are representative of three separate experiments.

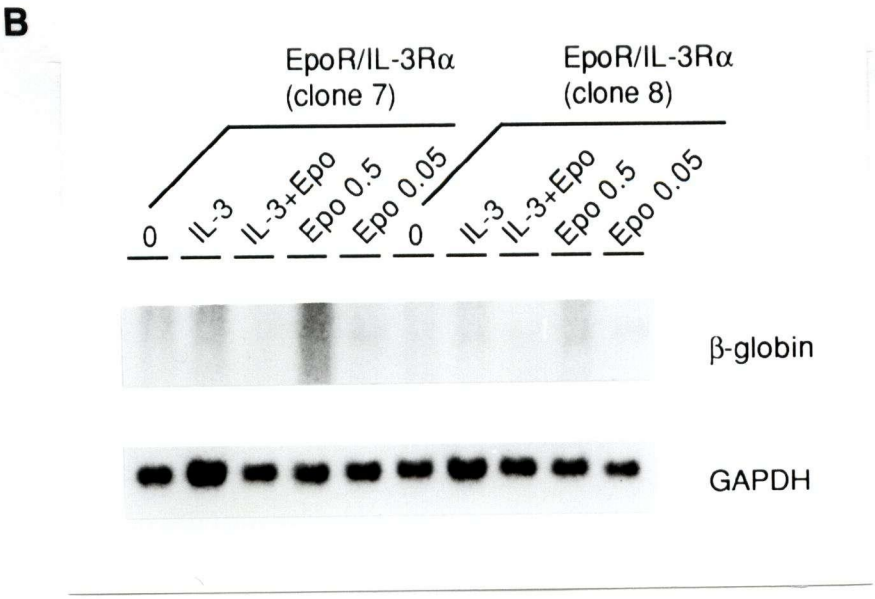

Figure 4.4.B absence of β-globin mRNA induction in Epo-stimulated EpoR/IL-3Rα cells.
Hybridization probes are listed on the right. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

To test whether the absence of the Epo-induced accumulation of β-globin mRNA by EpoR/IL-3Rα cells reflected an inability of this chimeric receptor to induce β-globin gene expression or an ability to actively suppress expression of the β-globin gene, two additional types of Ba/F3 clones were created. First, EpoR/IL-3Rβ cells, shown to accumulate high levels of β-globin mRNA in response to Epo (Fig. 4.2.B, clone 4), were engineered to coexpress the EpoR/IL-3Rα chimera, and several clones expressing 2 to 5-fold higher levels of cell surface EpoRs than the parental EpoR/IL-3Rβ cells were identified by Scatchard analysis. Ba/F3 clones coexpressing EpoR/IL-3Rβ and EpoR/IL-3Rα proliferated in response to Epo (Fig. 4.5) but failed to accumulate β-globin mRNA upon Epo stimulation (Fig. 4.7).

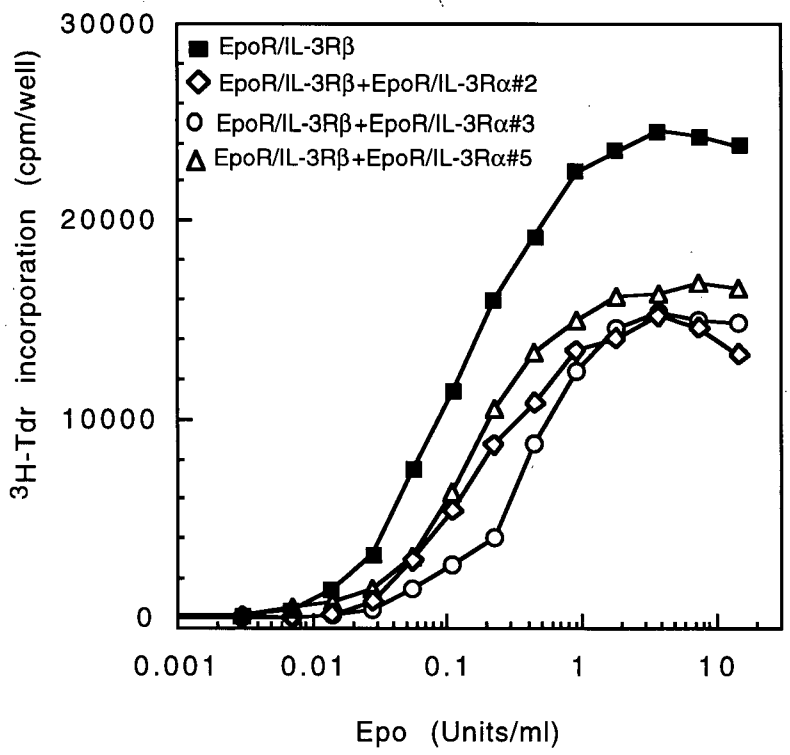

Figure 4.5., Epo-induced proliferative responses of Ba/F3 cells coexpressing EpoR/IL-3Rβ and EpoR/IL-3Rα chimeras.
3H-Tdr incorporation assay was performed as described for Fig. 2A. Results are representative of three independent experiments.

Regeneration of the IL-3R signaling complex thus prevented Epo-induced differentiation despite the capacity of the IL-3Rβ subunit alone to transduce a differentiating signal. Lastly, the capacity of the IL-3Rα subunit to inhibit β-globin gene expression was examined in WT EpoR cells engineered to coexpress the EpoR/IL-3Rα chimera. Several clones expressing 2 to 4-fold higher numbers of cell surface EpoRs than the parental WT EpoR cells were identified by Scatchard analysis (Fig.4.6.A, representative clone).

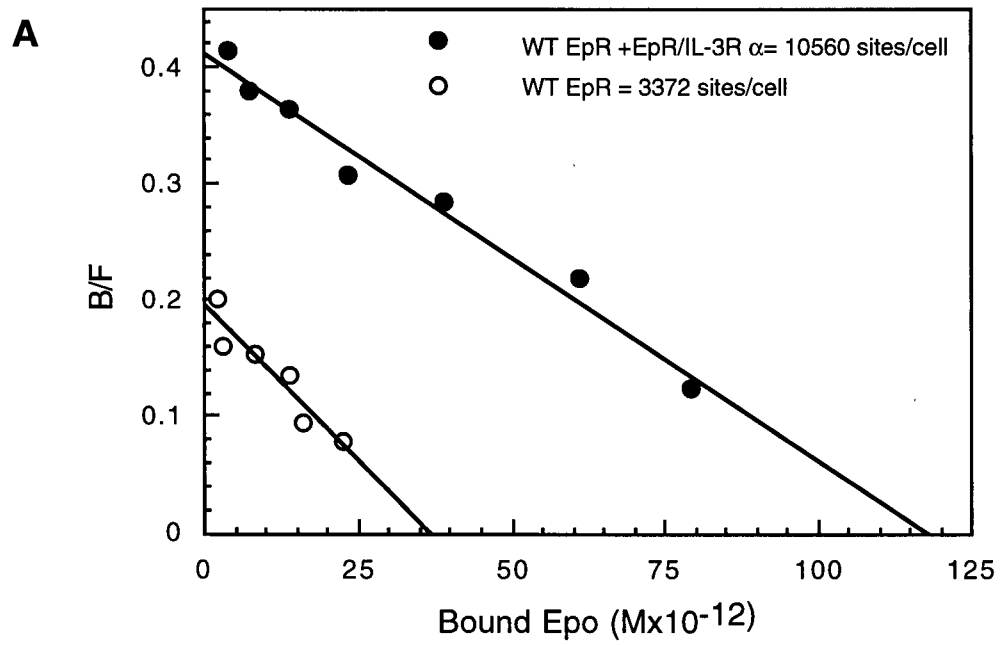

Figure 4.6.A. Scatchard analysis of the specific Epo binding by Ba/F3 cells co-expressing WT EpoR and EpoR/IL-3Rα chimera.
Cells were incubated for 4 hr with serial dilutions of ^{125}I-Epo, in the presence and in the absence of unlabelled recombinant Epo. Scatchard analysis of the specific ^{125}I-Epo binding was performed as described in Materials and Methods.

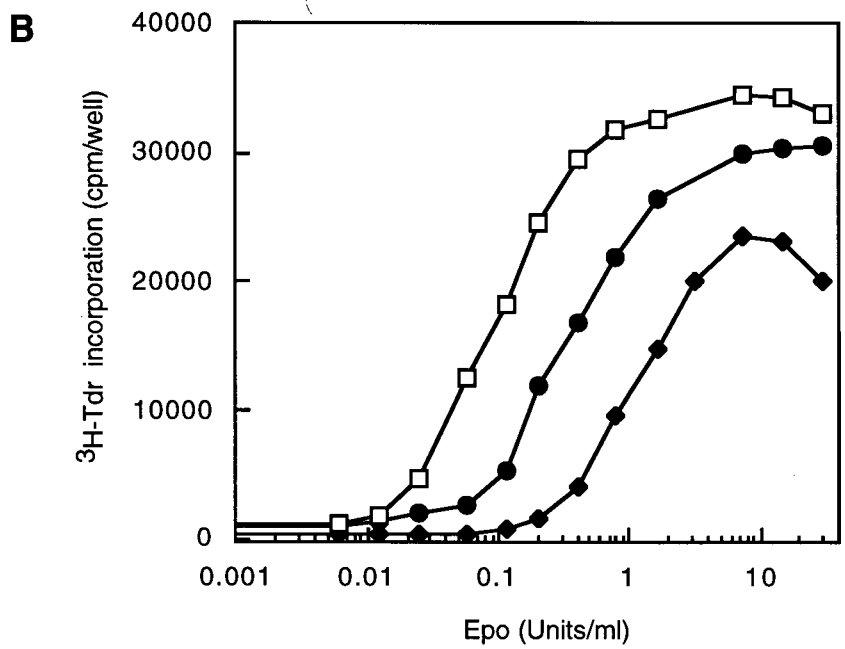

Figure 4.6.B. Epo-induced proliferative responses of Ba/F3 cells co-expressing WT EpoR and EpoR/IL-3Rα chimera.
Conditions for determining the Epo-induced incorporation of 3H-Tdr by WT and EpoR/IL-3Rα cells were as described for Fig. 2A. Results are representative of three separate experiments.

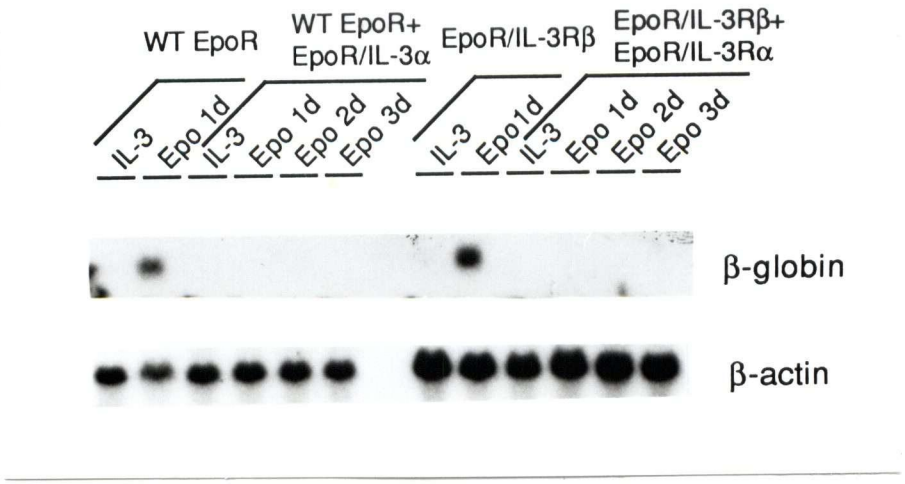

Figure 4.7. Northern blot analysis of β-globin mRNA levels upon IL-3 or Epo stimulation of Ba/F3 cells expressing WT EpoR or EpoR/IL-3Rβ chimera alone versus representative clones coexpressing EpoR/IL-3Rα chimera.
Ba/F3 cells expressing the WT EpoR or EpoR/IL-3Rβ alone (Fig. 4.3, clone 4) versus clones coexpressing the EpoR/IL-3Rα chimera. Growth factor-deprived cells were stimulated with 3 nmol/L of IL-3 or 0.5 unit of Epo/ml in RPMI 1640 containing 0.1% BSA for 1 day (WT EpoR and EpoR/IL-3Rβ or for 1-3 days as indicated for cells coexpressing EpoR/IL-3Rα. Each lane contains approximately 10 μg of total cellular RNA. Hybridization probes are listed on the right.(i.e. 3300 WT EpoRs/cell plus 7000-12,000 EpoR/IL-3Rα/cell).

These cells required 5- to 30-fold higher concentrations of Epo to achieve proliferation levels comparable with those obtained by the parental WT EpoR cells (Fig. 4.6.B). More importantly, cells coexpressing WT EpoR and EpoR/IL-3Rα ceased to accumulate β-globin mRNA in response to Epo (Fig. 4.7) suggesting that the IL-3Rα subunit inhibited the differentiating function of the EpoR.

4.4 Discussion

In this study, we expressed various mutant and chimeric EpoRs in the IL-3-responsive cell line, Ba/F3, to examine the functional roles of the EpoR and IL-3R in regulating β-globin gene induction in Ba/F3 cells. Surprisingly, EpoRs totally lacking in potential tyrosine phosphorylation sites (null EpoR) were found to be capable of inducing β-globin mRNA as well as the normal EpoR. This finding is compatible with our previously published results showing that Epo-induced differentiation was blocked by genestein (Chapter 2 and Krosl et al., 1995), since null EpoRs mediate tyrosine phosphorylation (and activation of Jak2) as well as WT EpoRs (Damen et al., 1995). Taken together, our data suggest that tyrosine phosphorylation of Jak2, but not the EpoR, is critical for Epo-induced differentiation. Null EpoRs, however, were severely compromised in their ability to promote proliferation of Ba/F3 cells, consistent with our previous results (Damen et al., 1995). It is conceivable that the reduced ability of the null EpoR to promote proliferation of Ba/F3 cells could create conditions permissive for differentiation. In this regard, Ba/F3 cells, although originally described as a pro-B cell line (Palacios and Steinmetz, 1985), express erythroid-specific transcription factors such as GATA-1 and NF-E2 (Liboi et al., 1993) and low levels of endogenous EpoRs (Damen et al., 1992) and may thus have evolved in culture toward an erythroid phenotype. The Epo-induced delay in progression through G1 of the cell cycle, shown previously for the WT (Carroll et al., 1995; Krosl et al., 1995) and demonstrated for the null EpoR may simply be triggering a predetermined erythroid differentiating program, as seems to be the case for MEL cells engineered to express p53 (Johnson et al., 1993). However, a simple delay in G1 of parental Ba/F3 cells does not induce β-globin

mRNA (Chapter 3) suggesting that induction of this gene depends on EpoR-mediated signaling.

The Epo-induced accumulation of β-globin mRNA in cells expressing the EpoR/IL-3Rβ chimera is consistent with the previously published findings of Carroll et al. (1994) and Chiba et al. (1993). This observation pointed to the possibility that Epo-specific signaling might depend on the interactions of the extracellular domain of the EpoR with a second as yet unidentified subunit of the EpoR complex. However, cells expressing high levels of a truncated $EpoR_{(-230)}$ lacking the cytoplasmic domain neither survived nor accumulated β-globin mRNA upon Epo stimulation, suggesting that the cytoplasmic region of the EpoR is indispensable for EpoR function. The differentiating capacity of the EpoR/IL-3Rβ chimera, however, also suggested that the cytoplasmic domains of the EpoR and the β_{IL-3} subunit of the IL-3R were interchangeable in providing for the differentiating signal, which argues against the existence of a differentiation-specific domain within the cytoplasmic region of the EpoR and points to a permissive rather than an instructive role for the EpoR in Epo-induced differentiation.

Our finding that the EpoR/IL-3Rα chimera was capable of supporting proliferation of Ba/F3 cells was somewhat surprising since Kitamura and Miyajima (Kitamura and Miyajima, 1992) reported that the human IL-3Rα subunit alone was unable to support proliferation of IL-2-dependent CTLL-2 cells. These cells proliferated in response to human IL-3 only when engineered to coexpress human IL-3Rα and murine IL-3R β_C suggesting that IL-3-induced mitogenic signaling depends on interactions between α and β subunits of the IL-3R complex likely mediated by their extracellular domains (Miyajima et al., 1993). It seems unlikely that a similar association occurs between the

extracellular domains of IL-3Rβ and EpoR. Our results are consistent with a steadily growing body of data suggesting that the membrane proximal region conserved among α subunits of receptors for IL-3, IL-5, and granulocyte macrophage colony-stimulating factor is essential for mitogenic signaling (Polotskaya et al., 1994; Sakamaki et al., 1992; Weiss et al., 1993) and Jak2 activation (Cornelis et al., 1995; Takaki et al., 1994). It is possible that the Epo-induced dimerization of EpoR/IL-3Rα chimeras results in activation of a mitogenic signal through the cytoplasmic domain of the IL-3Rα subunit that is not compatible with differentiation.

Several possible mechanisms could account for the observed inhibitory effect of the EpoR/IL-3Rα on the partial Epo-induced differentiation of Ba/F3 cells. First, EpoR/IL-3Rα chimeras could be forming unproductive heterodimers with EpoR/IL-3Rβ_{IL-3} or WT EpoRs. This mechanism seems unlikely since EpoR/IL-3Rα also inhibited the Epo-induced accumulation of β-globin mRNA in a clone expressing 3300 WT EpoRs and 7000 EpoR/IL-3Rα Random dimerization of cell surface EpoRs in this clone would be expected to yield 9% or approximately 1000 WT EpoR dimers/cell. Second, EpoR/IL-3Rα chimeras, when overexpressed, could shorten the duration of the G1 phase of cell cycle required for induction of β-globin mRNA. This was, however, not the case since exposure to Epo of all EpoR/IL-3Rα-expressing Ba/F3 clones led to a marked delay in G1 to S progression (data not shown). Thus, a delay in G1 is not sufficient for β-globin mRNA induction. Third, the Epo-induced di- or oligomerization of EpoR/IL-3Rα chimeras could activate IL-3-specific pathways involved in inhibition of β globin gene expression. Our results favor this last possibility and are consistent with the concept that the α subunits of the IL-3 (Miyajima et al., 1993), IL-5 (Takaki et al., 1994), and granulocyte macrophage

colony-stimulating factor (Eder et al., 1994) receptors initiate distinct ligand-induced events.

The data presented in this study thus suggest both a permissive role for Epo in inducing β–globin mRNA in Ba/F3 cells expressing EpoRs and an active role for the IL-3Rα subunit in the IL-3-induced inhibition of Epo-induced differentiation. Together these observations argue against an inductive role for Epo in inducing terminal erythroid differentiation proposed in Chapter 3. They rather indicate, that IL-3 R, and perhaps other cytokine receptors as well, actively inhibit hemopoietic cell differentiation, implying that terminal differentiation of committed progenitors depends on cessation of cytokine-mediated suppression, and not on the inductive activity of the lineage specific growth factors.

CHAPTER 5

ECTOPIC EXPRESSION OF EpoR IN PRIMARY HEMOPOIETIC CELLS LEADS TO DEVELOPMENT OF A LETHAL TRANSPLANTABLE MYELOPROLIFERATIVE DISEASE

5.1. Abstract

To examine the capacity of Epo and the EpoR to induce perturbations in the proliferative and differentiation behavior of early hemopoietic progenitor cells we introduced, using a myeloproliferative sarcoma (MPSV) based retroviral vector, the cDNA for normal murine EpoR and the neo^r gene into day 4 5-fluorouracil-treated bone marrow cells. Within 24 hr after co-cultivation infection very high levels of cell surface EpoR expression by bone marrow cells were detected using biotinylated Epo and flow cytometry. Clonogenic progenitor assays revealed that Epo alone supported formation of granulocyte, granulocyte-macrophage and multilineage-mixed colonies for the majority of the infected, G-418^r clonogenic progenitors. In suspension cultures Epo was capable of replacing IL-3, but not SCF in supporting the proliferation of the total population of EpoR-transduced bone marrow cells, as well as early lineage non-committed clonogenic progenitors (preCFU-GEMM). Epo appeared to promote the proliferation of the EpoR-infected cells directly through interactions with the transduced EpoR, as no growth promoting activity supporting proliferation of the neo^r control cells could be detected in cultures comprising high numbers of irradiated EpoR-infected cells. Irradiated recipients of the EpoR-transduced bone marrow cells developed a lethal myeloproliferative disease within 8-12 weeks after transplantation, characterized by anemia,

elevated numbers of peripheral neutrophils, accumulation of nondifferentiated blasts and splenomegaly. Concurrently, there was on average more than a 20-fold increase in total numbers of splenic erythroid and nonerythroid progenitor cells. Bone marrow and spleen cells recovered from the affected mice expressed high levels of cell surface EpoRs and formed distinct colonies in response to Epo alone. Moreover, upon transplantation into irradiated secondary recipients these cells induced development of acute myeloproliferative disease within 2-4 weeks. Constitutive over-expression of normal EpoR by pluripotent hemopoietic stem cells thus promoted proliferation of early hemopoietic progenitors without overt effects on their differentiation behavior, and induced a sequence of events leading to the development of a transplantable neoplastic disease.

5.2. Introduction

IL-3 dependent Ba/F3 cells engineered to express EpoR proliferate and accumulate β-globin mRNA in response to Epo (Carroll et al., 1994; Carroll et al., 1995; Chiba et al., 1993; Krosl et al., 1995; Liboi et al., 1993), suggesting that the Epo-induced signal can induce terminal differentiation events. Ba/F3 cells, however, already constitutively express erythroid lineage specific transcription factors such as GATA-1, NF-E2 and EKLF (Liboi et al., 1993) and low levels of EpoR (Damen et al., 1992), and therefore likely represent a permissive cell line competent to undergo erythroid differentiation in response to Epo.

If upregulation EpoR expression represented a key event leading to erythroid commitment, then expression of the EpoR by multipotent progenitor

cells might be anticipated to promote the differentiation of uncomitted cells towards erythroid lineage at the expense of other, non-erythroid lineages. For studying the effect of Epo on proliferative and differentiation decisions of multipotent hemopoietic progenitor cells, several groups have utilized retrovirus-mediated gene transfer to express the EpoR cDNA in primary mouse and human bone marrow cells. Epo alone was shown to be sufficient for supporting proliferation of the EpoR-transduced non-erythroid and multilineage progenitors (Dubart et al., 1994), indicating the presence within early hemopoietic cells of proteins capable of transducing EpoR-mediated signals. In several such studies the EpoR-transduced multipotent cells yielded apparently normal proportions of erythroid and non-erythroid progeny suggesting that Epo does not have a strong directive effect on the differentiation decisions taken by early cells (Dubart et al., 1994; Pharr et al., 1993; Pharr et al., 1994). Surprisingly however, Lu et al (1996) showed that Epo can enhance formation of erythroid precursors by the EpoR-transduced cord blood-derived multipotent progenitors. Moreover, a gradual shift towards production of erythroid cells was observed in Epo-supplemented long term bone marrow cultures of the EpoR transgenic mice (Kirby et al., 1997). The extent to which EpoR activity can influence the differentiation of early cells at the single cell level, as opposed to changes at the population level, has thus not yet been fully resolved.

Some of these studies may have been hampered by low expression levels of the transduced EpoRs. Recently, we have obtained very high level expression of the transduced EpoR using a myeloproliferative sarcoma virus-based vector system and have thus reexamined the role EpoR plays in regulation of stem cell behavior. Our results show that following transduction of normal EpoR, Epo was capable of replacing several growth factors normally required to promote proliferation of non-erythroid and multipotent progenitors,

but was not able to skew differentiation of multipotent cells the towards erythroid lineage. Moreover, recipients of the EpoR-transduced bone marrow succumbed within 6-8 weeks to myeloid leukemia suggesting that the aberrant expression EpoR by the responsive stem cells may lead to the rapid development of a neoplastic disease.

5.3. Results

5.3.1. Erythropoietin can support the proliferation of primitive hemopoietic progenitors engineered to express normal EpoR

In an effort to engineer high levels of EpoR expression we utilized a Jzen EpoR TKneo retroviral vector (Fig 5.1) to infect primary hemopoietic cells. Day 4 5-FU bone marrow cells were first incubated for 48 hr with IL-3, IL-6 and SCF and were subsequently exposed to the EpoR virus or control neo^r virus by 48 hour co-culture with viral producer cells. Flow cytometric analysis with biotinylated Epo (Fig.5.2) showed that within 48 hr of infection with the EpoR-containing retrovirus more than 50% of the recovered bone marrow cells express very high levels of surface EpoRs.

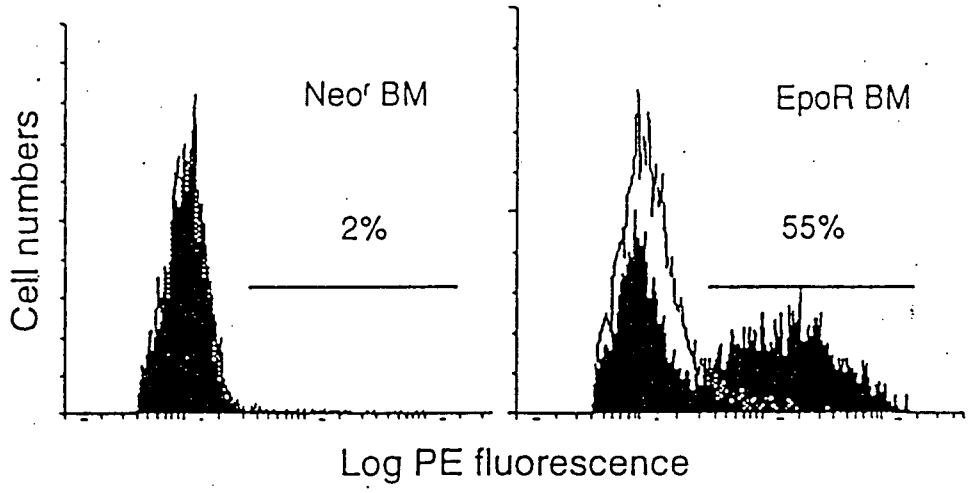

Figure 5.2. Expression of cell surface EpoRs by day 4 5-FU bone marrow cells 24 hours after coculture with the neo^r or EpoR viral producer cells.
Cells were first incubated with b-Epo (shaded peak) or with biotin-conjugated Epo (b-Epo) in the presence of a 100-fold molar excess of unlabelled Epo (unshaded peak), then with streptavidin-RPE, and analyzed by flow cytometry.

To determine the proportion and the phenotype of the Epo responsive clonogenic progenitors, infected cells were plated in methylcellulose cultures supplemented with Epo alone, with Epo plus SCCM, or without any added growth factors, in the presence or absence of G-418 (Table 5.1).

Table 5.1. Erythropoietin supports proliferation of nonerythroid EpoR-transduced cells.

		Colonies(2000 cells/dish)		
Cells	Stimulus	CFU-G+CFU-GM	CFU-GEMM	Total
EpoR	SCCM+Epo	81±7	22±3	111±5
	SCCM+Epo+G-418	62±9	17±2	85±6
	Epo	58±8	19±2	81±5
	DMEM	0	0	0
Neo	SCCM+Epo	76±7	15±2	93±9
	SCCM+Epo+G-418	55±4	13±1	79±5
	Epo	0	0	0
	DMEM	0	0	0

2000 cells recovered 24 hours after co-cultivation with viral producers were plated in methylcellulose containing 3 Units of Epo/mL and 2% SCCM with and without 1 mg/mL of G-418, or 3 Units of Epo/mL, or without added growth factors. Colonies were scored on day 14. Presented are mean values±SD for four separate experiments.

In seven independent experiments, the gene transfer efficiency assessed by G-418^r colony formation was 70-100% for the EpoR virus and control neo^r viruses. No colony formation was detected in the absence of growth factors showing that expression of the retrovirally transduced EpoR did not abrogate the growth factor dependency of clonogenic progenitors. Epo alone, however, supported development of virtually the same numbers of G-418^r granulocyte and granulocyte-macrophage (92±7%, mean value±SD for 4 experiments) and

multilineage mixed colonies (86±3%, mean value±SD for 4 experiments) as detected in response to Epo plus SCCM. Moreover, the EpoR-transduced cells gave rise to 50% (49±11, mean value±SD, 7 experiments) more mixed colonies in response to Epo than the G-418^r control cells gave in response to Epo plus SCCM, suggesting an Epo-associated activation of the EpoR-transduced multilineage progenitor cells.

One possibility suggested by these observations was that Epo directly activated mitogenic signals in the EpoR-transduced non-erythroid and multipotent cells. Another possibility was, however, that Epo stimulated enhanced production and/or presentation of growth factor(s) by a subpopulation of Epo-responsive cells engineered to over-express EpoR. To discriminate between these two possibilities varying proportions of EpoR- and neo^r-transduced cells were plated in Epo-supplemented methycellulose in the presence and in absence of irradiated EpoR-infected feeder cells ($1x10^5$/mL) (Fig 5.3.).

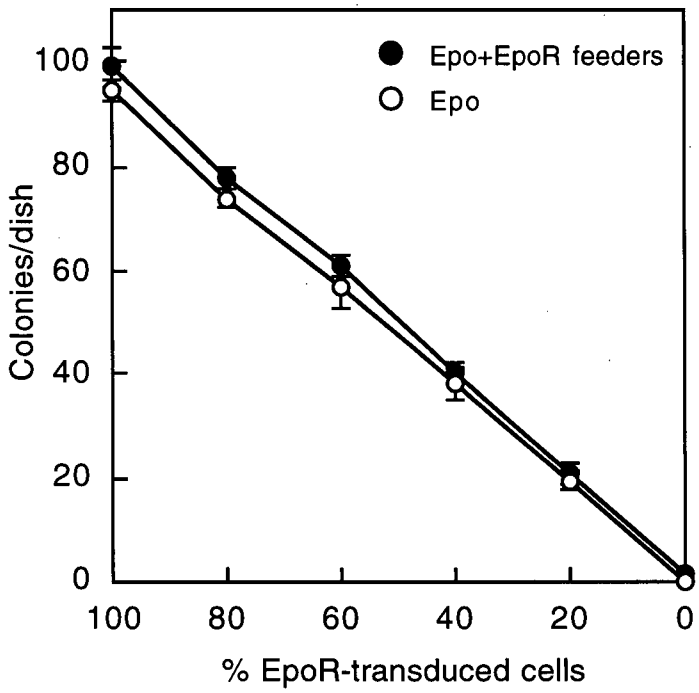

Figure 5.3. Colony formation in response to Epo in cultures comprising mixed populations of the EpoR- and neo^r-transduced cells.
24 hr after co-cultivation infection the EpoR- and neo^r-transduced cells were mixed at the indicated ratios and plated at 2000 viable cells/dish in methylcellulose containing 3 Units of Epo/mL, in the presence and in the absence of $1x10^5$ irradiated EpoR-transduced cells. Colonies were scored on day 14.

The numbers of colonies that developed in response to Epo correlated directly with the numbers of viable EpoR-transduced cells. Importantly, no differences could be detected between the numbers of colonies developing in cultures supplemented with Epo alone or Epo plus the EpoR-transduced feeder cells arguing against any significant release of growth factors by EpoR-transduced cells.

For the EpoR-transduced progenitors, Epo thus appeared to be as efficient as Epo plus SCCM in supporting colony formation. To assess the capacity of Epo to replace combinations of growth factors normally required to support proliferation of early hemopoietic cells the total yields of nucleated cells (Fig.5.4.) and multipotent progenitors (Fig 5.5) were compared for neo^r- versus

EpoR-transduced cells cultured for 8 days with G-418 in serum-free medium, supplemented with Epo, IL-3, or SCF in various combinations.

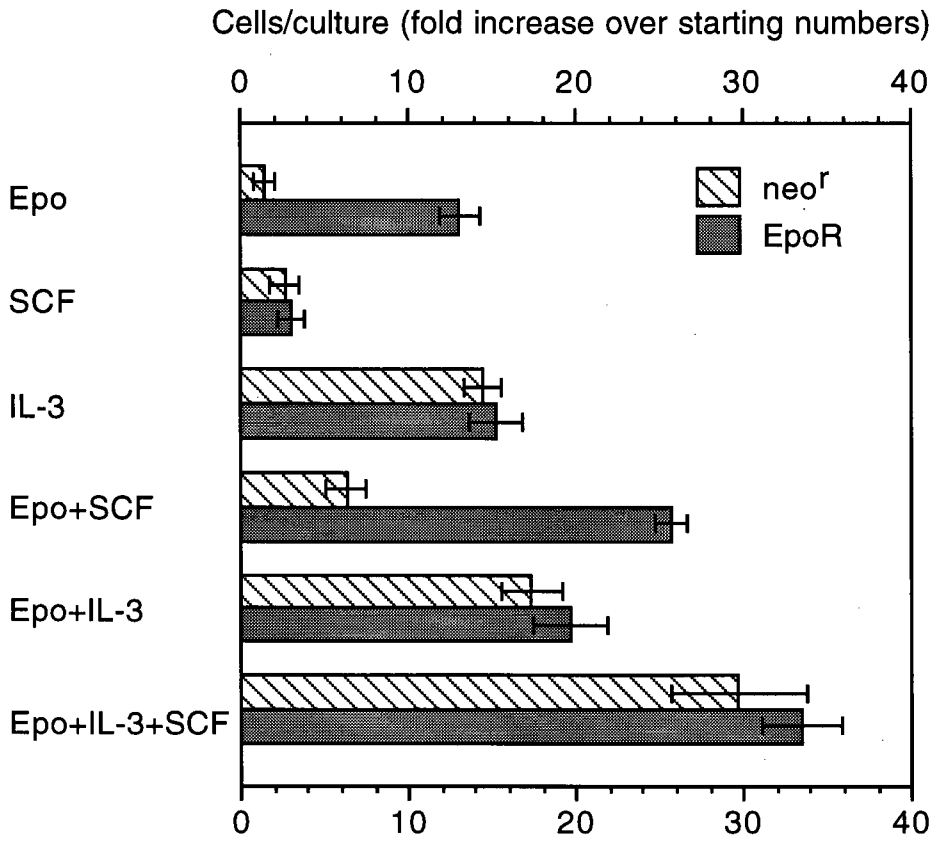

Figure 5.4. Epo can replace IL-3 in promoting proliferation of the EpoR-transduced cells in suspension culture.
EpoR- and neor-transduced cells were cultured for 8 days in serum free liquid medium with the indicated growth factors in the presence of G-418. Cell were counted and transferred to wells containing fresh medium every 48 hours, such that cell density never exceeded 4-5x10^5 cells/mL. Growth factor concentrations were: Epo, 1 Unit/mL; IL-3, 5 ng/mL; SCF, 25 ng/mL.

Consistent with results of clonogenic progenitor assays, Epo alone promoted did not significantly promote proliferation of neo^r-transduced cells. The numbers of the EpoR-transduced cells, in contrast, increased in response to Epo approximately 13-fold over starting numbers. Neither neo^r- nor EpoR-transduced cells proliferated in medium supplemented with SCF alone. IL-3 induced comparable, approximately 14-fold expansions of the starting neo^r and EpoR-transduced cell populations. Epo thus appeared to be equivalent to IL-3 in stimulating proliferation of the EpoR-transduced cells. Combination of

Epo plus SCF resulted in a 6-fold increase in the numbers of the neo^r-transduced cells, compared to a 26-fold increase in the numbers of the EpoR-transduced cells. Combination of Epo plus IL-3, or Epo plus IL-3 plus SCF gave equal responses for neo^r- and EpoR-transduced cells. Together these results suggested that Epo stimulation of the EpoR-transduced cells triggered proliferation responses overlapping with those induced by IL-3, but not by SCF.

In further effort to see if EpoR mediated signaling could act on more primitive cells, the recovery of CFU-GEMM from suspension cultures initiated with neo^r- and EpoR-transduced cells was assessed by plating aliquots of cells recovered from suspension culture in methylcellulose supplemented with Epo, IL-3 and SCF (Fig 5.5). After 8 days of liquid culture the numbers of the EpoR-transduced CFU-GEMM increased in response to all three growth factors approximately 11-fold compared to 7-fold for the neo^r-transduced cells. Moreover, approximately a 10-fold increase of the EpoR-transduced CFU-GEMM was also observed in response to Epo alone, whereas no generation of neo^r-transduced CFU-GEMM could be detected in Epo-supplemented cultures. Together, these results indicate that EpoR mediated signaling can support growth of non-erythroid and multipotent clonogenic progenitor cells.

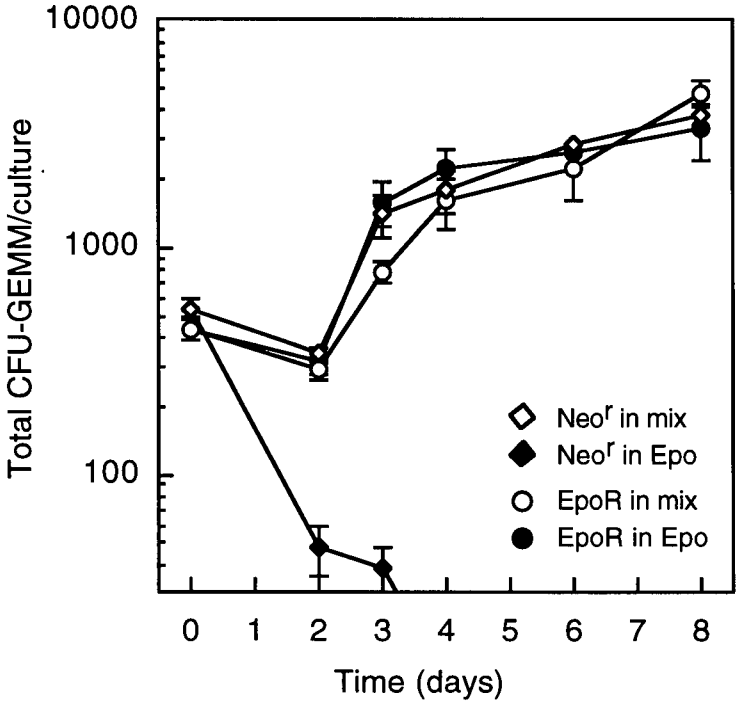

Figure 5.5. Epo supports proliferation of the EpoR transduced preCFU-GEMM in suspension culture.

The EpoR- and neo^r-transduced cells were cultured as described for Fig 4.4. At the indicated time points aliquots of cells grown in the presence of Epo, or Epo plus IL-3 plus SCF were plated in methylcellulose, supplemented with all three growth factors. Concentrations of growth factors were as described for Fig 4.4. Macroscopic bursts were scored on day 14.

5.3.2. Mice reconstituted with WT EpoR-transduced bone marrow develop a lethal transplantable myeloproliferative disease

To study the effects of ectopic WT EpoR expression on the proliferative and/or differentiation behavior of early hemopoietic stem cells and their progeny in vivo, EpoR- and control neo^r-transduced bone marrow cells were transplanted into lethally irradiated syngeneic recipients. A total of 23 recipients in four separate experiments were transplanted with of 0.2-1x10^6 infected bone marrow cells. Beginning 6-7 weeks after transplantation (range 6-14 weeks) all recipients of the EpoR-transduced bone marrow cells developed severe anemia

(hematocrit 20-25%) and leukocytosis (WBC counts 11 to 12 x10^4 cells/mm^3), became moribund and were sacrificed (Table 5.2). Recipients of the neo^r-infected bone marrow, in contrast, remained healthy and no gross hematological abnormalities could be detected during an 8 month observation period.

Table 5.2. Mice reconstituted with the EpoR-transduced bone marrow develop a lethal myeloproliferative disease.

			Differential cell counts (as % of total)			
Recipients	WBC (x 10^4/mL)	Hct (%)	Blasts	Gr	Mo	Ly
Neo Mean±SD, n=4	11±0.2	42±2	0	26±3	8±1	66±8
EpoR Mean±SD, n=9	117±0.3	23±2	40±7	38±5	10±2	9±2

Mice from four different experiments were sacrificed and analyzed 8-11 weeks after reconstitution. Total WBC counts were determined using hemocytometer, and the differential cell counts were determined for 200 cells on Wright stained blood smears. Gr, granulocytes; Mo, monocytes; Ly, lymphocytes.

For all affected EpoR animals, examination of Wright stained blood smears revealed elevated numbers of neutrophils, a reduction in peripheral lymphocytes, and accumulation of undifferentiated blasts. On autopsy, the most noticeable changes were enlarged spleens with weights of 0.4-1 gm compared to 0.1 gm in recipients of the neo^r-transduced bone marrow and normal controls. To assess the leukemogenic potential of the EpoR transduced bone marrow, four affected mice from two separate experiments were sacrificed and their bone marrow (2 x10^6 cells) or spleen cells (5x10^6 cells) injected together with 1x10^5 normal bone marrow cells into irradiated secondary recipients (three

recipients for each transplant). All secondary recipients (24/24) became moribund within 2-4 weeks after transplantation (Table 5.3) and were found at sacrifice to be anemic (hematocrit 20-22%) and have elevated peripheral leukocyte counts (12-16 $x10^4$ cells/mm^3) with greater than 40% undifferentiated blasts, consistent with an acute leukemia.

Table 5.3. The EpoR-associated myeloproliferative disease is transplantable.

				Differential cell counts (as % of total)			
Mice	Time (days)	Hct (%)	WBC ($x10^4$/mL)	Blasts (% of total)	Gr	Mo	Ly
Donors Mean±SD, n=4	83±4	27±2	10±3	40±4	48±5	6±2	2±1
Recipients Mean±SD, n=9	24±4	21±1	14±2	42±2	53±2	5±1	0
(B6C3)F1	-	51±2	1.1±0.2	0	18±4	3±1	71±3

$2x10^6$ bone marrow or $5x10^6$ spleen cells recovered from each affected mice were injected together with $1x10^5$ normal bone marrow cells into three to four irradiated secondary recipients. (B6C3)F1 values represent mean±SD of values determined for three normal mice. Peripheral blood leukocyte counts were determined as described for Table 2.

To demonstrate the presence of EpoR retroviral integration and to assess the clonality of hemopoietic reconstitution in recipients of EpoR-transduced bone marrow, Southern blot analysis of DNA from hemopoietic tissues of primary and secondary recipients was performed. Primary recipients of the EpoR-transduced bone marrow had individual proviral banding patterns with identical bands evident in DNA isolated from the bone marrow, spleen and

thymus of individual mice (Fig 5.6, representative result), consistent with repopulation with limited numbers of transduced lympho-myeloid stem cells. Within each group of secondary recipients identical banding patterns, characteristic for the donor derived hemopoietic tissues, was observed (Fig 5.7). Moreover, the polyclonal origin of the disease determined for the primary recipients #4 and #6 was preserved upon transplantation into secondary recipients, indicating that events leading to transformation of more than one HSC occurred in the primary recipients of the WT EpoR-transduced bone marrow.

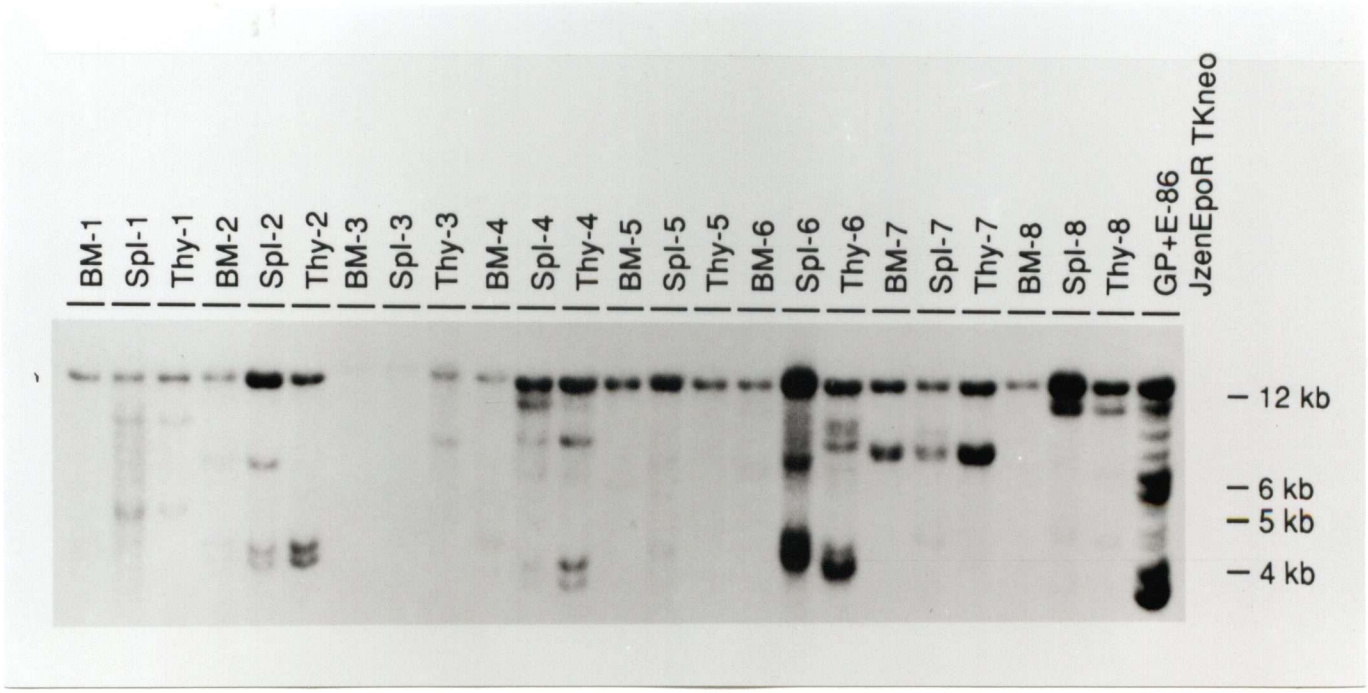

Figure 5.6. The EpoR-transduced HSC contributed to lympho-myeloid repopulation of the irradiated recipients.
DNA isolated from bone marrow, spleen and thymus was digested with EpoR I, which cuts the integrated provirus once and generates DNA fragments specific for each integration site. A 12 kb band derived from endogenous EpoR represents a single gene copy control of signal intensity. Probe used for hybridization was EpoR cDNA.

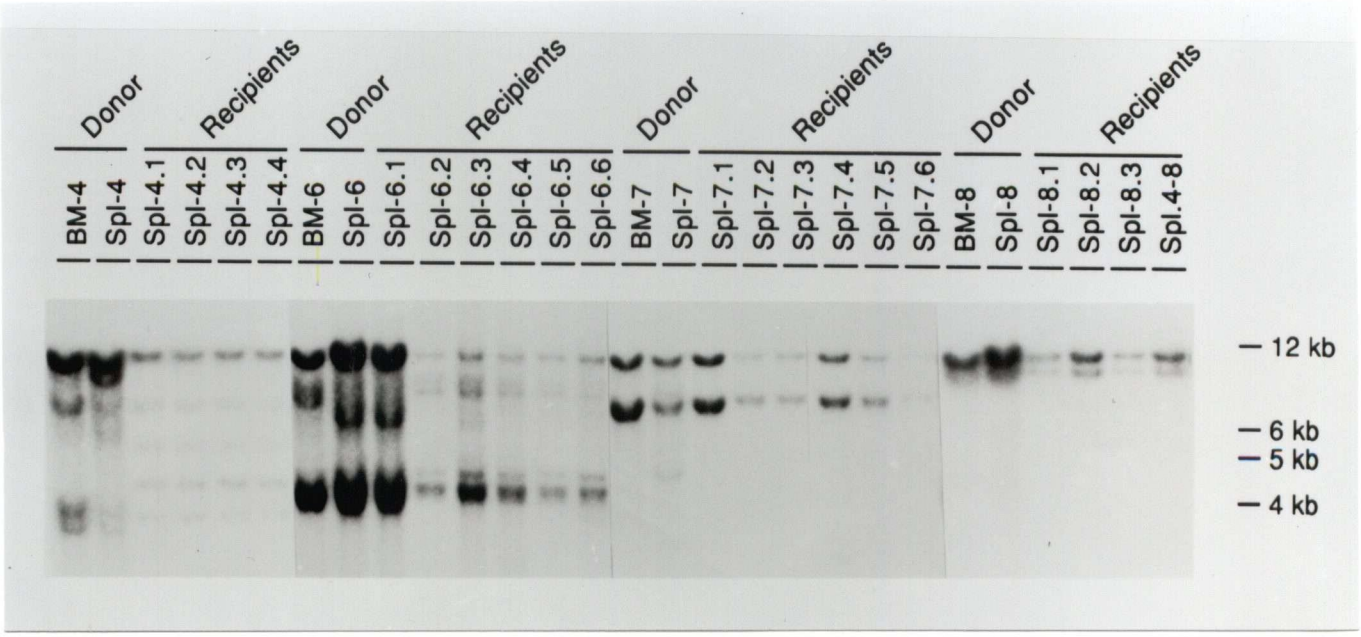

Figure 5.7. Polyclonal origin of acute leukemia developing in secondary recipients of the EpoR-transduced bone marrow.
Southern blot analysis was performed as described for Fig 5.6.

For all the affected animals examined approximately 10-fold increases in the numbers of WBC correlated with significant increases in proportion of the EpoR-expressing bone marrow and spleen cells. Flow cytometric analysis with biotinylated Epo (Fig 5.8, a representative analysis)) revealed that approximately 70% of the total bone marrow and spleen derived cell populations recovered from the affected EpoR mice expressed high levels of cell surface EpoRs compared to 5% of the Epo-binding cells detected in the bone marrow of a control mouse.

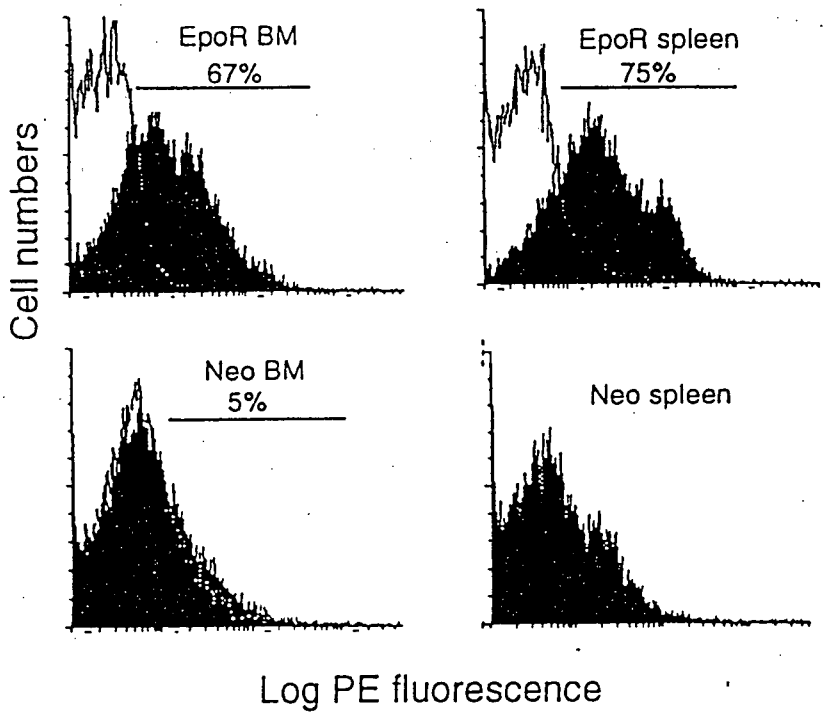

Figure 5.8. Bone marrow and spleen cells recovered from the EpoR-transduced bone marrow express high levels of cell surface EpoRs.
Flow cytometric analysis of the biotinylated Epo (B-Epo) binding by bone marrow and spleen cells isolated from recipients of the EpoR- and neo^r-transduced bone marrow was performed as described for Fig 5.2. Shaded peak, b-Epo only; unshaded peak, b-Epo and 100-fold molar excess of unmodified recombinant Epo.

Recipients of the EpoR-transduced bone marrow had 2- and 26-fold higher numbers of clonogenic progenitors in the bone marrow and spleen, respectively, than recipients of the neo^r marrow (Table 5.4).

Table 5.4. Mice reconstituted with the EpoR-transduced bone marrow cells have normal distribution of clonogenic progenitor classes.

		Bone marrow		Spleen	
Recipient	Time (weeks)	Erythroid ($x10^4$)	Nonerythroid	Erythroid ($x10^4$)	Nonerythroid
Neo.1	8	1.2	1.7	0.9	0.9
Neo.2	10	1.7	2.9	0.7	0.8
Neo.3	11	1.8	5.2	1.6	1.2
Mean±SD	-	1.6±0.2	3.3±0.8	1±0.2	1±0.1
EpoR.1	8	3.5	5.6	315	229
EpoR.2	10	2.5	5.5	358	254
EpoR.3	11	3.8	4.9	193	22.6
Mean±SD	-	3.3±0.3	5.3±0.2	29±4	23.6±0.7

Recipients of the EpoR- and neo^r-transduced bone marrow were sacrificed 8-11 weeks after transplantation. Bone marrow and spleen cells were counted and plated in methylcellulose supplemented with 3 Units of Epo/mL to determine the numbers of CFU-E and day 3 BFU-E, and in medium containing 3 Units of Epo/mL and 2% SCCM to determine the numbers of day 5 BFU-E/Meg, CFU-G, CFU-GM, and CFU-GEMM. The identity of colonies was verified by analyzing composition of 50 randomly chosen colonies. Presented are mean values±SD for three mice from three separate reconstitutions.

The increase was approximately equal for granulocyte/macrophage and erythroid progenitors (24- and 28-fold, respectively).

On average at least 65%-75% of clonogenic progenitors recovered from recipients of the EpoR- and neo^r-transduced bone marrow were derived from the retroviraly transduced cells as judged by their capacity to form colonies in the presence of G-418 (Table 5.5). Consistent with high levels of cell surface EpoRs, the majority of the G-418^r non-erythroid progenitors recovered from affected EpoR mice grew in response to Epo alone. Importantly, no colony formation was ever detected in the absence of added growth factors,

demonstrating that ectopic EpoR expression did not abrogate the growth factor dependency of clonogenic progenitors.

Table 5.5. Nonerythroid clonogenic progenitors recovered from mice reconstituted with the EpoR-transduced bone marrow proliferate in response to Epo.

Group	Cells	Stimulus	Plating efficiency (% of total)
EpoR-transduced	Bone marrow	SCCM+Epo	100 (76±4)*
		Epo	66±7
		DMEM	0
EpoR-transduced	Spleen	SCCM+Epo	100 (74±5)
		Epo	608
		DMEM	0
Neo-transduced	Bone marrow	SCCM+Epo	100 (73±5)
		Epo	0
		DMEM	0
Neo-transduced	Spleen	SCCM+Epo	100 (68±7)
		Epo	0
		DMEM	0

Bone marrow and spleen cells were seeded in methylcellulose supplemented with 3 Units of Epo/mL and 2% SCCM in the presence and absence of 1 mg/mL of G-418, or 3 Units of Epo/mL, or in the absence of added growth factors. Colonies were scored on day 14. Presented are mean values ± SD for seven mice from four different reconstitutions.
*- % of G-416 resistent progenitors

Together these results add further evidence that EpoR-mediated signaling can support growth of early hemopoietic progenitors without preferential erythroid differentiation. Moreover, in this bone marrow transplantation model, such signaling can confer a marked proliferative advantage leading to development of lethal myeloproliferative disease.

5.4. Discussion

In this study we have shown that the spectrum of cells responsive to Epo can be greatly expanded following retroviral transduction of the EpoR. The most striking effect detected was the capacity of Epo to replace other cytokines in promoting proliferation of essentially all (>85%) G-418^r nonerythroid and multipotent clonogenic progenitors (CFU-G, CFU-GM, CFU-GEMM), consistent with previously published observations that EpoR-infected CFU-GEMM are capable of forming colonies in response to Epo (Dubart et al., 1994). Our finding that Epo alone can support proliferation of EpoR-transduced CFU-GM and CFU-GEMM was somewhat surprising as several groups (McArthur et al., 1995; Pharr et al., 1993; Pharr et al., 1994) reported that non-erythroid and multipotent progenitor cells transduced with the mutant, constitutively activated EpoR (EpoR(R129C)) still depended for their proliferation on IL-3, IL-6 and SCF. The Epo sensitivity, however, appears to correlate with the levels of cell surface EpoRs (Youssoufian et al., 1993), and we were able to demonstrate very high EpoR expression levels on the EpoR-infected bone marrow cells, whereas no such information has been presented in studies utilizing the EpoR(R129C). It is possible therefore, that the apparent discrepancies between these and our studies result from the differences in expression levels of the transduced EpoRs.

Several possible mechanisms could account for the observed Epo-induced proliferation of the EpoR-transduced bone marrow cells. The EpoR-transduced monocytes and macrophages could enhance production and/or presentation of growth factors such as IL-1, G-CSF, IL-6 and SCF in response to Epo (Kittler et al., 1992; McArthur et al., 1995) and thus create a microenvironment supporting proliferation of other non-erythroid and multilineage progenitors. This mechanism seems unlikely to account for the

effects seen in this study, since no Epo-promoted proliferation of control cells could be detected in cultures containing excess irradiated EpoR-transduced cells. It is possible, however, that the EpoR-transduced cells responded to Epo by paracrine production of mitogenic factor(s), as reported for some cases of acute myeloid leukemias (Rogers et al., 1994). Lastly, Epo could generate the mitogenic signal directly, through activation of the transduced EpoRs. Our observations favor this last mechanism and are consistent with activation by the EpoR of at least some of the intracellular signaling pathways shared between the EpoR and other cytokine receptors (Ihle et al., 1995).

Consistent with results of our in vitro clonogenic progenitor assays, over-expression of EpoR also markedly affected the behavior of early hemopoietic cells in vivo. Importantly, ectopic expression of EpoR by HSC led to increased production of progenitor cells committed to all hemopoietic lineages without preferential erythroid differentiation of HSC, consistent with other factors playing a dominant role in erythroid lineage commitment. Our observations not only provide further evidence that multipotential progenitor have a potential to proliferate in response to Epo, but also suggest that the absence of EpoRs, and perhaps other cytokine receptors as well, on the surface of HSCs may be crucial for maintaining normal hemopoietic cell proliferation.

Development of myeloproliferative disease in recipients of the EpoR-transduced bone marrow was somewhat surprising, as Lacout et al (Lacout et al., 1996) detected no gross alterations in the hemopoiesis of mice reconstituted with EpoR-infected bone marrow. These mice exhibited only a transient albeit significant increase in numbers of CFU-GEMMs, consistent with the in vivo proliferative advantage conferred upon multipotent clonogenic progenitors by the transduced EpoR. Again, we speculate that in their study the expression

levels of the transduced EpoRs were too low to induce aberations in stem cell behavior. In mice infected with recombinant spleen focus-forming virus (SFFV) encoding constitutively activated (Epo independent) EpoR(R129C), in contrast, an initial polycythemia developed into growth factor independent erythroleukemia (Longmore and Lodish, 1991; Longmore et al., 1993). This development was thus entirely different from the myeloproliferative disease arising in the current study using the MPSV-based JzenEpoR retroviral vector. Insertional activation of erythroid specific factor fli-1 and inactivation of p53 appeared to represent key events enabling clonal expansion of growth factor independent cells in SFFV EpoR(R129C) infected mice, suggesting that the tropism of the SFFV based retroviral vector played a prominent role in determining the phenotype of the disease. It is possible that some insertional activations of putative oncogenes also occurred in the Jzen EpoR-transduced bone marrow cells, although the rapid development and polyclonal nature of disease argue that EpoR alone may have accounted for the observed aberrations in hemopoiesis.

In addition to stimulating proliferation of the multipotential progenitors, the transduced EpoR may have also had a suppressive effect on differentiation, as suggested by high numbers of undifferentiated blasts detected in affected mice. This interpretation is consistent with the reported capacity of Epo to inhibit the GM-CSF responsiveness of FDC-P1 cells engineered to express EpoR (Quelle and Wojchowski, 1991) and to decrease expression levels of the IL-3R β subunits in Ba/F3 cells (Liboi et al., 1993). As cytokines such as G-CSF and GM-CSF appear to play distinct roles in promoting functional maturation of hemopoietic cells (reviewed in Metcalf and Nicola, 1995), a decrease in expression levels of their corresponding receptors may have accelerated

accumulation of undifferentiated blasts in recipients of the EpoR-transduced bone marrow.

In summary, we have demonstrated that Epo was capable of promoting proliferation and suppressing differentiation of early hemopoietic progenitor cells engineered to over-express EpoR. Our results also indicated that the absence of EpoRs on cell surface of the HSC and the temporal restriction of the EpoR expression to late stages of erythroid differentiation may be crucial for maintaining normal hemopoiesis, and that aberrant expression of WT EpoR by HSC may lead to development of a neoplasia.

CHAPTER 6

GENERAL CONCLUSIONS

Epo, the major in vivo stimulator of mammalian hemopoiesis, exerts its action by binding to specific cell surface receptors on immature erythroid progenitor cells (Krantz, 1991; Sawada et al., 1990). Two plausible models exist for the role of Epo in erythroid differentiation. The stochastic model of stem cell commitment predicts that commitment of stem cells to any lineage, including the erythroid, displays a random pattern, and that growth factors such as Epo are required for the survival, proliferation and execution of differentiation programs once they have been irreversibly initiated (Till et al., 1964). According to the instructive model, Epo activates a distinct set of intracellular events which result in the commitment of cells to the erythroid lineage (VanZant and Goldwasser, 1979). Studies presented in Chapter 3 and reported by others (Carroll et al., 1995) indeed demonstrated distinct Epo-induced responses of the IL-3 dependent Ba/F3 cells engineered to express EpoR, such as transient growth delay (Fig.3.3.) and accumulation of β-globin mRNA (Fig.3.1, Fig.3.2, Fig.3.3.). Ba/F3 cells, originally described as pro-B cell line (Palacios and Steinmetz, 1985) were, however, reported to express erythroid-specific transcription factors NF-E2 and EKLF (Liboi et al., 1993) and low levels of endogenous EpoR (Damen et al., 1992). These cells therefore likely correspond to progenitors already committed to erythroid lineage, and as such may represent a model system for studying the events leading to terminal erythroid differentiation, but may be less suitable for analysis of commitment mechanisms.

Results presented in Chapter 3 are consistent with an active role for the EpoR-mediated signaling in promoting late stages of erythroid differentiation

and suggested that the proliferation and differentiation function of the EpoR can be uncoupled. Whether the differentiation and survival functions of the EpoR can also be uncoupled remain, however, an open issue. Several lines of evidence presented in Chapter 4 and elsewhere imply that the apparent differentiation capacity of the EpoR results primarily from its ability to prevent apoptosis. First, the EpoR expressing Ba/F3 cells die in the absence of IL-3 or Epo, but accumulate prior to apoptosis significant amounts of β-globin mRNA (Fig. 3.5. and Table 3.1). This indicates that, at least in this model system, cessation of the cytokine receptor-mediated signaling is sufficient for upregulation of β-globin gene expression. Similar conclusions were derived from studies presented by Fairbairn et al (Fairbairn et al., 1993). who showed that the Bcl-2-mediated suppression of apoptosis enabled terminal differentiation of multipotential FDCPmix cells in the absence of any added growth factors. Second, Ba/F3 cells expressing an EpoR/IL-3Rβ_{IL-3} chimera increased β-globin mRNA levels in response to Epo (Fig.4.3.B), suggesting that either the β subunit of the IL-3 receptor complex transmitted the Epo-specific differentiation signal or that the extracellular domain of the EpoR induced differentiation by activating additional components of the EpoR complex. The former possibility seems unlikely as IL-3 prevented accumulation of β-globin mRNA in all experimental conditions tested (Fig. 3.3 and Fig. 3.5), and the absence of any detectable biological activity of isolated extracelular domain of the EpoR (Fig.4.3.A and Fig 4.3.B) argues against the latter. Given that the α subunit of IL-3R complex appears to play an active role in inhibiting erythroid differentiation (Fig.4.7), and that in cells expressing EpoR/IL-3Rβ_{IL-3} chimeras, Epo activates only the cytoplasmic domains of the IL-3R β subunits, it is reasonable to assume that the IL-3Rβ is equivalent to EpoR in enabling survival and terminal differentiation of committed cells.

Our observation that the IL-3R α subunit plays an active role in inhibiting erythroid differentiation (Fig.4.7) is consistent with the concept that α subunits of the IL-3 (Miyajima et al., 1993), GM-CSF (Eder et al., 1994) and IL-5 (Takaki et al., 1994) receptors initiate distinct ligand-induced events. IL-3 or GM-CSF may thus simultaneously promote survival and/or proliferation and inhibit premature terminal differentiation of early erythroid progenitors (BFU-E). Activation of high numbers of EpoRs expressed at the CFU-E stage (Krantz, 1991) could, conversely, enable differentiation by suppressing the inhibitory activity of IL-3 and GM-CSF receptor α subunits, as Quelle et al (Quelle and Wojchowski, 1991) reported that Epo suppressed the GM-CSF responsiveness of FDC-P1 cells engineered to express EpoR. The mechanism of the IL-3 Rα subunit-mediated inhibition of erythroid differentiation has not yet been elucidated. Preliminary studies performed in Dr. G. Krystal's laboratory (M. Hughes and J. Damen, unpublished observations) suggest that chimeric EpoR/IL-3Rα receptor (Fig.2.1) is capable of activating Stat5. Activation of this signal transducer was reported to be incompatible with terminal differentiation of MEL cells (Merchav et al., 1995). and studies to identify genes activated by Stat5 are expected to provide more insight into molecular mechanisms involved in suppression of hemopoietic cell differentiation.

There is growing body of evidence suggesting that various cytokines utilize common mitogenic signal transducing pathways (Ihle et al., 1994). Growth factor responsiveness at any given stage of hemopoietic cell differentiation is thus likely determined by mechanisms which regulate expression of growth factor receptors. Consistently, multipotential cells, which normally depend for their proliferation on several growth factors (Szilvassy and Hoffman, 1995) can upon introduction of EpoR proliferate in response to Epo (Table 5.1, Fig. 5.3 and (Dubart et al., 1994). Development of myeloproliferative

disease in recipients of the EpoR-transduced bone marrow cells suggests, moreover, that the absence or low level of EpoR activity, and perhaps other cytokine receptors as well, is crucial for maintaining normal proliferative behavior of HSC. Deregulated expression of EpoR has not been implicated in development of human leukemias. Leukemic cell lines such as TF-1 and UT-7 (Chretien et al., 1994; Winkelman et al., 1995) are, however, characterized by rearrangements at the EpoR locus and high levels of EpoR expression, which may have contributed to development of neoplastic clone.

Normal hemopoiesis thus appears to depend on molecular mechanisms which regulate not only the types, but also the numbers of various surface receptors expressed at a given stage of hemopoietic cell differentiation. The most provocative conclusion derived from studies presented in this thesis is that cytokines may regulate hemopoiesis predominantly through inhibition of differentiation. The question remains, however, how the specificity of this suppression is achieved. Future studies directed towards identifying the combinations of intracellular signaling molecules involved in the activation of suppresor genes, and identification of these genes, will likely involve analyses of primary bone marrow populations highly enriched for the specific stage of hemopoietic cell differentiation. Identifying elements involved in the cytokine-dependent suppression of differentiation could enable designing specific antagonists, and lead to development of new modalities in treatments of leukemias.

Chapter 7

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