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ROLES FOR AN EPO-RECEPTOR PY343-STAT5 SIGNALING AXIS IN STRESS ERYTHROPOIESIS

A Thesis in Integrative Biosciences

By

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ABSTRACT

Erythropoietin (Epo) is a cytokine essential to the process of erythropoiesis and is currently in use as a therapeutic drug in human beings for treatment of anemia. Signals for erythroblast development are conducted via activation of JAK-2 and subsequently the cytoplasmic tyrosine sites in the erythropoietin receptor (EpoR). However, steady-state erythropoiesis seems to be controlled effectively by EpoR alleles that are either completely lacking, or truncated in terms of distal cytoplasmic phosphotyrosine sites. Present studies ascribe specific stress erythropoietic roles to the phosphotyrosine PY343 linked STAT-5 pathway in the Erythropoietin receptor (EpoR). Knock-in mice expressing a phosphotyrosine-null Epo receptor allele (EpoR-HM) exhibit defects in recovery from anemia due to hemolysis (Phenylhydrazine) or bone marrow suppression (5-Fluorouracil). In short-term transplantation experiments, donor EpoR-HM bone marrow cells also failed to efficiently rescue recipient mice from irradiation induced bone marrow ablation. In addition, Epo induced reticulocyte formation was attenuated in EpoR-HM mice. In each context, stress erythropoiesis was restored to wild-type levels upon the selective restoration of EpoR PY343 STAT-5 binding site (EpoR-H allele). In a newly developed ex vivo erythroid expansion system (SP34-EX), EpoR-HM erythroblasts exhibited marked stage-specific losses in Epo-dependent growth and survival. However, this defect was not observed in EpoR-H derived erythroid progenitor cells (EPCs). In order to characterize the downstream pathways employed by EpoR-HM and EpoR-H forms, signaling studies in response to Epo were conducted in bone-marrow derived EPCs. As an initial step, uniformity of JAK2 signaling was confirmed amongst the three alleles. As expected, STAT-5 was found to be activated via EpoR-H and wt-EpoR but not via EpoR-HM. For both EpoR-HM and EpoR-H, AKT and p70S6-kinase activation was decreased significantly. For Map kinases, JNK activation was minimal in the truncated

alleles. Interestingly, ERK was found to be hyperactivated uniquely via EpoR-HM allele. Also, in vitro studies with bone marrow derived EPCs demonstrate defects in EpoR-HM erythroblast differentiation (based on enucleation analysis, forward-angle light scatter profiles, and hemoglobinization) which were corrected upon MEK1,2 inhibition. These EpoR-HM–specific differentiation defects were not observed in EpoR-H EPCs. In addition, Epo-induction of the STAT-5 target genes *Pim-1* and *oncostatin-M* was found to be deficient in EpoR-HM derived erythroblasts. However a previously reported STAT-5 target gene *Bcl-x*, was not found to be modulated by Epo at the transcript level via wt-EpoR, EpoR-HM or EpoR-H alleles. Renal Epo transcripts were found to be slightly elevated in EpoR-HM kidney during steady-state erythropoiesis but were several fold higher than wt-EpoR or EpoR-H during stress erythropoiesis. In maturing $Kit^{neg}CD71^{pos}$ erythroblasts, oncostatin-M exerted anti-apoptotic effects, which likewise depended upon EpoR-PY343- mediated events. Stress erythropoiesis therefore requires EpoR-PY343-STAT-5 mediated pathways, certain of which converge and synergize with that of SCF and oncostatin-M. In addition, predominant signals emanating from EpoR-HM allele seems to involve ERK but is independent of STAT-5, STAT-1, p70S6-kinase, and JNK.

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ABBREVIATIONS

5-FU, 5-fluorouracil

AGM, Aorta gonads mesonephoros

Akt, Thymoma viral proto-oncogene 1

APCs, Antigen presenting cells

ATF-2, Activating transcription factor 2

B220, Protein tyrosine phosphatase, receptor type, C

Bak, BCL2-antagonist/killer 1

Bax, Bcl-2 associated X protein

Bcl-2, B-cell leukemia/lymphoma 2

Bcl-x, Bcl-2-like 1 protein

BFU-E, Burst forming unit-erythroid

bHLH, basic helix loop helix

Bim, Bcl-2 interacting mediator of cell death

BIT, Bovine serum albumin, insulin and transferrin

BKLF, Basic krupple like factor

BMP, Bone morphogenic protein

BrdU, Bromodeoxyuridine

BSA, Bovine serum albumin

Btg, B cell translocation gene

CBP, CREB binding protein

CD45, Cluster designation 45

CD71, Transferrin receptor

CFU-E, Colony forming unit-erythroid

CFU-meg, Colony forming unit-megakaryocytes

CIS-1, Cytokine inducible SH2 containing protein

c-Kit, SCF receptor

CLP, Common lymphoid progenitor

CMP, Common myeloid progenitor

myb, Cellular myeloblastosis oncogene

CNTF, Ciliary neurotrophic factor

CREB, cAMP responsive element binding protein 1

E2A, Transcripton factor 2 A

EGF, Epidermal growth factor

EKLF, Erythroid kruppel like factor

EPC, Eyrthroid progenitor cell

Epo, Erythropoietin

EpoR, Erythropoietin receptor

ERK, Extracellular regulated kinase

FACS, Fluorescence activated cell sorting

FALS, Forward angle light scatter

FL-1, Friend leukemia integration gene 1

FOG, Friend of GATA

Fos, FBJ osteosarcoma oncogene

GAB, Growth factor receptor bound protein 2-associated protein

Gas6, Growth arrest-specific 6

GATA-1 HRD, GATA-1 hematopoietic regulatory domain

GATA-1, GATA binding protein 1

GATA-2, Gata binding protein 2

G-CSF, Granulocyte colony stimulating factor

GFP, Green fluorescent protein

GM-CSF, Granulocyte macrophage colony stimulating factor

GMP, Granulocyte monocyte-macrophage progenitors

Gr-1, Lymphocyte antigen 6 complex, locus G

Grb2, growth factor receptor-bound protein 2

GSK3, Glycogen synthase kinase 3

HERF, Hematopoietic ring finger 1

HIF, Hypoxia inducible factor

IL-3, Interleukin 3

IL-7, Interleukin 7

IRS-2, Insulin receptor substrate 2

JAK, Janus kinase

JH, Jak homology

JNK, c-Jun N terminal kinase

Jun, Jun oncogene

Ldb1, LIM domain binding 1

LIF, Leukemia inhibitory factor

Lin, Lineage

LT-HSc, Long term hematopoietic stem cell

Lyn, Yamaguchi sarcoma viral (v-yes-1) oncogene homolog

Mac-1, Macrophage antigen alpha

MACS, magnetic activated cell sorting

Madh5, mothers against decapentaplegic homolog 5

Mcl-1, Myeloid cell leukemia sequence 1

MDS, myelodysplastic syndrome

MEK, mitogen activated protein kinase kinase

MEL, Murine erythroleukemia

MEP, Megakaryocyte-erythrocyte progenitors

MPP, Multipotent progenitor

m-sos, Murine homolog of Drosophila son of sevenless

mTOR, Mammalian target of rapamycin

NESP, Novel erythroid stimulating proteins

NFKB, Nuclear factor of kappa light chain gene enhancer in B-cells 1

Nix, BCL2/adenovirus E1B interacting protein 3-like

NK cells, Natural killer cells

onco-M, oncostatin-M

onco-MRβ, oncostatin-M receptor beta

p38MAPK, p38 mitogen activated protein kinase

PDK1, Phosphoinositide dependent kinase 1

PHZ, Phenylhydrazine

PI3K, Phosphatidylinositol 3-kinase

Pim, proviral integration site

PIP3, Phosphatidylindositol (3,4,5) triphosphate

PLC-gamma, phospholipase C gamma

P-S, phospho-serine

PTB-1B, Protein tyrosine phosphatase 1B

PU.1, SFFV proviral integration 1

PY, phosphotyrosine

Rb, Retinoblastoma gene

RBCs, Red blood corpuscles

rHuEpo, Recombinant human Epo

RON, c-met-related tyrosine kinase

SAPK, Stress activated protein kinase

Sca, Stem cell antigen

Shc, Src homology 2 domain-containing transforming protein C1

SHIP-1, Src homology 2 domain-containing inositol-5-phosphatase

SHP1, SH2 containing protein tyrosine phosphatase 1

SOCS, Supressor of cytokine signaling

SP-1, Trans-acting transcription factor 1

SP34-EX, SP34-containing serum-free erythroid expansion medium

STAT-1, Signal transducer and activator of protein 1

STAT-5, Signal transducer and activator of protein 5

ST-HSc, Short term hematopoietic stem cell

TAL1, T-cell acute lymphocytic leukemia 1

TNF-α, Tumor necrosis factor α

TRADD, TNF receptor type 1 associated death domain

TRAIL, Tumor necrosis factor (ligand) superfamily, member 10

TRPC, Transient receptor potential channel

TRPC2, Transient receptor potential cation channel, subfamily C, member 2

Tx, Transcripts

Vav, Vav1 oncogene

VHL, Von hippel landau

WT1, Wilms tumor antigen 1

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

A) HEMATOPOIESIS AND ERYTHROPOIESIS

Hematopoietic stem cells and lineages

Hematopoiesis (haima, blood + poiesis, formation) is a highly regulated complex series of events that are determined in part by hematopoietic growth factors and timely expression of transcription factors. The process of hematopoiesis is capable of giving rise to all cells that constitute the cellular component of blood (Figure 1.1). Erythrocytes or Red Blood Corpuscles (RBCs) are enucleated biconcave cells (7.5 u size) that contain hemoglobin, the major oxygen transporter in blood. RBCs primarily function to regulate oxygen and carbon dioxide transport between lung surface and deoxygenated tissues [1-3]. Granulocytes (Neutrophil, Eosinophil and Basophil) migrate among cells of various tissues and are predominantly involved in the processes of inflammation and phagocytosis. Platelets are extremely small enucleated cells that provide hemostasis on account of their ability to adhere, aggregate and activate the coagulation cascade [1-3]. Lymphocytes (B and T cells) render highly specific immunity either through production of antibodies (B-lymphocyte mediated) or through precise recognition of processed foreign antigens on cell surfaces (T- lymphocyte mediated) [1-3]. Macrophages are specialized cells derived from monocytes that function as antigen presenting cells (APCs) for the T-cells. Hematopoiesis begins early during embryogenesis and undergoes multiple changes as life progresses through its different stages including fetal and neonatal stages finally leading to adult hematopoiesis. The hematopoietic system is unique and dynamic in the sense that cells are

continuously being replenished and the rate of turnover is rapid (e.g. 24-48 hours in case of granulocytes) [1-3].

The definition of a hematopoetic stem cell is comparable to that of any stem cell; the ability to self-renew and give rise to all sub-lineages (in this instance, hematopoietic cells). As a result, hematopoietic stem cells are faced with a set of highly divergent options: to self-renew or differentiate. Once the multipotent hematopoietic stem cell commits to differentiation, it gives rise to two main cell types; 1) CMP (Common myeloid progenitor cell) which still retains a certain degree of pluripotency in terms of its ability to give rise to the GM (Granulocyte-monocyte-macrophage) or MEP (Megakaryocyte-erythrocyte) progenitor cells 2) CLP (Common lymphoid progenitor cell) which takes the lymphoid path to generate B and T cells as well as natural killer cells (NK cell) [2,3].

Embryonic and Adult erythropoiesis

Erythropoiesis (erythro, red cell + poiesis, formation) is the process of generation of mature circulating red blood cells. Erythropoiesis in the bone-marrow, spleen and liver consists of developmentally heterogenous erythroid progenitor cells at different stages of maturation. The whole mass of these erythroid cells has been functionally defined as an erythron consisting of different staged progenitors, from commited stem cell derivatives to the fully mature RBC [4]. Erythropoiesis is a tightly regulated process of proliferation, differentiation and survival that is influenced by physiological and stress requirements of tissue oxygenation. Post commitment, erythroid progenitor cells can be sub-divided into different populations based on specific gene

FIGURE 1.1

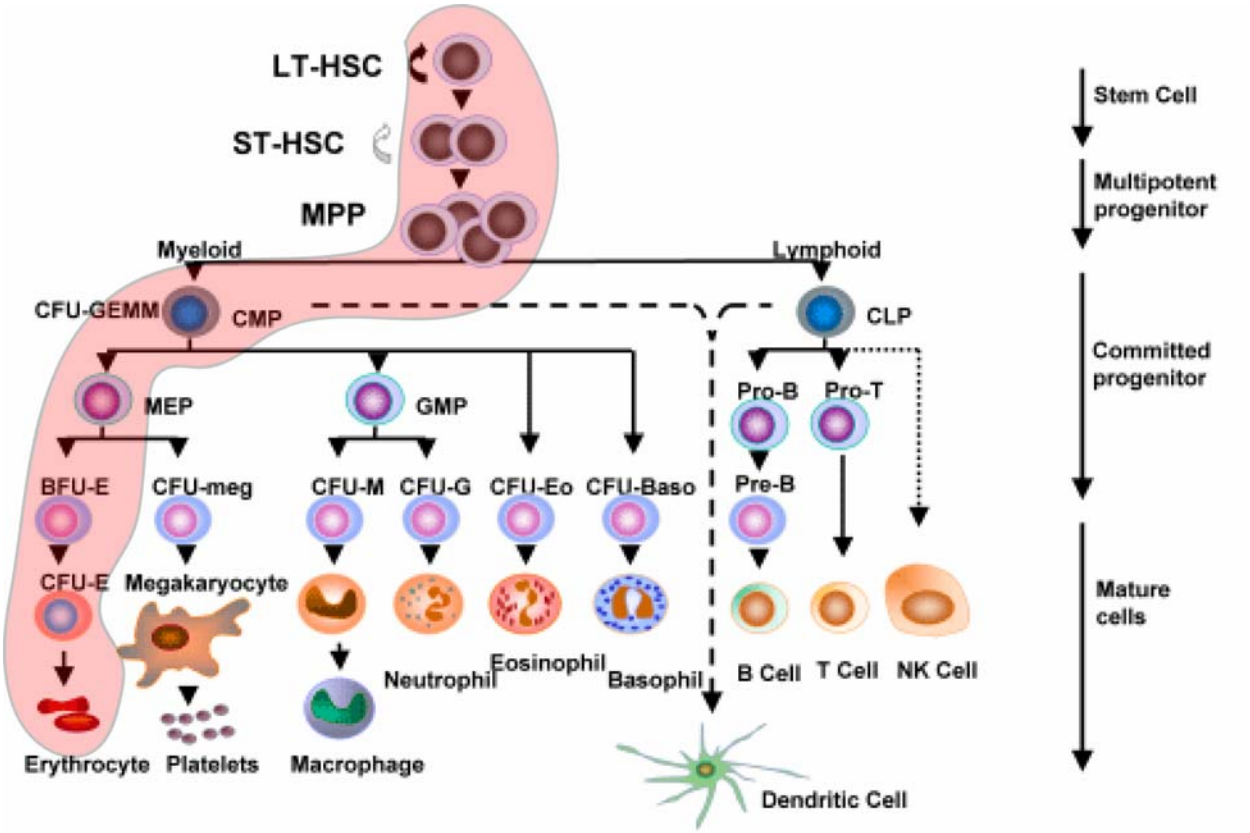

Figure 1.1. Hematopoietic lineages and cell-progression along the erythroid lineage.

Illustrated here is the step-wise development of various lineages of blood (Erythrocyte, Platelets, Macrophage, Neutrophil, Eosinophil, Basophil and lymphoid cells) from a hematopoietic stem cell). The shaded red portion demonstrates the formation of erythrocytes from a Long term hematopoietic stem cell (LT-HSC).

(Lund University, http://www.molmed.lu.se/hematopoiesis_p00.htm)

expression profiles and morphological patterns. Resident to the bone-marrow are the earliest functionally defined progenitors, BFU-E and CFU-E and their morphologically staged derivatives, erythroblasts (proerythroblast, basophilic erythroblast, polychromatophilic erythroblast and orthochromatic erythroblast) [1]. Cells released into circulation following enucleation are called reticulocytes, which achieve the status of a fully functional RBC after 48 hours.

The process of commitment of a hematopoietic progenitor cell along the erythroid lineage is presently unclear and three major theories seem to explain this event; 1) Stochastic theory or random commitment of cells to a specific lineage, 2) Instructive theory or determination of lineage commitment by an inductive micro-environment surrounding the hematopoietic stem cells, although its unclear what determines the formation or regulation of this environment around a cell and finally, 3) hematopoietic regulatory factors which act at later stages for cell-fate determination [1].

The earliest morphologically distinct erythroid precursor cells called erythroblasts are derived from erythroid progenitor cells called CFU-E (Colony forming unit-erythroid) and BFU-E (Burst forming unit-erythroid). These cells are not morphologically distinct, but are capable of giving rise to colonies of erythroblasts in semi-solid culture media in vitro. Under the influence of erythropoietin, these cells proliferate, differentiate, and form colonies in relatively precise time-intervals [5-7].

Burst-forming unit (BFU-E) is the least mature commited erythroid progenitor cell. Morphologically, they are highly related to multipotent hematopoietic stem cells by virtue of

their cell size, density and low percentage of cells in S phase [8,9]. In semi-solid assays, under the influence of Epo, SCF and IL-3, it gives rise to distinct hemoglobinized colonies containing 500-5000 erythroblasts in 5-7 days for mouse cells and 14-16 days for human cells derived from bone marrow [8,9]. Morphologically, they appear as highly immature blast cells with oval, lightly basophilic cytoplasm with occasional pseudopods, highly fine chromatin and large nucleoli. On electron microscopy, they seem to have abundant cytoplasm and polyribosomes, and the nucleus contains small amounts of clumped heterochromatin and prominent nucleoli [8,9]. The frequency of BFU-E is approximately 115 (human) and 40 (mouse) per 1 x 10^5 bone-marrow cells [10,11].

Colony-forming unit (CFU-E) is a functional cell very similar in structure and characteristics to proerythroblast – the earliest identifiable erythroid progenitor cell [12]. In semi-solid in vitro assay, on Epo exposure, they give rise to hemoglobinized colonies containing 8-50 cells in 2 days for murine cells and 5-8 days for human cells derived from bone-marrow [5-7]. Under light microscopy, purified CFU-Es appear as immature cells with fine nuclear chromatin, well defined large nucleolus, high nuclear-cytoplasmic ration and basophilic cytoplasm with pseudopods [8]. On electron microscopy, these cells appear as a highly primitive blast with fine nuclear chromatin, prominent nucleolus, and clear cytoplasm containing mitochondria and abundant pinocytic vesicles [8].The frequency of CFU-E is approximately 31 (human) and 170 (mouse) per 1 x 10^5 bone-marrow cells [8]. The size and number of the CFU-E compartment is directly proportional to the circulating levels of Erythropoietin. Either anemia leading to indirect increase in Epo or exogenous Epo administration leads to expansion of the CFU-E compartment [13]. It has been established that CFU-E is the most Epo responsive cell by virtue of functional assays and highest density of Epo receptors on cell-surface [14,15].

Various groups have successfully separated the BFU-E compartment from CFU-E using unit gravity sedimentation and centrifugal elutriation [16,17]. Recent efforts by Suzuki et al. and Terszowski et al. have further characterized these cells based on cell-surface markers and transgenic gene-reporter models [16,17]. Using GFP tagged mouse under the control of GATA-1-HRD domain, it was shown that BFU-E like cells can be identified as a GATA-1 and c-Kit expressing subpopulation with low transferrin (CD71) expression, whereas the CFU-E like cell was GATA-1^{pos}c-$Kit^{pos}$$CD71^{high}$ [18]. Further characterization by Terszowski et al. ascribed the following markers to the CFU-E cell; lin^{neg}c-Kit^{pos}Sca-1^{neg}IL-7$Ralpha^{neg}$IL-3$Ralpha^{neg}$$CD41^{neg}$$CD71^{pos}$ [19].

The nucleated erythroblasts which exhibit progressive decrease in size, increased condensation of nucleus and increased cytoplasmic to nucleus ratio as they mature can be sequentially defined as proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatic erythroblasts (Figure 1.2 and 1.3). **Proerythroblasts** are round or oval cells (14 to 19 um) with large nucleus and a rim of light basophilic cytoplasm. Nucleus has a very fine chromatin network and nucleoli may be prominent. They contain very little hemoglobin, although, electron microscopy analysis has exhibited traces of ferritin molecules. **Basophilic erythroblasts** are characterized by lack of nucleoli, decreased cell size (12 to 17 um) and condensation of chromatin. The nucleus may assume a characteristic wheel-spoke arrangement and the cytoplasm is deeply basophilic because of heightened ribosomal content, hence the name. **Polychromatophilic erythroblasts** exhibit the first signs of hemoglobinization and a decrease in ribosomal content, which gives the cytoplasm its characteristic pinkish-blue hue.

Irregular masses of chromatin, lack of nucleoli and a smaller size (11 to 15 um) characterize this cell. **Orthochromatic erythroblasts** are defined by maximum acidophilic staining due to increased hemoglobin content. This cell is the immediate precursor of reticulocytes and as the cell prepares for enucleation, the nucleus undergoes pyknotic degeneration, heightened chromatin condensation and nuclear shrinkage. **Reticulocytes** are formed after nuclear extrusion from orthochromatic erythroblasts. They are approximately the same size as RBCs and contain cytoplasmic organelles like ribosomes, mitochondria and Golgi complex. Reticulocytes are more adhesive than RBCs and seem to retain some amount of transferrin on surface. **Red blood corpuscles (RBCs or erythrocytes)** are formed from circulating reticulocytes after 48-72 hours. They have a unique biconcave shape (7.5um), lack cell organelles including ribosomes and are incapable of synthesizing any additional hemoglobin. Unlike reticulocytes, they do not contain mitochondria and consequently, do not utilize oxidative phosphorylation for their metabolic needs. The oxygen-transport protein, hemoglobin constitutes more than 95% of cytoplasmic proteins in erythrocytes.

Previous studies of erythropoiesis included ex vivo semi solid assays for BFU-E and CFU-E, morphological analysis and hemoglobin assays. The advent of flow cytometry and identification of various cell-surface markers allowed for proper delineation of sub-populations in a heterogeneous group of cells based on maturity and cell-stages. The most frequent cell-surface markers currently used in the study of erythropoiesis includes c-Kit, Transferrin receptor (CD71) and Ter119 [20-22]. Figure 1.3 depicts the different staged cells during the process of erythropoiesis and their corresponding cell-surface markers. **c-Kit** is the Stem cell factor (SCF) receptor and is broadly expressed in hematopoietic stem cells and early progenitors of all commited lineages [21].

In analysis of bone marrow cells, it was found that half of c-kit^{pos} cells do not express lineage markers like Mac-1, Gr-1, TER-119, and B220, while the remaining half coexpress myeloid lineage markers such as Mac-1 and Gr-1 [21]. After c-kit^{pos} cell depletion, the remainder of the cells were unresponsive to IL-3, GM-CSF, or M-CSF and failed to give rise to spleen colonies in marrow-transplantation context [21]. In our ex vivo expansion studies we use c-Kit expression (in conjunction with CD71) to demarcate immature erythroid progenitors that correspond mostly to proerythroblasts [23]. **Transferrin receptor (CD71)** is a membrane spanning glycoprotein responsible for the uptake of transferrin into the cell and consequently is involved in iron transport [22]. The level of transferrin receptors is directly proportional to the iron requirements of the cell. As a result, even though its expression is found in various cell-types, erythroid cells express maximum numbers of this receptor [22]. CD71 expression begins around the CFU-E stage (~300,000), reaches its maximum at the orthrochromatic erythroblast stage (~800,000) and decreases to about 100,000 on reticulocytes [1]. CD71 expression is absent on RBCs by virtue of absent hemoglobin synthesis [1]. **Ter119** antigen is a 52-kDa molecule associated with glycophorin A on erythroid cells in embryonic yolk sac, fetal liver, newborn liver, adult bone marrow, adult peripheral blood, and adult lymphoid organs [20]. Ter119^{pos} cells do not co-express cell-surface markers of other lineages (Mac-1, Gr-1, B220) and Ter119 is the only specific marker for late erythropoiesis (post proerythroblast stage) [20]. Ter119 expression is absent in early erythroid progenitor cells like BFU-E, CFU-E and some proerythroblasts [20]. Hence, a combinatorial analysis using Ter119+CD71 and c-Kit+CD71 using flow cytometry results in a fairly accurate interpretation of erythroid cell-staging.

The process of erythropoiesis occurs in two waves that are defined as primitive and definitive. Primitive erythropoiesis, so called because of similarities with that of earlier species like reptiles, birds and fish, begins in the mesoderm of yolk sac in specialized regions called blood islands [24,25]. Yolk sac erythropoiesis is predominant during the earlier phase of embryo development, through 6-8 weeks of gestation in humans, and 10-11 days of gestation in mice [24,25]. The second wave of erythropoiesis begins in liver, as it becomes the primary site of erythropoiesis around mid gestation (E 11.5) [26,27]. Erythropoiesis in the liver is believed to begin with seeding of cells originating from the aorta-gonads-mesonephros region [24,28], although some studies have indicated that yolk sac erythroid progenitor cells might provide for the first wave of definitive erythropoiesis leading to seeding of progenitors in the fetal liver [29]. There are several differences between primitive and definitive erythropoiesis. First, primitive erythrocytes have been shown to be larger than adult red cells, possess nucleus and contain more hemoglobin [30]. Interestingly, via co-staining for cells expressing embryonic βH1-globin, kingsley et al. have demonstrated enucleation in primitive murine erythrocytes in the peripheral blood stream between E12.5 and E16.5 [31]. However, in terms of enucleation, the major difference between primitive and definitive erythropoiesis is the actual site of this phenomenon, adult erythrocytes enucleate extravascularly in blood islands in close proximity to macrophages whereas primitive erythrocytes seem to undergo enucleation (specifically between E12.5 and E16.5) in the circulation. Second, primitive and adult erythrocytes express different subsets of hemoglobin. Primitive erythroblasts express both embryonic (ζ, βH1, and εy) and adult (α1, α2, β1, and β2) globins during cell maturation whereas the definitive erythrocytes express only α and β globins [32]. By E16-E18 most of the nucleated erythrocytes disappear from the circulation and RBCs are

predominantly enucleated [33].. In the neonatal and adult period, erythropoiesis shifts to predominantly bone-marrow in humans, and bone-marrow plus spleen in mice [34].

Figure 1.2. Electron microscopy images and cytospins of erythroid progenitor cells, reticulocyte and mature RBC. Depicted here are electron microscopy images of maturing erythroblasts (**A**, Proerythroblast,; **B**, Basophilic erythroblast; **C**, polychromatophilic erythroblast; **D**, Orthochromatic erythroblast). Also shown are cytospins of **E**, Proerythroblast; **F**, Basophilic erythroblast; **G**, early and **H**, late Polychromatophilic erythroblast; **I,** Orthrochromatic erythroblast. Note the progressive decrease in size, increased condensation of nucleus and increased cytoplasmic to nucleus ratio. Electron micrscopy images of **J**, Reticulocyte and **K**, RBC are also demonstrated. (Adapted from Wintrobe's Clinical Hematology textbook, Lippincott Williams and Wilkins)

FIGURE 1.2

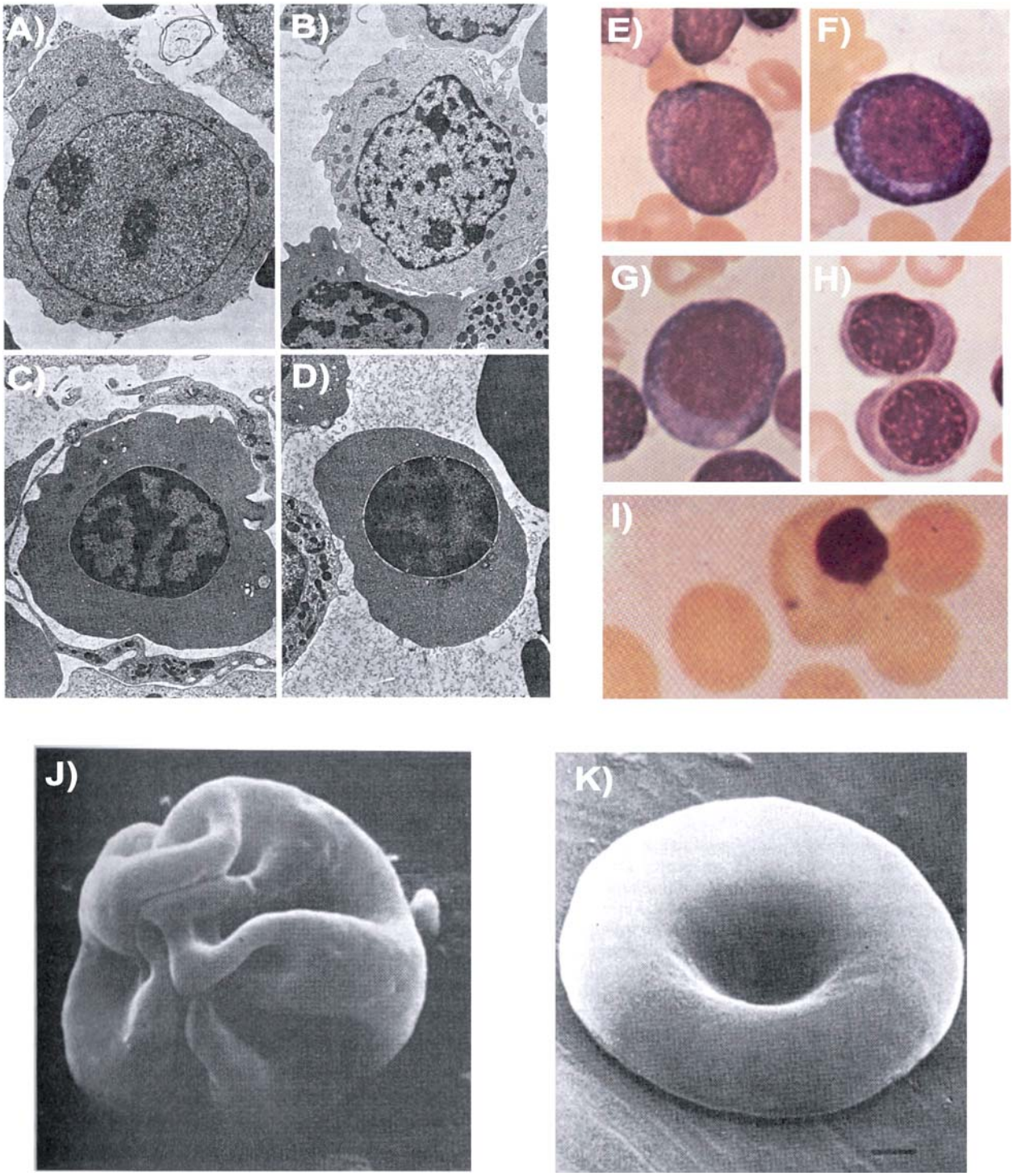

FIGURE 1.3

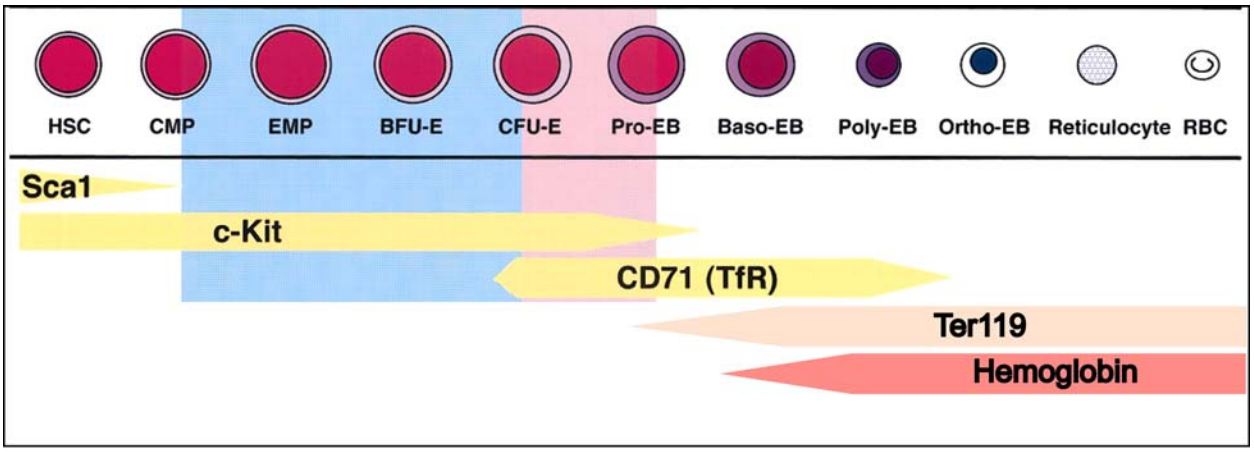

Figure 1.3. Summary of cell-surface markers and hemoglobinization at different stages of erythropoiesis. Depicted here are the expression profiles of c-Kit, CD71 and Ter119 and the corresponding erythroid cell-types along with levels of hemoglobinization.
(HSC, Hematopoietic stem cell, CMP, Common myeloid progenitors, EMP, Erythroid/megakaryocytic cells, BFU-E, Burst forming unit-erythroid, CFU-E, Colony forming unit-erythroid, Pro-EB, proerythroblast, Baso-EB, Basophilic erythroblast, Poly-EB, Polychromatophilic erythroblast, Ortho-EB, Orthochromatic erythroblast, c-Kit, Stem cell factor receptor, CD71, Transferrin receptor) (Adapted from Suzuki et al. Blood, 15 November 2003, Vol. 102, No. 10, pp. 3575-3583).

Transcription factor mediated regulation of hematopoiesis and erythropoiesis

A fairly well-defined subset of transcription factors regulate the process of self-renewal vs. differentiation for hematopoietic cells. For erythropoiesis, this involves a multitude of transcription factors that determine survival, proliferation and lineage commitment of multipotent hematopoietic progenitor cells along the erythroid component of hematopoiesis. These transcription factors function either alone or as a component of a greater transcriptional regulator complex. Some of these transcription factors include Stem cell leukemia (SCL), LIM-only protein 2 (LMO2), GATA binding factor-2 (GATA-2) and myeloblastosis oncogene (Myb). Factors that are predominantly involved in regulating erythropoiesis include GATA binding factor-1 (GATA-1), Friend of GATA (FOG), Erythroid Kruppel-like factor (EKLF), Basic Kruppel-like factor (BKLF), Friend leukemia integration gene-1 (Fli-oncogene), SFFV proviral integration 1 (PU.1), Hematopoietic RING finger 1 (HERF1), CREB-binding protein (CBP) and Signal transducer and activator of transcription (STAT-5) [35-37].

SCL (a basic helix-loop helix transcription factor) forms complexes with E proteins, like E2A and binds to conserved canonical sequences (CAGGTG) [38,39]. It is also responsible for forming a transcriptional factor complex with GATA-1, LIM domain binding 1 (Ldb-1), E12/E47 and LMO2 [40]. In a hematopoeitic context, SCL is expressed in all hematopoetic progenitor cells but at a more mature stage, it remains persistently expressed only in the erythroid, megakaryocytic and mast cell lineages [38,41]. In the erythroid lineage, expression of SCL remains persistent throughout but is maximum at the CFU-E stage [42]. The known gene targets for SCL in erythroid cells include glycophorin A and c-kit (Stem cell factor receptor) [43,44]. $SCL^{-/-}$ mice embryonic stem cells are unable to contribute to any hematopoetic lineage in a chimeric mice model, and

disruption of SCl gene in mice resulted in embryonic lethality around day E8.5 with a predominant absence of yolk sac hematopoiesis [45-47]. These studies confirm the central and indispensable role that SCL plays during hematopoiesis. Also, over-expression of SCL enhanced erythroid proliferation and differentiation in various cell lines and primary human CD34+ hematopoietic progenitor cells [48,49]. Continuation of SCL expression along the erythroid lineage seems to be vital for erythropoiesis, as demonstrated by failure of a SCL transgene (expressed under a stem-cell enhancer present in hematopoietic progenitors but absent in erythroid cells) to rescue erythropoiesis in SCL knockout embryos [50]. However, recent studies by Hall et al. using conditional $SCL^{-/-}$ mice have demonstrated that although these mice were mildly anemic and exhibited decreased proliferative potential in their progenitor cells, they were capable of forming fully functional mature RBCs (albeit without Ter119 expression) in response to erythropoietin and hemolytic stress [51]. Thus, in the light of conflicting studies, it seems unclear whether SCL is indispensable for erythropoiesis or whether a differential requirement exists in terms of embryonic vs. adult erythroid development.

LIM-only protein 2 (LMO2), a cysteine-rich LIM domain containing protein, has been implicated in the pathogenesis of T-cell acute lymphoblastic leukemia [36,37]. Even though LMO2 is expressed in a variety of tissues, maximum expression has been noted in hematopoietic tissue [52]. Although LMO2 is incapable of binding DNA on its own, it is capable of forming a complex with other DNA-binding transcription factors such as SCL and GATA-1 [36,37]. The critical role of LMO2 in hematopoiesis is evidenced by studies in $LMO2^{-/-}$ which demonstrate embryonic lethality around E9 because of absent yolk sac hematopoiesis [53]. Also, in a chimeric mice model, ES cells with deficient LMO2 fail to generate any hematopoietic lineages [54]. With regards to

erythropoiesis, LMO2 seems to have unique roles depending on the transcription factors it complexes with, e.g. with SCL and GATA-1 it promotes erythroid differentiation in xenopus pole explants [55] whereas a pentameric complex of LMO2, SCL, E2A, Lbd1 and pRb represses erythroid differentiation [56].

GATA-2 is a zinc finger domain containing protein which binds to a conserved consensus sequence (T/A GATA A/G) found in promoter elements of erythroid genes like globins, band 3, EKLF, FOG, Epo receptor and heme enzymes [36,37]. GATA-2 expression is limited to undifferentiated ES cells, immature hematopoietic progenitors, erythroid progenitor cells, megakaryocytes, mast cells, and endothelial cells [2,57]. GATA-2 expression seems to be vital for erythroid progenitor cell proliferation, albeit, at the expense of differentiation [58]. GATA-$2^{-/-}$ mice exhibit embryonic lethality around E10-11 due to failed yolk sac hematopoiesis and severe embryonic anemia [37]. Ex vivo differentiation assays of GATA-2 null ES cells demonstrated severe deficiency of primitive mast cell and erythroid colonies along with pronounced decrease in proliferation and survival of immature hematopoietic progenitors [59]. A delicate balance exists between the GATA family of transcription factors, with a specific requirement of GATA-2 downregulation followed by GATA-1 upregulation, to enforce differentiation of early erythroid progenitor cells [58]. In recent ex vivo studies using mouse ES cells with a Tetracycline inducible GATA-2 and/or PU.1, it was demonstrated that GATA-2 in partnership with PU. 1 is capable of inhibiting macrophage differentiation and promoting commitment along the erythroid and megakaryocytic lineage [60]. GATA-2 functions, therefore, seem to highly complex and not only involve survival and proliferation but possible lineage determination roles.

Myb is a proto-oncogene with heightened expression in immature hematopoietic progenitors (erythroid, myeloid and lymphoid) [37]. Myb null mice demonstrate normal primitive hematopoiesis but fail to progress beyond E15 due to failed definitive hematopoiesis [61]. The levels of all circulating blood cells are decreased in the $Myb^{-/-}$ mice with the exception of megakaryocytes, granulocytes and monocytes [37,61]. Unique roles for Myb in determining erythroid lineage commitment have been demonstrated in recent studies utilizing fetal livers from $Myb^{-/-}$ mice [62]. The results from these studies also indicate a growth arrest at the CFU-E stage and c-kit as a transcriptional target of Myb [62].

GATA-1 is a zinc finger containing transcription factor that is vital for erythropoiesis. Its expression is limited to erythroid cells, megakaryocytes, mast cells, eosinophils, multipotential hematopoietic progenitor cells and testis [55]. A 5' enhancer and a first intron element regulate GATA-1 expression in erythroid cells and together they are called the hematopoeitic regulatory domain or GATA-1-HRD [63]. GATA-1 is capable of forming both activating as well as repressing transcriptional complexes depending on the interacting partners [64]. GATA-1 is known to activate erythroid genes like α and β-globin, band 3 or EKLF and repress genes like GATA-2, myc and myb [65,66]. In addition, GATA-1 has been shown to transactivate the erythropoietin receptor gene [67]. Interestingly, recent studies indicate that Erythropoietin is also capable of phosphorylating and activating GATA-1 through the PI3-kinase/Akt pathway [68]. GATA-1 null mice are embryonic lethal (E 10.5) due to failed primitive and definitive erythropoiesis and failure to progress beyond the proerythroblast stage [69,70]. In addition, $GATA\text{-}1^{-/-}$ ES cells exhibit defects in survival at the proerythroblast stage in ex vivo assays and fail to give rise to mature RBCs in chimeric mouse models [71]. Recent studies in patients with Downs syndrome with both transient

myeloproliferative disorder and acute megakaryoblastic leukemia have led to the discovery of a short form of GATA-1 which is incapable of supporting normal erythropoiesis but is fully capable of supporting megakaryopoieisis [68].

Friend of GATA (FOG) is a close interacting partner of GATA-1 with similar expression profiles [72]. FOG protein consists of nine proposed zinc fingers four of which interact with amino zinc fingers of GATA-1 [73]. A mutated form of GATA-1, which is incapable of interacting with FOG, was found to be deficient in supporting erythropoiesis [74]. $FOG^{-/-}$ mice are embryonic lethal (E 10.5) due to failed erythropoiesis at a stage similar to that of $GATA\text{-}1^{-/-}$ mice [72]. In contrast, $FOG^{-/-}$ mice also exhibit severe deficiencies in megakaryopoiesis [72]. Thus, FOG seems to have GATA-1 independent roles during hematopoiesis.

Erythroid Kruppel like factor (EKLF) is a zinc finger containing protein responsible for the transcriptional regulation of β-globin -the predominant adult globin in hemoglobin [36,75]. The expression of EKLF is restricted to erythroid, megakaryocytic and mast cells [76]. EKLF null mice die around E16 due to severe anemia and β-globin deficiency [77]. However, expression of embryonic globins (ε globins), fetal globins (Aγ and Gγ) and α globins was normal [77]. Roles have been ascribed to EKLF as a factor involved in the switch from fetal to adult globin expression by virtue of elevated γ-globin expression in $EKLF^{-/-}$ mice with a complete human β-globin locus [77]. Indeed, overexpression of EKLF in mice results in a premature switch from gamma to beta globins [78]. In addition, in humans, naturally occurring mutations have been discovered in the consensus binding motif of EKLF resulting in β-globin deficiency and β-thalassemia along with elevated γ-globin levels [79,80].

CREB-binding protein (CBP) is a histone acetyltransferase (HAT) protein that interacts predominantly with GATA-1 through its zinc finger, in the context of erythropoiesis [81]. CBP not only aids GATA-1 transcriptional activity by acetylation of histone but is also responsible for acetylating GATA-1 itself on conserved lysine residues [82,83]. This modification apparently improves GATA-1 transcriptional activity [82,83]. It has also been demonstrated that mice with haplo-insufficiency of CBP have aplastic anemia and increased incidence of hematological malignancies [84].

PU.1, an ETS family member, is important for development of granulocytic, monocytic and lymphoid lineages [36]. In the erythroid lineage, PU.1 seems to anatagonize GATA-1 function and vice-versa [85,86]. Recently, it was shown that PU.1 directly binds to GATA-1 at the promoter of target genes, creates a repressive complex of proteins and shuts down the erythroid program [86]. Transgenic mice expressing PU.1 are more susceptible to erythroleukemias and retroviral expression of PU.1 in bone marrow erythroid cells leads to their immortalization and inhibition of differentiation along the erythroid lineage [85,87,88]. In addition, $PU.1^{-/-}$ mice fetal liver derived erythroblasts exhibit proliferation arrest, increased apoptosis and premature differentiation emphasizing the role of PU.1 in the self-renewal of immature erythroid progenitor cells [89].

STAT-5 belongs to the the STAT (Signal transducer and activation of transcription) family and is the predominant STAT activated through the Erythropoietin receptor. Its role in erythropoiesis is discussed in detail below (See Chapter 4).

B) ERYTHROPOIETIN (Epo)

Overview of Erythropoietin

Erythropoietin, a 34,000 Da glycoprotein, is the major humoral regulator of erythropoiesis and is essential for the proliferation, differentiation and survival of erythroid progenitor cells [90-92]. The existence of this molecule was discovered almost a century back by Carnot and Deflandre who named it "hemopoietine", by virtue of the ability of an anemic rabbit's plasma to induce reticulocytosis when injected into normal recipient rabbit [93]. Purification of tryptic fragments of Epo from human urine and the deciphering of their amino acid sequences has allowed the isolation and cloning of human genomic DNA encoding erythropoietin [94,95]. Shortly thereafter, results of a successful trial of recombinant Epo in patients with anemia of chronic renal failure was published [96]. The Epo gene is located on chromosome 7, as a single copy in a 5.4 kb DNA fragment which encodes a polypeptide chain of 193 amino acids [97]. The gene also encodes a 27 amino acid signal peptide (leader sequence) which is eliminated during Epo secretion [97]. Erythropoeitin has four glycosylated chains that are absolutely necessary for its in-vivo activity and it has been shown that asialated and non-glycosylated Epo are non-functional [98,99]. Presently, the Food and Drug Administration (FDA) has approved Erythropoietin for treatment of anemia of chronic disease including chronic renal failure, chemotherapy and irradiation induced anemia, and in patients scheduled for elective noncardiovascular surgery.

In fetal stage, liver is the major site of Epo synthesis [100], although there is evidence of Epo gene expression in mammalian mesonephric kidney during early gestation [101]. In other studies, murine Epo transcripts were detected in the fetal liver around 14 days of gestation and Epo mRNA

appeared in the kidney around E 20 [102]. Shortly after birth, the kidney assumes the role of the major Epo producing organ, although studies have indicated that adult liver is capable of secreting about 7% of total Epo [103,104]. Induction of anemia leads to increase in Epo transcript levels in liver and kidney of rats within 1 hour [105,106]. In another experiment, anemia due to bleeding led to a 1000 fold increase in Epo transcripts in the kidney [106]. Various studies have indicated that the site of Epo production in the kidney rests in the interstitial cells of the kidney cortex. In addition, via in-situ hybridization studies and immuno-histochemistry, Epo transcripts were further co-localized to cells expressing 5 'ectonucleotidase- the peritubular interstitial fibroblasts [107]. These findings were confirmed by subsequent studies using transgenic mice expressing SV40 T-antigen under Epo gene regulatory sequences [108]. Immunohistochemistry assays detected T-antigen in Ito cells, which are fibroblast like renal interstitial cells [108]. It is still unclear whether Epo transcription in response to anemia is due to increase in the number of Epo-producing cells or due to increase in Epo-transcripts on a per-cell basis. Within the liver, in situ hybridization studies have revealed the source of Epo to be hepatocytes surrounding the central veins [109].

Regulation of Erythropoietin

The initial perception was that Epo production was a direct result of decrease in hematocrit and that the body had sensing and feedback mechanisms in place for this function. However, it is now known that Epo transcript regulation is consequent to the levels of oxygenation of the body. Production of Epo is predominantly in response to different kinds of hypoxia. These include hypoxic hypoxia (tissue hypoxia in combination with either decreased alveolar O_2 or arterial O_2) and anemic hypoxia (tissue hypoxia without decreased arterial O_2) [110]. The production of Epo is

directly related to the amount of transcripts produced but it might also relate to increased transcript stability [111]. Specific DNA sequences containing putative regulatory region of Epo were expressed in transgenic mice to study Epo regulation [112]. These techniques as well as other studies were capable of identifying certain promoter sequences, which in isolation are capable of oxygen-dependent Epo regulation [112,113]. One such sequence that was identified was a transcriptional enhancer region in the 3' region of Epo. When this specific sequence was excised and tagged with reporter genes, it conferred oxygen dependent transcription of these genes in transfected cells [113]. Subsequent studies identified Hypoxia inducible factor (HIF) as a regulatory protein which binds to this sequence [114]. Since then, this consensus motif has been described as "hypoxia inducible element" and is found in a variety of genes where it carries out transcript regulation in an oxygen dependent way [115]. HIF protein is a heterodimer consisting of alpha and beta subunits which belong to the basic helix-loop-helix protein (bHLH) super family [116]. Studies have indicated that HIF-1ά is the predominant Epo regulatory HIF isoform. Under normoxic conditions, HIF-1α is hydroxylated by the enzymes proly-hydroxylases which use oxygen as their substrate [117]. The protein VHL (Von Hippel Lindau protein) then binds to the hydroxylated form of HIF-1α and targets it for proteasomal degradation [117]. Under hypoxic conditions, this interaction is minimal or absent, hence, HIF-1α is stabilized and available to bind the hypoxia inducible elements of Erythropoietin gene. Recent studies, however, have characterized HIF-2 α as a major oxygen dependent regulator of Epo transcription by virtue of specific expression in Epo-producing cells in liver and kidney. Also, siRNA studies in specific cell-lines revealed that HIF-2 α inactivation but not HIF-1α resulted in blunting of hypoxia mediated Epo response [118,119]. Recently, it was demonstrated by Dame et al. that WT1 (Wilms Tumor 1) is capable of activating erythropoietin gene expression in a tissue-specific manner [120]. In addition, GATA

binding sites have been demonstrated on the erythropoietin gene promoter. Specifically, hGATA-1, 2 and 3 have been demonstrated to inhibit erythropoietin gene expression in Hep3B cells {Imagawa, 1997 #762}. Also, GATA-4 has been shown to enhance erythropoietin gene transcription specifically in fetal liver hepatocytes {Dame, 2004 #759}. Interestingly, in embryonal carcinoma cell lines P19 and F9, Retinoic Acid enhances Epo expression in an oxygen-independent manner {Kambe, 2000 #760}.

Role of Erythropoietin in a non-hematopoietic context

Distinct from its role in erythropoiesis, recent evidences point to Epo's role in diverse nonhematopoietic functions. Epo and EpoR expression has been demonstrated in a wide variety of tissues e.g., heart, liver, bone marrow, gastro-intestinal tract, pancreas, reproductive orgran, prostate, brain and endothelial cells of vascular system [121]. Of all these tissues, role of Epo in brain and vascular system remains most extensively researched. In these tissues, Epo primarily acts to promote survival through PI3-Kinase/Akt, STAT-5, NFkB pathways and by upregulating the anti-apoptotic protein Bcl-xl [121]. In various in vitro and in vivo assays, Epo is shown to act as a survival and anti-inflammatory agent (by blocking the effects of IL-6 and TNF-α) predominantly in the context of cerebral ischemia and neuronal injury [122,123]. Evidence also points to the role of Epo in brain in a developmental context [124]. In the vascular system, Epo plays a dual role by maintaining the integrity of endothelial cells and promoting new vessel growth (angiogenesis) [121,125]. In various oxidative stress injury models, Epo is shown to protect endothelial cells by decreasing free radical production and caspase activity [121]. Also, after ischemic brain injury, Epo has been shown to improve circulation to ischemic cells by causing proliferation of endothelial cells and neo-vascularization [126].

Because of Epo's role in promoting survival, reducing inflammation and enhancing angiogenesis some degree of skepticism exists regarding the use of Epo in anemia associated with malignancies. A plethora of studies have indicated the presence of either Epo or EpoR in various malignancies e.g., hepatoblastomas, lung cancer, melanoma, cerebral astrocytomas and medulloblastomas, pancreatic cancer, prostate cancer, sarcomas, thyroid cancer, bladder cancer, gynecological malignancies (ovarian, endometrial and cervical), GI malignancies, wilms tumor of kidney, head and neck cancers and breast carcinoma [127]. Recently, a clinical trial of Epo in breast carcinoma was terminated due to higher mortality in the Epo group as compared to placebo [128]. In addition, clinical trials of Epo in head and neck cancer patients revealed reduced survival rates in the Epo group [129]. Various studies have indicated functional Epo-EpoR signaling in multiple primary tumors and tumor cell lines [127]. Interestingly, the expression of EpoR splice variants was also demonstrated in cancer cell lines, which could probably influence Epo signaling [130]. In at least two studies, investigators have been able to reduce tumor proliferation, survival and angiogenesis by using either neutralizing Epo antibodies or JAK2/STAT-5 inhibitors (the downstream signaling component of EpoR) [131]. In addition, Epo also is capable of increasing tumor cell survival by inducing the anti-apoptotic genes bcl-xl, bcl-2 and mcl-1 as well as by modulation of the NF-kB pathway [132,133]. Thus, a generalized approach of administering Epo in anemia associated with all malignancies and/or chemotherapy-radiotherapy is probably not judicious and Epo therapy needs to be individualized depending on the type of malignancy.

Other erythroid stimulating proteins

After the initial cloning of the Epo gene and establishment of its role in therapy as recombinant human Epo (rHuEpo), various groups developed newer compounds that either mimic rHuEpo or function better. Two groups in 1996 succesfully developed novel peptides that act as epo mimetics [134-137], In addition, they also studied the x-ray crystallophic structure of the Peptide-EpoR complex and determined that these Epo mimetics achieved perfect dimerization of the EpoR [134]. Also, Naranda et al. were successful in synthesizing a peptide with a unique binding region in EpoR distinct from that of endogenous Epo [138]. This peptide was capable of activating the JAK-Stat pathway in a synergistic fashion with endogenous Epo [138]. Quereshi et al. have been successful in developing non-peptide compounds fully capable of binding to the Epo receptor and activating downstream components [139].

Later in 2001, Amgen researchers successfully developed a longer-acting, more stable form of Epo called Darbepoietin or Novel erythroid stimulating protein (NESP) [140,141]. It is known that glycosylation of Epo is vital for its in vivo function. NESP has additional sialic acid containing carbohydrate chains that were added in such way that it did not hinder the ability of the protein to bind the EpoR (Figure 1.4) [142]. Subsequent studies revealed that this hyperglycosylated form of Epo has higher in vivo activity, longer serum half-life and is more stable [140,141,143]. It is known that inspite of reduced affinity of hyperglycosylated Epo for the EpoR, the dissociation rate of darbepoietin is higher than rHuEpo [143,144]. This in turn translates to decreased degradation and longer half-life.

FIGURE 1.4

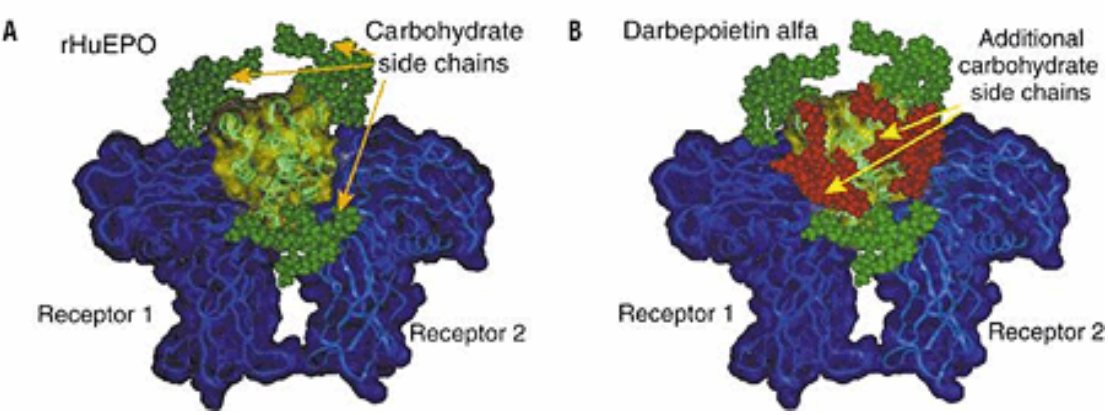

Figure 1.4. Three dimensional structural analysis of rHuEpo and NESP (Darbepoietin alpa). A) represents a complex between rHuEpo and Epo receptor whereas **B)** represents the complex between NESP and Epo receptor. The Epo receptors are shown in blue, the n-linked carbohydrates and the artificially added carbohydrates are shown in green and red respectively. (Elliott S, Lorenzini T, Asher S, et al.. Enhancement or therapeutic protein in vivo activities through glycoengineering. *Nature Biotechnology***.** March 2003).

C) ERYTHROPOIETIN RECEPTOR (EpoR) and EpoR SIGNALING

Hematopoietic growth factor signaling and EpoR overview

Intercellular communication in the hematopoietic and immune system is mediated through soluble factors called cytokines. Based on specific amino acid sequences and conservation of specific sequences, the cytokine receptors have been classified into either Type I (class I) or Type II (class II) cytokine receptor family [145-147]. Most of the receptors involved in hematopoetic signaling belong to the Type I receptor superfamily. These include receptors for erythropoietin, thrombopoietin, G-CSF, GM-CSF, IL2 (γ chain), IL3-7, IL9-11, LIF and oncostatin M [145-147]. This family also includes receptors that function outside the hematopoietic and immune system e.g. CNTF, prolactin and growth hormone [145-147]. Structurally most cytokine receptors consist of a multi-unit protein complex with an extracellular domain (ligand binding subunit), transmembrane domain and a cytoplasmic domain (signal tranducing unit). The extracellular domain has a length of approximately 200 amino acids and is highly conserved amongst the different cytokine receptors. It contains four positionally conserved cysteine residues in the amino-terminal region and a WSXWS motif at the carboxy terminal. The cytoplasmic subunit of cytokine receptors is less conserved excluding two regions of highly conserved amino acid residues in the membrane-spanning region (Box 1 and Box 2). In addition, the cytoplasmic subunit has various tyrosine motifs that recruit downsteam signaling proteins. The most notable feature in cytokine receptors is the lack of a kinase domain in their cytoplasmic domain [145-147]. Upon binding of a ligand to the extracellular domain, the receptor molecules form homo-dimers or hetero-dimers (in a few cases also hetero-trimers). But in some instances e.g. Epo receptor, they exist as preformed dimers. Ligand binding leads to recruitment and activation of protein

tyrosine kinases like JAK and Src-kinases, which phosphorylate the tyrosine motifs in the cytoplasmic tail and activate downstream signaling pathways [145-147].

Erythropoietin receptor (EpoR) is a member of the Type I cytokine superfamily [91]. After radiolabelled Epo was made available, existence of Epo receptors was seen in normal as well as transformed erythroid cells [148]. The complete gene representing EpoR was isolated using a cDNA probe from a MEL (Murine erythroleukemia cell) library [149]. The EpoR gene has eight exons and is approximately 5000 base pair long [149]. EpoR expression has been demonstrated on erythroid cells and a wide-variety of non-hematopoietic tissues (normal and malignant) (see above) [8,121,150]. Maximum expression of Epo receptor is observed primarily between the CFU-E stage and proerythroblast stage [150]. Studies using radio-labelled Epo have revealed small number of receptors on BFU-E but these cells exhibited a substantially lower number of Epo receptors as compared to more mature cells and a weaker response to Epo [8]. In primary human erythroid system, the number of Epo receptors on cell-surface gradually decreases during differentiation (post proerythroblast stage) and reticulocytes and erythrocytes have minimal to no expression of Epo receptors [150]. Recently, it was demonstrated by Heinrich et al. using a knock-in mouse model expressing GFPcre fusion protein under the control of endogenous erythropoietin receptor (EpoR) promoter that maximum expression of GFP is obtained in the Stem cell antigen –1 Sca-1^{neg} c-kit^{pos} stage and expression gradually starts decreasing at $Ter119^{pos}$ stage [151].

The indispensable nature of Epo and EpoR in erythropoiesis has been demonstrated via gene knockout strategies [152,153]. Both Epo and Epo receptor knockout mice exhibit embryonic lethality around day 13 due to failed definitive erythropoiesis [152]. The embryos were visibly pale and

EpoR$^{-/-}$ mice had smaller fetal livers [152] (Figure 1.5 and Figure 1.6). While the wt-EpoR fetal liver had both non-nucleated erythrocytes and few yolk-sac derived nucleated erythrocytes, the EpoR$^{-/-}$ liver preparations showed only proerythroblasts and yolk-sac derived nucleated erythrocytes [152]. However, these studies revealed that committed BFU-E and CFU-E cells were present in both Epo$^{-/-}$ and EpoR$^{-/-}$ fetal livers, indicating that Epo is not required for lineage commitment [152]. Epo$^{-/-}$ fetal liver cells were capable of forming CFU-E and BFU-E colonies in the presence of Epo while EpoR$^{-/-}$ fetal liver cells transfected with wt-EpoR retrovirus also formed colonies in presence of Epo that were comparable to wt-EpoR efficiencies in size and number. However, in untransfected EpoR$^{-/-}$ fetal liver liver cells, no BFU-E or CFU-E colonies were detected in the presence of Epo [152]. Thus, it seems that BFU-E and CFU-E progenitors develop independent of Epo, but their survival, proliferation and eventual differentiation depends on Epo. Regarding primitive erythropoiesis, EpoR$^{-/-}$ E 7.5 yolk sac seems to contain normal number of primitive erythrocytes that expressed near normal levels of embryonic globins, although, after E 9.5 they were reduced in number and exhibited decreased proliferation ex vivo [152,153]. Thus, studies indicate either a partial requirement of Epo or a specific subset of cells requiring Epo, in the context of primitive erythropoiesis [152,153]. Recently, it was demonstrated that erythroid specific expression of EpoR via a GATA-1 HRD domain rescues the embryonic lethality suggesting that lack of EpoR in other non-hematopoietic tissues like blood vessels, brain and heart is non-contributory to the embryonic lethality [154]. Interestingly, Yu et al. successfully demonstrated that expressing the human EpoR in the EpoR$^{-/-}$ mice fully rescued embryonic lethality and subsequent normal adult erythropoiesis [155].

Transcriptional control of EpoR seems to depend predominantly on the transcription factor GATA-1 and SP-1 binding sites in the 5' flanking region of the EpoR [156]. It was also shown that GATA-1 was capable of transactivating the EpoR and transcriptional activation is enhanced by SP-1 or SP-1 like proteins [156]. Subsequent studies, however, revealed that a CCACC motif in the +79 to +135 fragment of the human erythropoietin receptor negatively regulated EpoR transcription [157]. It was also demonstrated that SP-1 weakly bound to this site and EpoR transcription inhibition was directly proportional to the binding affinity of SP-1 [157]. Studies in the erythroid cell line UT-7 have demonstrated that EpoR and GATA-1 expression is closely related to proliferative cell-cycle status (S and G2/M) and that EpoR mRNA levels closely corresponded to the DNA binding activity of GATA-1 to the EpoR promoter [158].

FIGURE 1.5

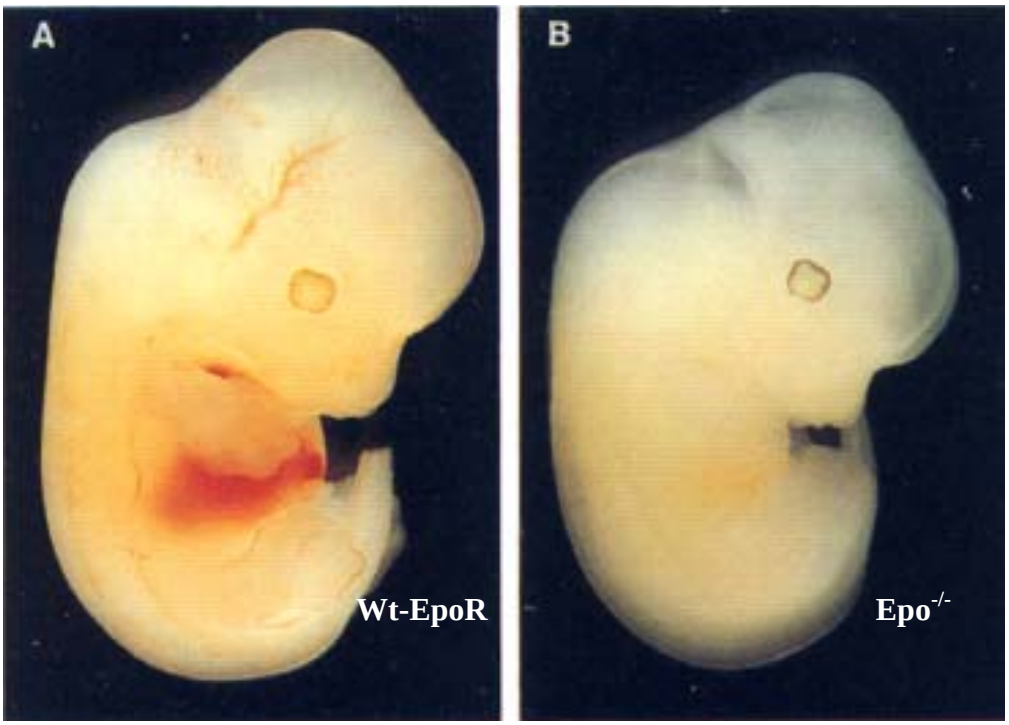

Figure 1.5. Embryos of E13 littermates from Epo +/- heterozygotes. Wt-EpoR embryo is shown on the left (A) while $Epo^{-/-}$ embryo is shown on the right with significant reduction of circulating red cells and smaller and paler liver. (Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995;83:59-67)

FIGURE 1.6

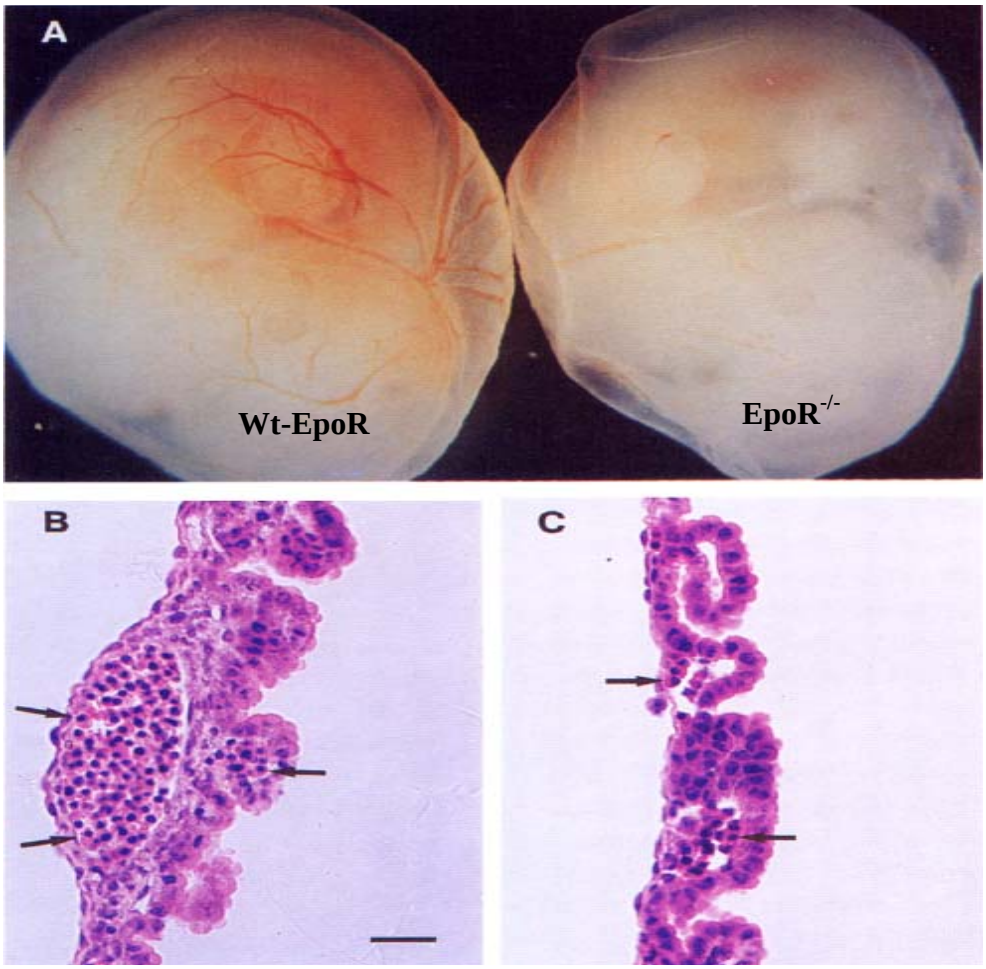

Figure 1.6. whole embryos and embryo sections from E 11 wt-EpoR and EpoR$^{-/-}$ mice. A) The EpoR$^{-/-}$ embryo appears pale with decreased number of circulating RBCs as compared to wt-EpoR. Also, Yolk sac sections reveal nucleated RBCs (see arrows) within the vessels in wt-EpoR sections (B) whereas EpoR$^{-/-}$ sections reveal empty vessels and occasional nucleated RBCs (see arrows) (C). **(**Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995;83:59-67)

Erythropoietin receptor structure and activation

EpoR is a single-transmembrane receptor belonging to the Type 1 family of cytokine receptors [91,145]. It has a conserved 225 amino acid extracellular ligand-binding domain consisting of fibronectin III-like subdomains, a transmembrane segment as well as a 235 amino acid cytoplasmic domain with no intrinsic kinase activity [90-92]. The cytoplasmic segment contains a β-chain cytoplasmic box 1 motif which is the binding site for non-receptor tyrosine kinases like JAK kinases [159]. The structure of the extracellular domain of the EpoR was deciphered via analysis of crystal structure of a EpoR complex with a non-related agonist peptide [134]. The extracellular domain consists of two subdomains, termed D1 (amino acids 11-113) and D2 (amino acids 118-218) [90]. Conservation and homology in the extracellular domain is by virtue of presence of four conserved cysteines (two in each D domain) that form two disulfide bonds and the WSXWS motif in the membrane spanning region [91]. Almost all mutations in the WSXWS motif inhibit protein folding in the endoplasmic reticulum and hinder surface expression of EpoR except one (A234E) which results in more efficient processing and surface expression [92,160]. At assembled EpoR complexes, Epo binding occurs via Epo high affinity αA, αB, and αD site-1 and low-affinity αA and αC site-2 helix domains to the hydrophobic flat region of EpoR (Phe 93, Met 150, Phe 205) [161,162] (Figure 1.7). Contrary to initial beliefs that Erythropoietin receptor exists as monomers that homodimerize in response to Epo binding, studies incorporating x-ray crystallography and in vivo protein fragment complementation have revealed that EpoR exists as a preformed dimer [163,164]. In its unliganded native state, EpoR extracellular domains are in a configuration which holds the intracellular domain with the JAK2 molecules separated from each other and therefore, in an inactive status. Binding of Epo to EpoR causes a conformational change bringing the two D2 subunits closer, which in turn brings the intracellular domains and

JAK2 molecules in close proximity to each other (Figure 1.8) [164]. Subsequently, JAK2 molecules are capable of cross-phosphorylating and activating each other as well as other tyrosine subunits in the cytoplasmic tail of EpoR [164-166].

Inspite of unsuccessful attempts to crystallize the full-length EpoR, mutagenesis studies have revealed the critical motifs in the transmembrane and juxtamembrane regions that are vital for EpoR function. Studies have revealed that EpoR dimerization depends on leucine residues at position 240 and 241 in the transmembrane region [90,167]. It is important to note that impaired dimerization depends on mutation of both 240 and 241 leucine residues and it seems that substitution of only one site with alanine is incapable of hindering EpoR dimerization or function [167]. In addition, Seubert et al. have described various dimeric transmembrane orientations which either impart full activity to the cytosolic domain (JAK-Stat pathway and MAP kinase), partial activity (activation of MAP kinase only) or total inactivity [168].

Intracytoplasmic domain of EpoR lacks enzymatic activity and consists of conserved domains; Box1 (amino acids 257-264), Box 2 (amino acids 303-313) and the region between Box 1 and Box 2 (amino acids 265-302) [92]. Several residues in the conformationally rigid hydrophobic motif of cytoplasmic domain of EpoR have been found to be important for activation of the non-receptor tyrosine kinase JAK2 (L253, D287 and Box 1 motifs -L257 and W258) [162]. In addition, Miura et al. have demonstrated that mutations either at position W282 (between box 1 and box 20 or LEVL motif of box 2 impaired Epo mediated EpoR activation [92,169]. It was also shown that the W282 motif in the membrane proximal region is vital for a physical association between JAK2 and EpoR leading to subsequent JAK2 phosphorylation/activation [165]. Studies by Wu et

al. and Kapur et al. have demonstrated that Erythropoietin receptor contains an extended box 2 (residues 329-372) that is essential for physical interaction of EpoR with the tyrosine kinase receptor Kit. Interestingly, SCF (Kit ligand) was found to transphosphorylate EpoR and the physical interaction between the two receptors was deemed necessary for this function [170,171]. The region identified as important for EpoR internalization is downstream of Box 1 between amino acids 267-276 [172]. This region further was characterized as containing an endocytosis motif and was required for Epo mediated proliferation [173]. In addition, intracytoplasmic segment of EpoR contains eight highly conserved tyrosine sites that are phosphorylated by the EpoR/JAK2 pathway [90,174,175]. Subsequently these tyrosine sites recruit a variety of signaling molecules that activate different pathways (see below).

Role of JAK2 and other non-receptor tyrosine kinases

As mentioned above, EpoR does not have a cytoplasmic kinase domain, so it recruits non-receptor kinases, the predominant of which is JAK2 [159,164-166]. Converse to previous lines of thought, JAK2 seems to be bound to the Epo receptor even during the unliganded state although, the receptor conformation does not allow the two JAK2 molecules to come in contact with each other (see above and Figure 1.8) [163,164]. The sites in the Epo receptor that are important for binding to JAK2 are discussed above. JAK2 has seven conserved unique domains, termed JAK-homology (JH) domains [145]. JH1 is the catalytically active subunit of JAK2 and the adjacent JH2 is termed as the pseudokinase domain because it lacks any observable kinase activity inspite of considerable homology to the tyrosine kinase domains [145]. Both the JH1 and JH2 domains are located in the C-terminus of JAK2 (Figure 1.9) [145]. There is some evidence that the pseudokinase domain might have inhibitory roles in the function of JAK2 and it was demonstrated that

tyrosine phosphorylation in the JH2 domain at Y570 was found to inhibit JAK2 signaling [176,177]. The other domains (JH3-JH7) are located in the N-terminus of JAK2 (Figure 1.9) [90,145]. The N-terminal domain were shown to important for both physical assocication with EpoR as well as appropriate Golgi processing and surface expression of Erthropoietin receptor [178]. Specifically, it was postulated that JH4-JH7 which is termed as the FERM (band4.1, ezrin, radixin, moesin-homology region) domain was responsible for proper golgi-trafficking of the EpoR-JAK2 complex [90]. Previous studies indicated that Y1007 auto/trans-phosphorylation in the JH1 domain of JAK2 was responsible for initiating the catalytic activity of JAK2 [166]. However, it was demonstrated recently that JAK2 autophosphorylation at Y221 can also increase its catalytic activity [177]. In addition, YXX[L/I/V] was determined to be the consensus motif for JAK2 activated targets [177]. However, amongst eight cytoplasmic tyrosine residues in the Epo receptor, only four of them (Y343, Y401, Y429, Y431) contain this consensus motif [90]. The mechanism of phosphorylation of other cytoplasmic EpoR tyrosine sites following JAK2 activation is presently unclear and might involve one or more intermediaries.

$JAK2^{-/-}$ mice are embryonic lethal and die around E 12.5 due to failed definitive erythropoiesis and embryonic anemia [179,180]. The phenotype of $JAK2^{-/-}$ mice is very similar to that of $Epo/EpoR^{-/-}$ mice except that JAK2 null fetal livers do not contain CFU-E or BFU-E [179,180]. Even though $JAK2^{-/-}$ and $Epo/EpoR^{-/-}$ mice share considerable similarities, deficiency of JAK2 seems to result in early lethality both in terms of gestational age as well as decreased representation of progenitor cells at an earlier stage [179,180].

Various factors are known to negatively regulate JAK2 [90]. The protein LNK recently was shown to inhibit EpoR and JAK2 signaling and $LNK^{-/-}$ mice exhibit elevated number of erythroid progenitors, increased CFU-E responsiveness to Epo and enhanced splenic erythropoiesis [181]. Interestingly, other factors that negatively regulate JAK2 seem to be activated by EpoR/JAK2 pathway and may represent a negative feedback mechanism. Some of them include Suppressor of cytokines signaling (SOCS) factors, Cytokine inducible SH2-containing protein 1 (CIS-1), SH2 containing protein tyrosine phosphatase 1 and 2 (SHP1 and SHP2), Protein tyrosine phosphatase 1B (PTB-1B) and Cluster designation 45 (CD45 phosphatase) [90,182-186]. These factors are discussed in more detail below.

Other non-receptor tyrosine kinases involved in transducing EpoR mediated signals include Lyn, Btk and Src. Initial studies in J2E cells had indicated that **Lyn**, a Src kinase family member, is required for erythroid differentiation [187]. In addition, Lyn was found to activate STAT-5 via association with EpoR PY694 upon Epo activation [188]. Lyn deficiency in $Lyn^{-/-}$ mice has been linked to deficient GATA-1, EKLF and STAT-5 expression [189]. $Lyn^{-/-}$ mice exhibit increased splenic erythropoiesis as evidenced by increased frequencies of BFU-E and CFU-E [189]. Also, these mice recover more quickly from anemic stress but exhibit defective clearing of senescent RBCs [189]. Recent studies by Karur et al. in Lyn deficient mice indicate that Lyn is needed for differentiation and survival of late stage erythroblasts [190]. **Btk** belongs to the family of Tec kinases and has been demonstrated to be rapidly activated via EpoR/JAK-2 pathway [191]. $Btk^{-/-}$ erythroid progenitors exhibit delayed activation of STAT-5 and increased differentiation at the expense of proliferation [191,192]. In addition, Btk deficient cells were found to be more susceptible to apoptosis induced by the death ligand TRAIL [191]. **Src** has been demonstrated to be important

for Epo mediated differentiation of erythroid K562 cells as well as robust generation of CFU-Es from murine bone marrow [193,194]. In addition, even though a physical association with the Epo receptor has not been established, Src has been shown to activate STAT-5 and PI3-kinase in response to Epo stimulation [193,194].

Positively regulating signal transduction factors (STFs)

As mentioned above, Erythropoietin receptor contains eight conserved tyrosine sites (PY) that bind a variety of SH2 and/or PTB signal transduction molecules in response to binding of Epo to EpoR and subsequent JAK2 (or other non-receptor protein kinases) activation [91,174,175]**.** Various cell-line, transgenic and knock-in/knockout studies have stressed the importance of these PY-sites and their downstream effectors [90-92,175,195,196]. The following section is a description of various positively modulating factors mediated through the EpoR with either known EpoR cytoplasmic PY specificities or presently undiscovered PY-binding sites.

PI3-Kinase/AKT pathway is one of the primary mediators of proliferation and survival in erythroid cells [197-199]. Phosphatidylinositol-3 Kinase (PI3-Kinase) is a heterodimer consisting of regulatory SH2 domain containing P85 subunit and catalytic p110 subunit which phosphorylates membrane phosphoinositides at the inositol ring [200]. Through immunoprecipitation studies, initial results had indicated a physical association between p85 subunit and Epo receptor in response to Epo stimulation [201,202]. Subsequently, Klingmuller et al. demonstrated by retrovirally transducing EpoR mutants into $EpoR^{-/-}$ fetal liver cells that Erythropoietin receptor PY479 is the primary site responsible for binding the p85 subunit of PI3-kinase [199]. Mutant EpoR with a single PY479 site was fully capable of supporting the proliferation and differentiation of CFU-E cells derived from

fetal EpoR$^{-/-}$ livers [199]. In addition, studies in Day-14.5 p85alpha$^{-/-}$ revealed pale embryo with decreased representation of the erythroid CFU-E and BFU-E compartment [203]. Subsequent studies have revealed other mechanisms of activation of PI3-Kinase through association with proto-oncogene Vav, GAB family proteins (Gab1 and Gab2) and Insulin receptor substrate-2 (IRS-2) [204-207]. In addition, PY343 and PY401 sites in the EpoR were found to be principally responsible for activating GABs in response to Epo, which in turn led to a physical interaction between the activated GABs and p85 subunit of PI3-Kinase [205]. Recently, RON, a receptor tyrosine kinase, was found to associate with JAK2 and EpoR in response to Epo and get phosphorylated [208]. Once activated, RON was demonstrated to bind and phosphorylate GAB1 [208]. Thus, this pathway might be potentially another mechanism leading to PY479 independent activation of AKT. One of the primary downstream targets of PI3-kinase is a Serine-threonine kinase called AKT, which binds to the phosphorylated phosphoinositides in the membrane and gets activated through another set of kinases called phosphoinositide dependent kinases 1 and 2 (PDK 1 and 2) [200]. AKT is primarily responsible for regulating cell-survival, cell-cycle progression, mRNA translation and metabolism through a wide variety of downstream targets [200]. In the context of erythropoiesis, AKT's action seems to be primarily mediated through Forkhead proteins like FOXO3A, Glycogen synthase kinase-3 (GSK3) and ribosomal protein p70S6-kinase [197,209-211]. FOXO3A is predominantly a pro-apoptotic factor that positively reinforces the transcription of apoptosis causing ligands like Fas ligand, TRAIL (TNF-related apoptosis-inducing ligand) and TRADD (TNF receptor type 1 associated death domain) [200]. In addition, it also enhances the transcription of other members of the pro-apoptotic machinery like Bim (bcl-2 interacting mediator of cell death), a pro-apoptotic Bcl-2 family member and Bcl-6 [200]. Initial studies in the FOXO3A$^{-/-}$ mice revealed anemia and compensatory reticulocytosis but

a more thorough study might reveal additional defects [212]. In the context of preventing apoptosis, AKT is primarily responsible for phosphorylating FOXO3A leading to its cytoplasmic retention and nuclear exclusion [213]. Recently, B cell translocation gene 1 (BTG) was demonstrated to be a novel target of FOXO3A in an erythroid cell line [214]. These studies also indicated that FOXO3A levels increase upon erythroid differentiation and that premature expression of FOXO3A leading to increased BTG, had anti-proliferative and pro-differentiation effects [214]. GSK3 is another target of AKT in erythroid cells, and it has been demonstrated that Epo mediated activation of AKT leads to phosphorylation of GSK3 and subsequent inactivation. Upon Epo withdrawal, accelerated cell death is noted due to increase in GSK3 activity and a subsequent activation of the pro-apoptotic molecule BAX [210]. Ribosomal protein p70S6-kinase, a target of AKT, is predominantly involved in the translational machinery and regulation of cell cycle progression [92,197,211]. Interestingly, recent studies demonstrate that a constitutively active form of AKT was capable of restoring wild type differentiation profiles to fetal liver cells from $JAK2^{-/-}$ mice [215]. In addition, these studies indicate that AKT might also have a role in differentiation that is unique and unrelated to its survival function [215]. Also, recent studies have revealed that PI-4-phosphatase II is a Epo-responsive gene that mediates negative regulation of the PI3K/AKT pathway in the context of a possible feedback loop [216].

MAP Kinase pathway members known to play a role in erythropoiesis consist of ERK, p38 map kinase and JNK. ERK or Extracellular regulated kinase activation is because of the Ras-Raf-MEK pathway**.** The activation of Ras-Raf-MEK-ERK pathway can be achieved via two SH2-domain containing adaptor proteins, Grb2 and Shc [217-220]. Receptors that utilize Janus kinases typically depend upon receptor beta-chain PY site coupling to a Grb2-mSos route to Ras-Raf-MEK-ERK signaling. Direct Grb2-EpoR interactions have not been demonstrated for EpoR, but

Epo can stimulate Shc and Grb2 phosphorylation, and Shc can complex with Grb2 [217]. Shc phosphorylation also can occur independently of EpoR PY sites via JAK-2 route [218], but Epo activation of Raf-1 and hence, ERKs can occur via Shc-independent mechanisms [221]. The other known routes for Grb2 engagement involves SHIP-1 or Shp2 and PY401 has been demonstrated to be required for recruitment and activation of Shp2 [219,220]. Recently, PY499 mediated recruitment of PLC-gamma followed by ERK1/2 activation was demonstrated [222]. In cytokine RTK systems, by comparison, coupled G-proteins can also modulate Ras (in part via Shc-dependent mechanisms) [223]. One related mechanism proposed for EpoR stimulation of Ras/Raf/MEK/ERKs involves Gi protein binding to an EpoR C-terminal domain as studied in CHO cells [224]. There is a growing body of evidence that ERK plays a very critical role in regulating the process of erythroid proliferation vs. differentiation [225-227]. It was recently demonstrated that a constitutively active MEK/ERK pathway leads to a block in erythroid differentiation, which was corrected by the addition of UO126 (MEK inhibitor) [225]. Similarly, the ligand TRAIL was found to exert its anti-differentiative effects on cord-blood derived erythroblasts predominantly through the ERK route [226]. Recently, Epo mediated activation of Raf-1-ERK pathway was shown to maintain proliferation and inhibit differentiation via downregulation of the death ligand Fas which has pro-differentiation roles in erythropoiesis [227]. In addition, SCF (Stem cell factor) seems to act in a synergistic fashion with Epo to activate ERK1/2 in purified human erythroid colony-forming cells [228].

p38 map kinase is one of the members of the SAPK (Stress activated protein kinase) family [229]. P38 alpha is known to activate its downstream targets primarily ATF-2 and CREB (indirectly through ribosomal SK kinases) which in turn, are involved in transcriptional regulation [229].

It has been demonstrated that p38 map kinase activation can be achieved by Epo and is involved in promoting proliferation [230,231]. While some p38 alpha$^{-/-}$ mice exhibit early embryonic lethality due to placental defect, most survive until E 14.5, but subsequently die of severe anemic and defects in definitive erythropoiesis [232]. Specifically, the p38 alpha null mice have decreased representation of later-stage erythroblasts, however, the representation of the BFU-E and CFU-E compartment seems to be normal [232]. Further studies revealed that erythroid defects in these mice were a direct result of reduced expression of Epo in fetal liver of p38 alpha$^{-/-}$ mice. Thus, a role for p38 map kinase in either Epo expression or Epo transcript stabilization was proposed [232].

JNK- c-jun N-terminal kinase belongs to the SAPK (Stress activated protein kinase) family and has been demonstrated in various studies to be activated by Epo and subsequently modulate proliferation, survival and differentiation of erythroid cell [231,233-235]. Activated JNK is responsible for phosphorylating AP-1 which is heterodimer of c-jun and Fos proteins [236]. In addition, AP-1 DNA binding and transcriptional activity have been demonstrated to increase in response to Epo [237]. Specifically, activated JNK is known to phosphorylate the N-terminal of c-Jun and increases its transactivation potential [238]. In SKT-6 cell lines, anti-sense oligos against JNK inhibited Epo induced hemoglobinization and differentiation [233]. In addition, it was demonstrated that osmotic or heat shock treatment of these cells resulted in activation of JNK (and p38 map kinase) which resulted in Epo-independent differentiation [231]. Epo induced JNK activation also seems to be important for cell proliferation as studied in three erythroid cell line studies and it was clearly demonstrated that apoptosis induced by Epo withdrawal was JNK independent [234,235]. Also, Jacobs-Helber et al. recently demonstrated that proliferation of murine BFU-E depended on JNK activity, although this effect might not be completely Epo-dependent by virtue of low numbers of

Epo receptors on BFU-E cell surface [235]. In addition, studies in MEK kinase 1 (activating kinase for JNK) null mice revealed embryonic anemia due to a failure of definitive erythropoiesis [219].

Regulation of calcium concentrations in erythroid cells seems to be mediated via Epo mediated phosphorylation of PY460 [239]. Subsequent studies revealed that Epo was capable of modulating calcium levels in cells via members of the transient receptor potential channel (TRPC), TRPC2 and TRPC6 [240,241]. Recent studies demonstrated that TRPC2 mediated calcium influx is via phospholipase C gamma and inositol 1,4,5-trisphosphate [242]. Calcium has been implicated both in differentiation of erythroid cells as well as triggering apoptosis in mature erythrocytes [91,243]. Proteins belonging to the STAT (Signal transducers and activator of transcription) family are very important positive mediators activated by the Epo/EpoR pathway and are discussed in detail below.

Negatively regulating signal transduction factors (STFs)

Epo is capable of recruiting some negative regulatory factors like SOCS family proteins, Cytokine inducible SH2-containing protein 1 (CIS-1), SH2 containing protein tyrosine phosphatase 1 and 2 (SHP1 and SHP2), Protein tyrosine phosphatase 1B (PTB-1B) and Cluster designation 45 (CD45 phosphatase) [90,182-186]. Recruitment of these inhibitory factors might constitute a Epo mediated feedback mechanism.

*Suppressors of cytokine signaling (SOCS***)** family of proteins have been implicated as negative factors for various cytokine receptors [244-247]. SOCS proteins consists of a unique N-terminal region, central SH2 domain and a c-terminal SOCS box that targets proteins for degradation via

ubiquitin-protesomal pathway [244-248]. Role of three SOCS family proteins namely cytokine-inducible SH2 domain containing protein-1 (CIS-1), SOCS-1 and SOCS-3 have been characterized in erythroid cells [248]. CIS-1 is known to associate through its SH-2 domain with the Epo receptor PY401 and has been shown to negatively regulate EpoR-JAK2-STAT-5 signaling [249,250]. Studies have demonstrated that CIS-1 is an early immediate response gene of STAT-5 and is involved in regulating proliferation and survival of erythroid cells [249-251]. However, CIS-1 transgenic mice do not seem to exhibit any erythroid phenotype [252]. SOCS-3 has been shown to bind to both EpoR and JAK-2 to negatively regulate EpoR signaling. EpoR PY401 and the newly identified higher affinity PY429/PY431 are the PY sites on the Epo receptor that bind to SOCS-3. SOCS-3 deficient mice exhibit embryonic lethality because of placental defects while at the same they exhibit marked erythrocytosis with increase proliferative capacity of the erythroid progenitor cells [253]. SOCS-1 has been shown to be activated by Epo and phosphorylation of PY1007 in JAK2 is needed for SOCS-1 binding and ubiquitin-proteosomal pathway degradation [182]. However, SOCS-1 deficient mice demonstrate low hematocrits and aberrant erythroid differentiation [254]. In addition, SOCS-$1^{-/-}$ mice derived CFU-E exhibit hypersensitivity to Epo [255].

Tyrosine phosphatases involved in negative regulation of EpoR signaling include the SH2-domaining containing phosphatases (Shp1 and Shp2), cluster designation 45 (CD45) and protein tyrosine phosphatase 1B (PTP-1B) [90,182-186]. Shp1 has been shown to bind to EpoR at PY429 and PY431 and terminate JAK2 signaling [184]. Erythroid cells with mutated EpoR PY429 exhibit increased proliferative response to Epo and prolonged Epo mediated JAK-2 phosphorylation [184]. Also, erythroid progenitor cells from *motheaten (me)* mouse, which has germline mutations in Shp1, exhibits Epo hypersensitivity and Epo-independent proliferation [256]. Shp2 is rapidly

phosphorylated in response to Epo and is recruited to the PY425 site of EpoR [220]. However, this PY site in Epo is linked to proliferation and this might be a result of Shp2 mediated Grb2 recruitment and subsequent ERK activation [220]. In addition, ES cells deficient in Shp2 exhibits decreased differentiation along the erythroid lineage [257]. Deficiency of CD45, a JAK phosphatase, in mice results in predominant defects in the lymphoid compartment [186]. However, these mice also have increased representation of BFU-E in the bone marrow compartment [186]. Also, it was demonstrated in CD34+ human progenitor cells that CD45 was involved in inhibiting differentiation along the erythroid lineage and this effect was primarily a result of LYN inactivation [258]. Through substrate-trapping mutants, it was recently demonstrated that Protein tyrosine phosphatase (PTP-1B) predominantly targets Tyk2 and JAK-2 [185]. However, its role in erythropoieis needs to be explored further.

Inositol phosphatases involved in negative regulation of EpoR signaling includes SH2-inositol phosphatase (SHIP-1) [259]. It is predominantly involved in dephosphorylating Phosphatidylinositol (3,4,5)-triphosphate (PIP3) which is engaged in a number of pathways including PI3-kinase. SHIP-1 has been demonstrated to be recruited to PY401 of EpoR and SHIP-$1^{-/-}$ mice exhibit elevated levels of BFU-E and CFU-E [259,260].

Regulation of apoptosis in erythropoiesis (EpoR and non-EpoR mediated)

The major players regulating apoptosis in erythroid cells include the PI3-Kinase-AKT pathway, Bcl-2 family members (Bcl-xL, Mcl-l, Bax, Nix and Bak) and the death ligands (Fas, TNF and TRAIL). The PI3-Kinase-AKT pathway has been clearly demonstrated to play a very important anti-apoptotic role in erythroid progenitor cells and is discussed in detail in the previous section

(see above). B cell lymphoma-xL (Bcl-xL) belongs to the Bcl-2 family and it's a predominant anti-apoptotic factor pertaining to erythropoiesis based on several studies in cell-lines and primary models [261-263]. The Bcl-xl knockout mice is embryonic lethal at E12.5 due to marked apoptosis in brain and severe embryonic anemia [264]. In addition, conditional disruption of Bcl-xL gene in the hematopoietic lineage resulted in hemolytic anemia and markedly decreased survival of circulating reticulocytes [262]. However, in both mouse models representation of early erythoid progenitor cells (BFU-E and CFU-E) remains unperturbed. It seems likely that Bcl-xL plays an anti-apoptotic role only in the later stages of erythropoiesis as also evidenced by markedly higher protein expression at more mature stages of differentiation [265]. In addition, exogenous expression of Bcl-xL seems to eliminate the dependence of erythroid progenitor cells on Epo for differentiation [266]. Initial investigations had indicated the regulation of Bcl-xL via JAK-2/STAT-5 pathway at a transcriptional level, but newer studies (including some from our lab) have brought this theory into question and indicate possible regulation of Bcl-xl by Epo at a post-transcriptional or post-translational level [196,267]. This issue is discussed in further detail in the subsequent section (see below). Also, studies in mouse and human erythroblasts have demonstrated that Epo induces up- regulation of other members of the Bcl-2 family e.g. Bak (pro-apoptotic) and Mcl-1 (anti-apoptotic) [261]. In human erythroblasts, the conformational isoform of Bax (pro-apoptotic Bcl-2 family member) associated with apoptosis has been shown to be repressed through Epo induced PI3 kinase activation [210]. Also, an additional pro-apoptotic member of the Bcl-2 family, Nix, has been shown to be co-expressed with Bcl-xL during terminal differentiation of erythroid cells [265]. However, its precise role in modulating apoptosis during erythropoiesis needs to be elucidated further. Thus, a complex interplay between the pro-

and anti-apoptotic members of the Bcl-2 family seems to be in place for regulation of Epo induced proliferation and differentiation.

Fas ligand and Fas belong to the Tumor necrosis factor (TNF) family [268]. Expression of both Fas and Fas ligand has been detected on erythroid progenitor cells, although, levels of Fas ligand expression is substantially higher in more mature cells [269,270]. These death ligands seem to induce apoptosis in earlier progenitor cells by activating Caspases 3, 7 and 8 leading to the cleavage of essential erythroid transcription factors GATA-1 and TAL1 [268,271]. Interestingly, on Epo exposure, Fas ligand expression on more mature cells is either abrogated or reduced [269]. This leads to the hypothesis that at physiological states (low Epo), this mechanism leads to a check in the production of RBCs. However, on exposure to increased Epo as seen during need for accelerated erythropoiesis, down-regulation of Fas in the more mature cells might be a way to prevent apoptosis in the earlier progenitor cell. Also, recent studies indicate that downregulation of Fas/Fas ligand via Erythropoietin is essential in splenic erythroblasts for accelerated erythropoiesis during anemia states [272]. In addition, Fas has been linked to various erythroid disorders like Myelodysplastic syndrome (MDS), multiple myeloma and β-thalassemia [273-275].

Other pro-apoptotic members of the TNF family which modulate erythropoiesis include TNF-α and TRAIL [276-278]. TNF-α was demonstrated to have different effects on normal erythroid progenitor cells vs. erytholeukemic cell lines [278]. In erythroid progenitor cells, it was found to inhibit proliferation and induce apoptosis, whereas in erythroleukemic cell lines, TNF-α promoted proliferation [278]. TRAIL belongs to the TNF family of death ligands and it modulates a variety of functions like apoptosis, inflammation and immune responses [279,280]. TRAIL has been

demonstrated in the human CD34+ cell system to reduce the size and number of erythroid colonies as well as survivability of erythroblasts [276]. However, the effect was very stage specific and more mature glycophorin A positive cells were unresponsive to the effects of TRAIL [276]. Recently, Trail mediated apoptosis in erythroid cells was demonstrated to be hindered via Epo mediated induction of PKCζ [277].

FIGURE 1.7

Figure 1.7. Crystal structure of Epo-EpoR complex. Shown here is the crystal structure of a complex between Erythropoietin and Erythropoietin receptor with circled Site 1 (high-affinity and Site 2 (low-affinity) interfaces. (From Stroud lab structural gallery, www.msg.ucsf.edu/ stroud/gallery_epo.html).

FIGURE 1.8

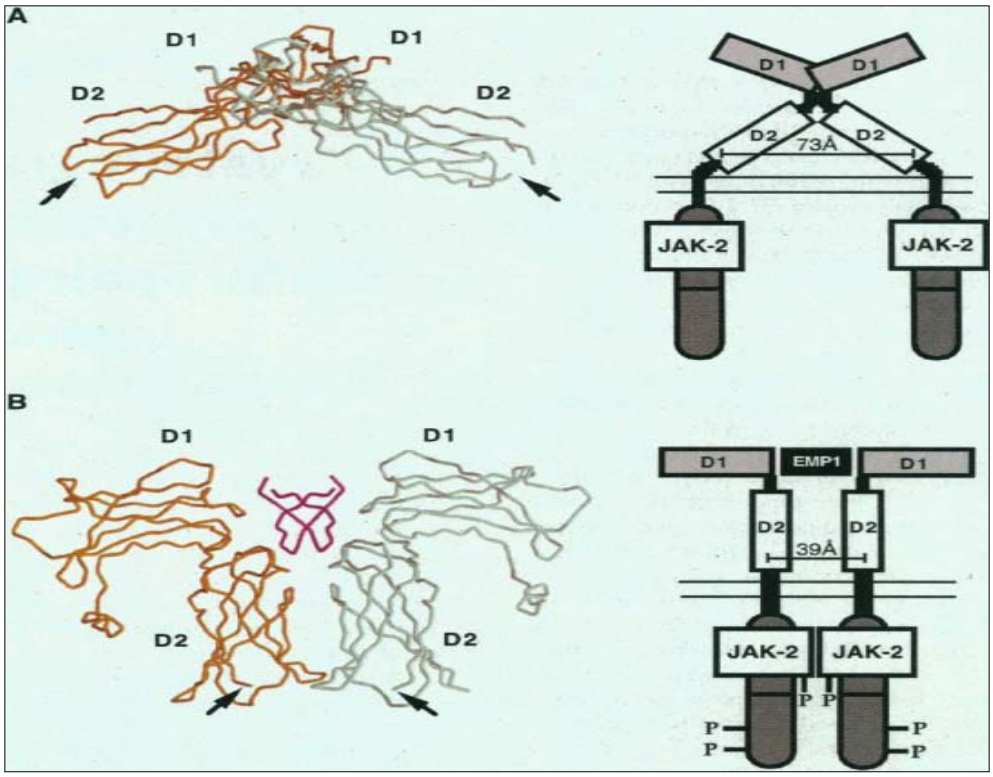

Figure 1.8. Comparison of unliganded and liganded state of Epo-EpoR complex. A) Unliganded inactive state- The two individual extracellular domains of the EpoR dimer are shown in gold and silver. In the unliganded state, the membrane proximal regions of D2 subunits are separated from each other. This leads to separation between the intracellular units and JAK2. **B)** Active liganded state- In this state, binding of Epo or EMP1 leads to a conformational change bringing the D2 subunits closer to each other. This in turn, brings the cytoplasmic domain and JAK2 molecules in close contact. This leads to autophosphorylation of JAK2 and subsequent phosphorylation of intra-cytoplasmic tyrosine sites. (Livnah O, Stura EA, Middleton SA, Johnson DL, Jolliffe LK, Wilson IA. Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. Science. 1999;283:987-990).

FIGURE 1.9

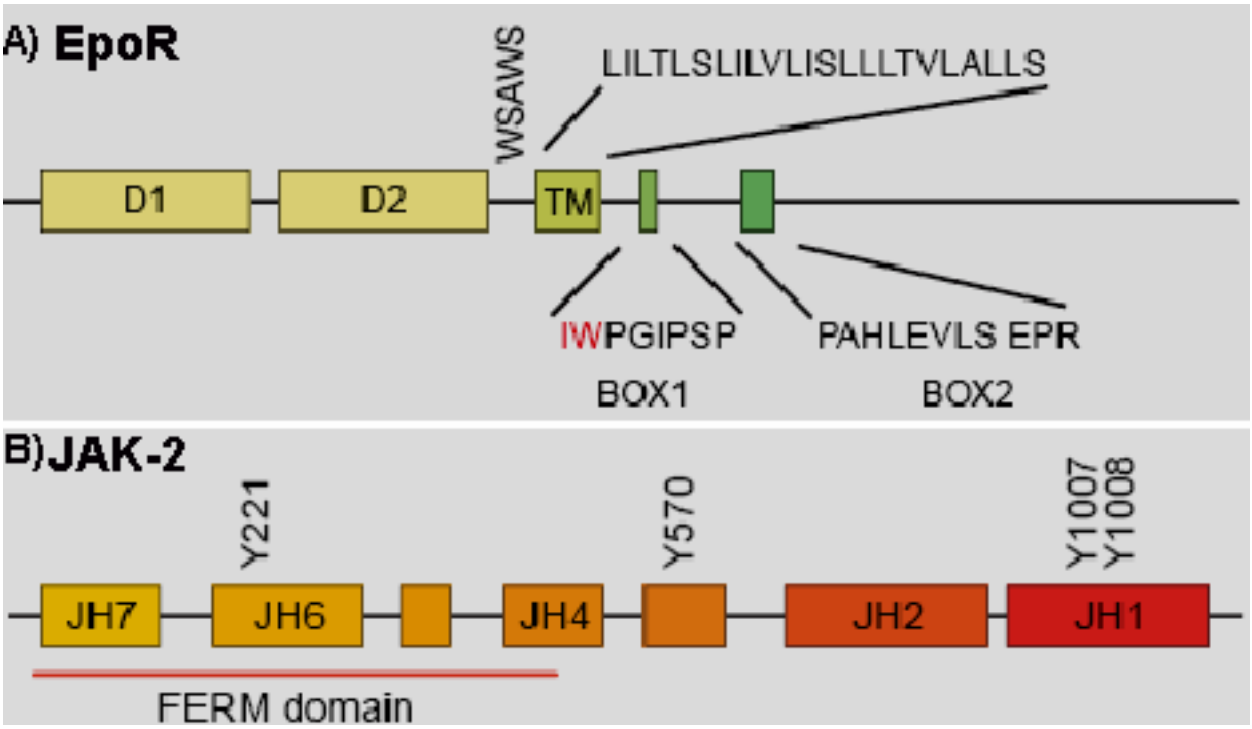

Figure 1.9. Structure of Erythropoietin receptor (EpoR) and Janus Kinase 2 (JAK2). A) The structure of EpoR is shown with extracellular domain (conserved D1 and D2 subunits), transmembrane domain (TM) and conserved Box 1 and Box 2 domains in the juxta-membrane cytoplasmic domain. **B)** Structure of JAK2 is shown with the conserved seven domains. The c-terminal JH1 constitutes the kinase domain and the phosphorylation sites are shown. JH2 represents the pseudokinase domain and has regulatory effects on the kinase domain. JH4-JH7 represents the FERM domain, which is postulated to have roles in the golgi trafficking of the EpoR-JAK2 complex. Positions Y221 and Y570 indicate recently identified phosphorylation sites involved in JAK2 regulation. (Adapted from Richmond TD, Chohan M, Barber DL. Turning cells red: signal transduction mediated by erythropoietin. Trends Cell Biol. 2005;15:146-155)

FIGURE 1.10

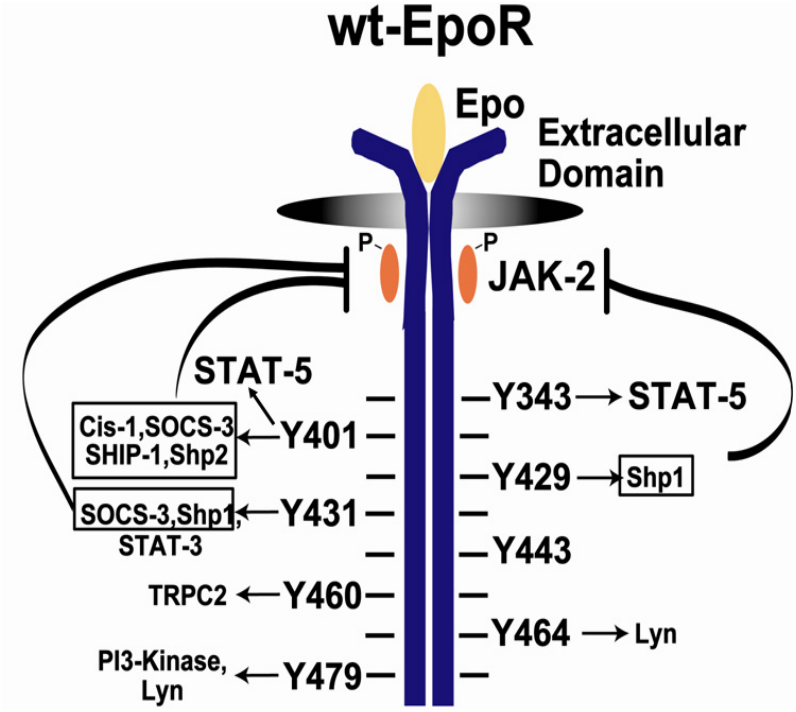

Figure 1.10. EpoR phosphotyrosine sites and corresponding signal transduction factors. Illustrated here are the different signal transduction factors that bind to the eight cytoplasmic tyrosine residues of EpoR. PY343 predominantly binds to STAT-5 (Signal transducer and activator of transcription-5). PY401 binds Cis-1 (Cytokine inducible SH2-domain containing protein-1, SOCS-3 (Suppressor of cytokine signaling-3), SHIP-1 (SH2-inositol phosphatase-1) and Shp2 (SH2-domaining containing phosphatase 1). PY431 binds to SOCS-3, Shp1 (SH2-domaining containing phosphatase 1), and STAT-3 (Signal transducer and activator of transcription -3). PY460 binds to TRPC2 (transient receptor potential channel 2) and links to calcium activation. PY464 links to LYN kinase activation. PY479 binds the p85 subunit of PI3-kinase as well as LYN kinase. Cis-1, SOCS-3, SHIP-1, Shp1 and Shp2 act in a negative feedback manner to inhibit JAK-2 signaling.

F) EPOR-PHOSPHOTYROSINE-343 (PY-343) AND STAT-5 IN ERYTHROPOIESIS

Structure and function of STAT-5

STAT-5 belongs to the Signal transducer and activator of transcription family and is one of the predominant STATs to exert effects in erythroid development [145,281-287]. STAT-5 consists of two isoforms STAT-5a and STAT-5b. Binding of Epo to EpoR results in activation of JAK-2 and phosphorylation of the PY sites in the cytoplasmic tail. STAT-5 is one of the signal transduction factors recruited to specific PY sites (PY343 and PY401) in the EpoR [281-284,288]. The STAT family of proteins have five conserved domains; C-terminal transactivation domain, SH2 domain, putative SH3 domain, DNA binding domain and a N-terminal domain (Figure 1.11) [145]. The N-terminal is involved in protein-protein interactions and stabilization of polymeric structures at the transcriptional regulatory site [145]. DNA binding domain is highly conserved amongst STATs and recognizes conserved GAS (TTNCNNNAA) sequences on target promoters [289]. The function of putative SH3 domain is presently unclear. The SH2 domain is essential for recruitment of STAT-5 to the phosphorylated EpoR where it recognizes the YXXQ motif [290]. Once recruited to EpoR, STAT-5 is phosphorylated at PY694 of its c-terminus which is vital for its activation, dimerization and translocation to the nucleus [290]. Activated STAT dimers then recognize and bind GAS sequences on the promoters of a fairly well defined subset of genes regulating survival, proliferation and differentiation (Figure 1.12) [145,290]. In vitro assays have demonstrated physical JAK-2/STAT-5 interactions, suggesting that STAT-5 might be a direct substrate for JAK-2 [291]. In addition, a Serine site exists in the C-terminal domain of STAT-5, the phosphorylation of which greatly enhances its transcriptional activity [292]. Some of the known

transcriptional targets of STAT-5 include Pim1, oncostatin-M, SOCS-3, Cis-1 and Bcl-x [145,293-296].

STAT-5 null mice survive to adulthood but demonstrate multiple defects in lactation, growth and peripheral T cell-function due to defective prolactin, growth hormone and IL-2 signaling [297,298]. Studies pertaining to erythropoiesis in these mice, however, are conflicting. Initial studies indicated no erythropoietic defects except splenomegaly [297,298]. However, subsequent studies indicated multiple defects including embryonic anemia, defective PHZ-induced stress erythropoiesis, increased apoptosis in erythroid progenitor cells and defects in erythroid differentiation [296,299]. However, Zang et al. demonstrated by crossing the STAT-5 null mice into a $RAG2^{-/-}$ deficient background that the erythroid defects, including splenomegaly were due to auto-immune extrinsic defects [300]. In studies by Socolovsky et al., Bcl-xL levels were found to be lower in $CD71^{high}$ erythroid progenitor cells [296]. In combination with the mapped STAT-5 consensus binding site in the first intron and demonstration of Epo mediated STAT-5 binding in HCD57 cell lines, a case was made that Bcl-xL was an immediate response gene of STAT-5 [296]. However, this issue is presently unclear because of conflicting results from other studies demonstrating that 1) Bcl-xL levels were not lower in erythroid cells derived from STAT-5 null mice [300]and 2) Bcl-xL induction is independent of Epo/JAK-2/STAT-5 pathway [90,267,300]. Recently, a different strategy of creating the STAT-5 null mice where both isoforms were deleted together at the germ-line level and conditionally in the hematopoietic lineage resulted in embryonic lethality due to marked defects in erythropoiesis [301].

Other STATS regulated by EpoR

In addition to STAT-5, Epo has been demonstrated to activate STAT-1 and STAT-3 in erythroid cell-line (UT-7) and primary erythroid cells [302,303]. Epo mediates strong activation of STAT-1 in spleen cells from mice treated with phenylhydrazine [303]. However, the activation of STAT-1 in bone-marrow derived erythroid progenitor cells is minimal [303]. Studies in STAT-1 null mice have clearly elucidated its role in erythropoiesis. These mice exhibit decreased bone marrow derived CFU-Es and but a compensatory increase in the splenic BFU-Es and CFU-Es [303]. In addition, early progenitor cells also exhibit increased apoptosis in STAT-1 deficient mice. Interestingly, splenic erythroid cells from phenylhydrazine primed STAT-1 null mice exhibit hyperactivation of STAT-5, ERK1/2 and AKT [303]. Activation of STAT-3 in response to Epo has also been shown in UT-7 cells and PY431 has been demonstrated as the primary binding site [302]. However, its clear role in erythropoiesis in a primary mouse model remains to be elucidated. In addition, Both JAK-2 and Fes tyrosine kinases have been assigned roles in activation of STAT-1 and STAT-3 [302].

Role of EpoR phosphotyrosine -343 (PY343) in erythropoiesis

Regarding the function of PY-343 in erythroid cells, it is clearly established through studies employing either tyrosine-mutated or truncated Epo receptors in cell lines [281-284], murine fetal liver [287], murine bone marrow and spleen erythroid cells [285 286] that signals emanating from PY343 leading to STAT-5 activation links to development of erythroid cells. Studies of EpoRs missing all tyrosine residues in erythroid cell-lines (e.g., FDCW2) demonstrated that they functioned at 25 % proliferative efficiency and needed 5-10 fold more Epo as compared to cells expressing full-length wild type EpoR (wt-EpoR) [281,282]. In addition, cells expressing EpoRs with

only PY343/STAT-5 site exhibited proliferative capacities similar to that of wt-EpoR [281,282]. In addition, PY343/STAT-5 site has been demonstrated to be vital for the proliferative synergistic effects mediated by Epo and SCF [281,304]. Also, BAF-3 erythroid cell lines transfected with EpoRs lacking cytoplasmic tyrosine residues exhibited a marked reduction in proliferative capacity, although, they were able to produce β-globin transcripts at equal efficiency as compared to wt-EpoR [305]. However, subsequent studies in another erythroid cell line SKT-6 demonstrated that PY-343 site in EpoR linking to STAT-5 is crucial for hemoglobinization [306]. In addition, Wu et al. demonstrated by transducing $EpoR^{-/-}$ fetal liver cells with different mutant receptor forms that EpoR with a single PY-343 supports formation of CFU-E colonies at 70 % efficiency as compared to that of wt-EpoR [287]. Studies by Miller et al. in transgenic mice expressing chimeric EpoRs with extracellular domain of EGF and intracellular EpoR domain clearly established positive and indispensable roles for EpoR PY343. Transgenic mice with EpoR chimeras expressing phosphotyrosine null (PY-null) EpoR form were capable of supporting CFU-E formation but the colonies were reduced in size and number [285]. In addition, bone-marrow cells derived from these mice exhibited defective erythroid maturation as measured by percentage of Ter119 positive cells [285]. However, mice with a single restored PY-343 only form functioned with efficiencies similar to that of wt-EpoR [285].

Based on the above mentioned studies linking PY-343 to proliferation/differentiation and PY479 and PY343 to survival, clear roles seemed to have been established for their function in erythroid development [199,281-287]. In addition, based on studies linking various signaling transduction molecules to the cytoplasmic tyrosine residues in EpoR, it could be predicted that mice expressing PY-null EpoR from the endogenous EpoR locus might be severely deficient in

erythropoiesis if not embryonic lethal. Recently, Zang et al. developed knock-in mice with truncated EpoR either lacking all cytoplasmic tyrosine residues (EpoR-HM) or retaining a single PY-343 site (EpoR-H) (Figure 1.13) [300]. Interestingly, the PY-null EpoR-HM mice survive to adulthood and exhibit relatively normal erythropoiesis (Figure 1.14). Both EpoR-HM and EpoR-H mice exhibited comparable frequencies of CFU-E and BFU-E from fetal liver and bone-marrow as compared to that of wt-EpoR. Also, hematocrits from EpoR-HM mice were essential similar to that of wt-EpoR and EpoR- H (~ 80%) [300]. Interestingly, BFU-E and hematocrits in EpoR-H were slightly higher as compared to that of wt-EpoR, which is consistent with the ascribed negative roles for the distal EpoR cytoplasmic domain [300].

FIGURE 1.11

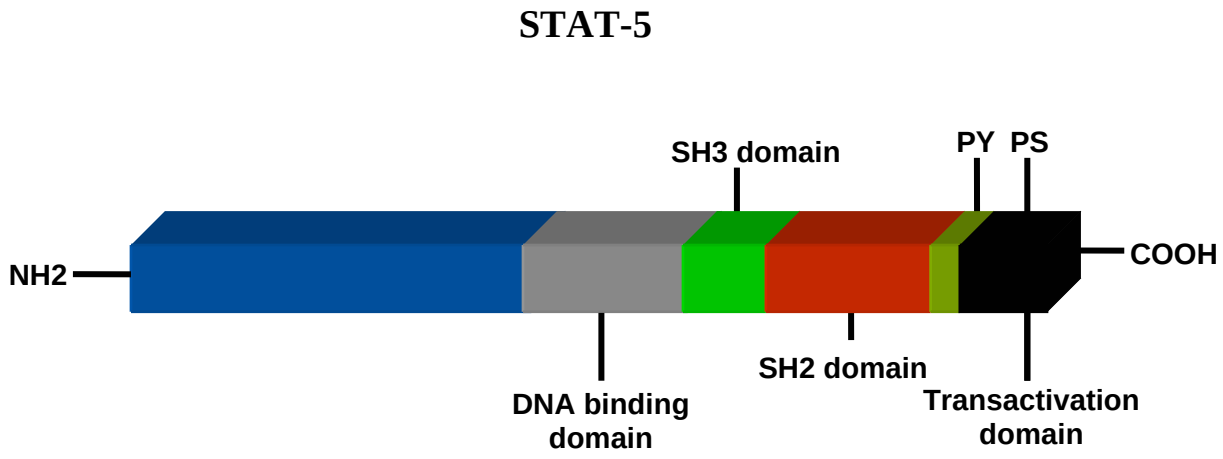

Figure 1.11. Structure of STAT-5 and role of conserved domains. Illustrated here is the protein structure of STAT-5. It consists of N-terminal domain involved in dimerization of STAT-5, DNA binding domain which recognizes the conserved GAS sequences and regulates transcription, SH3 domain and SH2 domain which is recruited to to the EpoR and C-terminal transactivation domain. Also shown is important tyrosine site Y694 involved in STAT-5 activation and dimerization.

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Figure 1.12. Epo mediated activation of JAK-2 leading to phosphorylation of PY343 and STAT-5. Epo binds to the EpoR leading to JAK-2 activation. Activated JAK-2 is responsible for phosphorylating the eight cytoplasmic tyrosine sites in the cytoplasmic tail of EpoR including PY343. Once phosphorylated, PY343 recruits STAT-5 through its SH2 domain where JAK-2 is possibly responsible for directly phosphorylating STAT-5 at Y694. Activated STAT-5 forms homodimers, translocates to the nucleus, and transcribes a well-defined set of genes by direct DNA binding to the conserved GAS sequences in the regulatory regions.

FIGURE 1.12

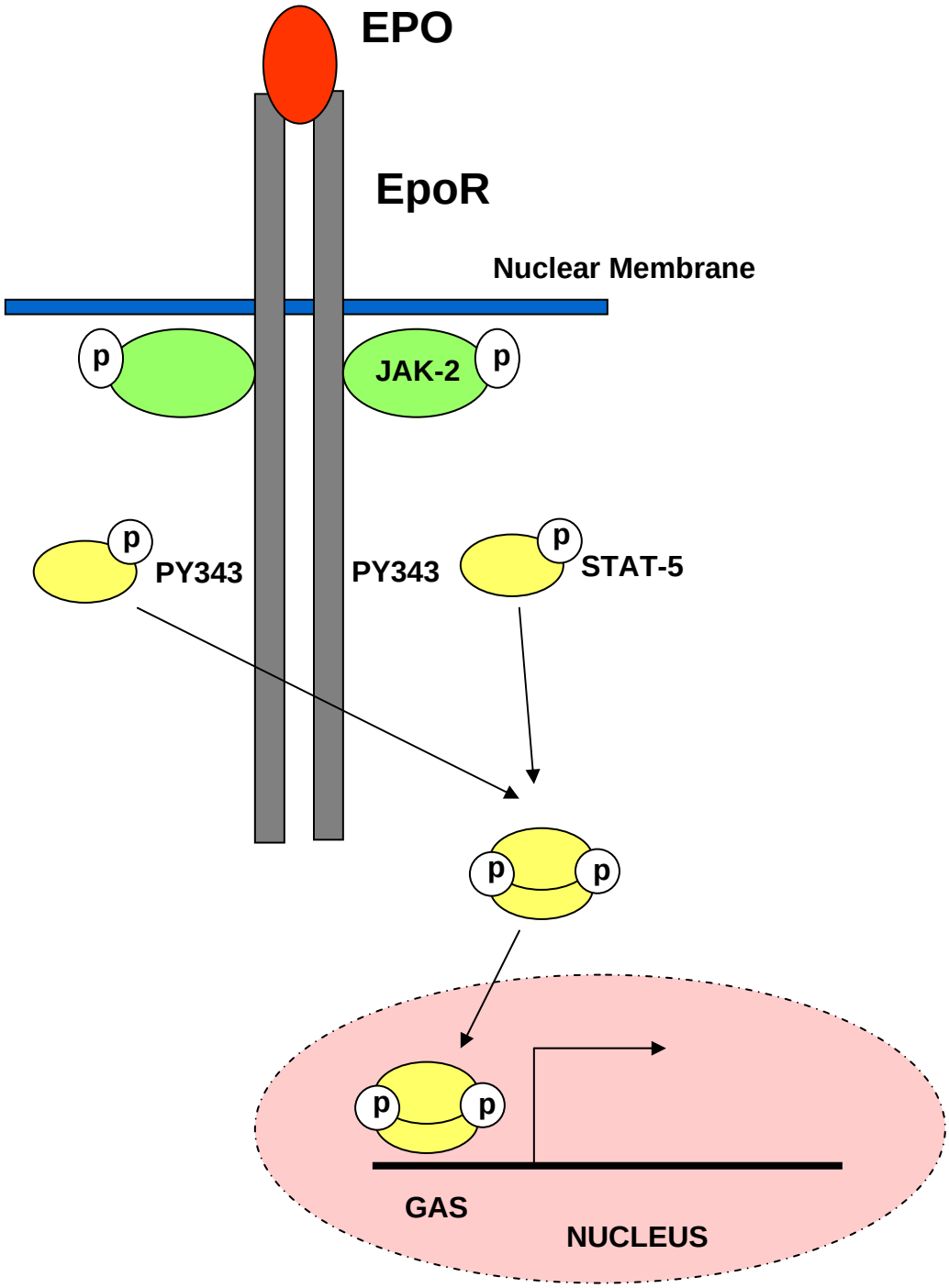

EPO
EpoR
Nuclear Membrane
p
JAK-2
p
p
PY343
PY343
p
STAT-5
p
p
p
p
GAS
NUCLEUS

FIGURE 1.13

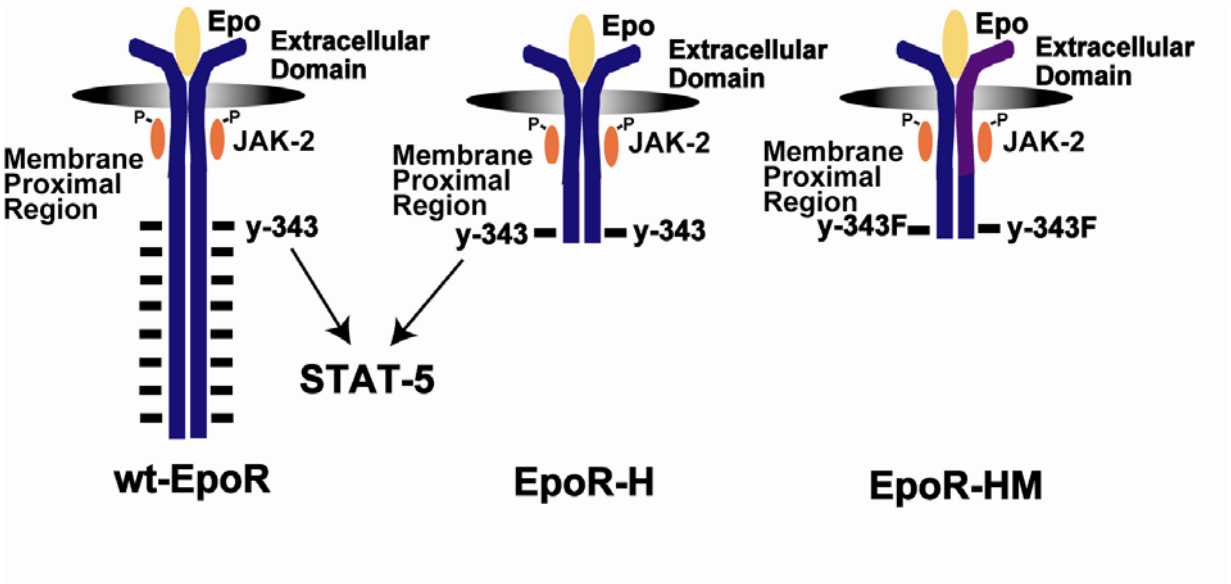


Figure 1.13. Truncated Erythropoietin receptor forms and wild type EpoR. An illustration of wt-EpoR with intact 8 tyrosine residues is shown along with EpoR-HM which retains the membrane proximal region linking to JAK-2 but is devoid of distal cytoplasmic domain containing the tyrosine residues. Also shown is EpoR-H, which retains a singly phosphotyrosine site PY343 linking to STAT-5 but lacks the rest of the distal cytoplasmic domain.

FIGURE 1.14

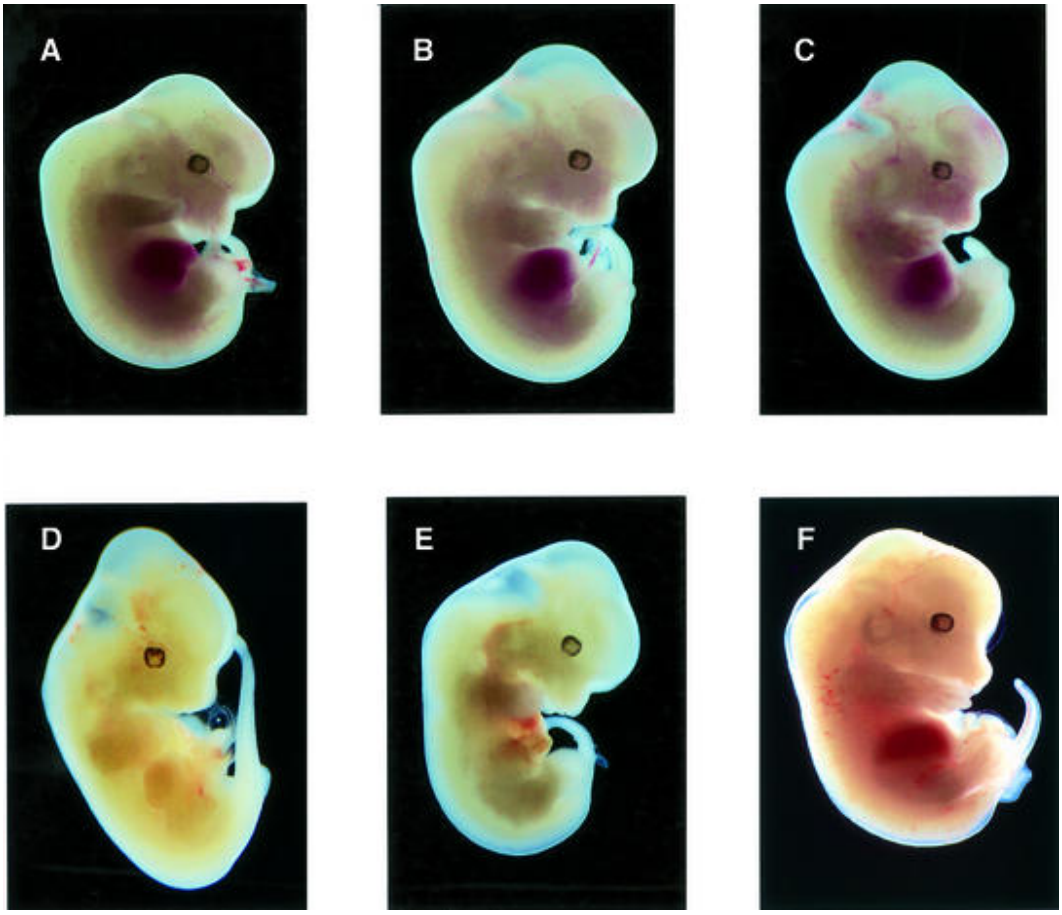


Figure 1.14. Morphology of EpoR-HM and EpoR-H mice embryos. Photographs of day 12.5 EpoR-H (**B**) and EpoR-HM (**C**) is shown. For comparison, representative photographs of mice embryos from wild type (**A**), JAK2$^{-/-}$ (**D**), EpoR$^{-/-}$ (**E**) and STAT-5a/b$^{-/-}$ (**F**) are also demonstrated.

(Zang H, Sato K, Nakajima H, McKay C, Ney PA, Ihle JN. The distal region and receptor tyrosines of the Epo receptor are non-essential for in vivo erythropoiesis. Embo J. 2001;20:3156-3166.)

E) HYPOTHESIS AND AIMS

The present studies focus on the hypothesis that Epo dependent stress erythropoiesis depends upon Erythropoietin receptor mediated activation of JAK2/EpoR-PY343/STAT-5 signaling axis and one or more key STAT-5 target genes.

SPECIFIC AIMS:

A] WHAT EPO-DEPENDENT DEFECTS MIGHT EXIST DURING THE *IN VITRO* DEVELOPMENT OF ERYTHROID PROGENITOR CELLS (EPCS) FROM MICE WITH KNOCKED IN PY-NULL (EPOR-HM) ERYTHROPOIETIN RECEPTOR ALLELE?

A.1] Develop serum-free culture system for expansion of bone-marrow derived erythroid progenitor cells and quantitative analysis of the *in vitro* development of erythroid cells.

A.2] Test the proliferation, survival and differentiation capacities of wt-EpoR, EpoR-HM and EpoR-H derived EPCs.

B] WHAT ARE THE PHENYLHYDRAZINE- (PHZ), 5-FLUOROURACIL- (5-FU), AND BONE MARROW TRANSPLANT- (BMT) INDUCED STRESS ERYTHROPOIETIC CAPACITIES OF EPOR-HM AND EPOR-H ALLELES?

B.1] Assess the stress erythropoietic response of EpoR-HM and EpoR-H mice to PHZ-induced hemolysis, and 5-FU induced bone marrow suppression.

B.2] Test the reconstitution abilities of hematopoietic progenitor cells from EpoR-HM mice in lethally irradiated recipient mice via short-term bone-marrow transplant assays.

C] WHAT SIGNAL TRANSDUCTION EVENTS (AND PRO/ANTI APOPTOTIC REGULATORY EVENTS) AND KEY STAT-5 TARGET GENES ARE MEDIATED BY WT-EPOR, EPOR-HM AND EPOR-H ALLELES?

C.1] EPO RECEPTOR COUPLED STFS (SIGNAL TRANSDUCTION FACTORS):

i) JAK2 and STAT-5, 3 & 1.

ii) PI3-kinase/AKT and its downstream target p70s6K.

iii) ERK1/2, p38map kinase and JNK.

iv) phospho-SRCs

C.2] APOPTOTIC FACTORS:

i) Anti-apoptotic factors**:** Bcl-xl

ii) Pro-apoptotic factors: Bax and Dapk2.

C.3] STAT-5 TARGET GENES: *Pim-1, Oncostatin-m, Socs-3, Cis and Bcl-x.*

D] WHAT ARE THE EFFECTS OF EITHER ONCOSTATIN-M OR RAPAMYCIN ON THE *IN VITRO* DEVELOPMENT OF WT-EPOR, EPOR-HM AND EPOR-H EPCS?

D.1] Can Oncostatin M correct the compromised *in vitro* development of EpoR-HM EPCs?

D.2] Do EpoR-HM EPCs exhibit increased sensitivity to rapamycin (an mTOR inhibitor)?

CHAPTER 2
AN EX-VIVO SYSTEM FOR MURINE BONE-MARROW DERIVED ERYTHROID PROGENITOR CELL (EPC) EXPANSION

A) INTRODUCTION

The generation of erythroid cells from a mixed cohort of different lineage progenitors in the bone-marrow is a complex process which is highly cytokine dependent. A decision to self-renew or differentiate is also dependent on the presence of absence of specific cytokines. The erythroid progenitor cells, BFU-E and CFU-E, as mentioned above, are the earliest in the erythroid lineage. Their quantification and ex-vivo generation is well-characterized [1]. Generation of BFU-E is dependent on cytokines like SCF, Epo and IL3 whereas CFU-E colonies are formed ex vivo under the influence of Epo and SCF [1]. To recapitulate the process of erythropoiesis ex vivo, in a stage-specific manner, precise cytokine concentrations and timings need to be optimized. Some vital initial studies by other groups paved the way in our lab for development of highly optimized ex vivo media for generation of different-staged erythroblasts culminating in the terminal step of erythropoiesis; formation of enucleated cells. The following few paragraphs will discuss the various constituents in the media called SP34-EX.

As mentioned above, Epo is an indispensable cytokine in the process of erythropoiesis and relays signals for survival, proliferation and differentiation through EpoR [90]. Initial studies in the EpoR$^{-/-}$ mice suggest that generation of BFU-E and CFU-E cells is Epo-independent [152]. However and importantly, the subsequent survival and maturation of these erythroid progenitor cells is highly Epo dependent [152]. SCF or Kit ligand also plays essential roles pertaining to erythropoiesis in terms of growth and survival [171]. Studies in Dominant *White Spotting (W)* and *Steel (Sl)* which

carry homozygous mutations in the Kit receptor and SCF-Kit ligand (soluble and membrane – associated) respectively exhibit multiple defects in erythropoieis and other organ systems [171,307-311]. c-Kit receptor belongs to the receptor tyrosine kinase family and upon binding of SCF, it dimerizes, autophosphorylates and recruits a variety of signaling molecules, notably phospholipase Cγ (PLC- γ), phosphatidylinositol 3-kinase p85 subunit (PI-3Kinase), Ras GTPase activating protein, SHP2 phosphatase, Src kinases, Grb2, Grb7 and Shc [171]. Various studies have suggested that Kit acts in synergy with Epo to further erythropoiesis [170]. Upon SCF administration, c-kit receptor has been demonstrated to physically associate and transphosphorylate the Epo receptor [170]. In addition, Tan et al. recently demonstrated that PY567 and PY569 sites in the Kit receptor is important for regulating the EpoR phosphorylation and synergistic effects [312]. However, Cui et al. have shown that even though SCF is capable of phosphorylating the EpoR, it is incapable of transducing EpoR mediated signals required for cell survival and growth in the absence of Epo [228]. Studies have indicated that the STAT-5 pathway and MAP Kinase pathway might be potential downstream effectors of the synergistic interaction between Epo and SCF [228]. Recent studies by Boer et al. have demonstrated that SCF can enhance Epo mediated STAT-5 activation through PKA/CREB pathway [313]. Interestingly, SCF has also been demonstrated to increase the expression of EpoR at the transcript level and the Sp1 and GATA-1 sites in the promoter are essential for this function [314]. The study of Avian erythroblastosis virus (AEV) products v-ErbA and v-ErbB in maintaining oncogenesis and growth factor dependent erythropoiesis has led to the discovery of comparable factors in mammalian cell systems [315]. The steroid hormones, Dexamethasone and Estradiol have been demonstrated to aid the proliferation of erythroid progenitor cells in avian, human and murine erythroid progenitor cells [316,317]

[318-320]. Both Dexamethasone and Estradiol seem to assist the process of self-renewal and inhibit differentiation of erythroblasts [316,317]. Estrogen in combination with TGF α has been shown to promote proliferation of chicken erythroblasts [317]. Dexamethasone acts through the glucocorticoid receptor (GR) and has been demonstrated to induce self-renewal and inhibit differentiation primarily through induction of myb in chicken erythroid progenitor cells [318]. Also, this effect requires DNA binding and ATF2-transactivation domain of glucocorticoid receptor [318]. Studies in mice lacking GR or having mutations in dimerization domain ($GR^{dim/dim}$) have demonstrated deficient proliferation of fetal erythroid cells [318]. In addition, these mice exhibit multiple defects in erythropoiesis under hypoxic and hemolytic stress, underlying the critical nature of glucocorticoids in stress erythropoiesis [318].

Several optimized media systems have been generated by various groups for ex vivo expansion of erythroid cells. Panzenbock et al. developed one of the first optimized media for expansion of cord blood derived human erythroblasts [319]. This media generated Erythropoietin and SCF responsive erythroid cells, which could be induced to differentiate in presence of Epo and Insulin [320]. Subsequently, Von Lindern et al. developed a similar media with minor modifications to support the growth of erythroid cells derived from peripheral human blood [320]. Dolznig et al. also developed a serum free media which is capable of supporting the expansion of fetal liver erythroblasts (> 10000 fold) for 21 days [321]. Studies by these groups greatly aided us in optimizing the media components for expanding erythroid cells from murine bone marrow. Initial pilot experiments dealt with determining the concentration of media components, cell-stagedness at different time-points and frequency of feeds. Morphological analyses of cells, flow

cytometry, proliferation and hemoglobin assays helped us determine the in vitro growth and differentiation of murine bone marrow erythroid cells.

B) METHODS

Bone marrow isolation and expansion strategies

To isolate bone marrow, Femurs and tibiae from C57BL6 or B6129PF2J mice were collected, and marrow cells were gently flushed from cavities using 21ga (femur) or 23ga (tibia) needles and 10 mL of Iscove's Modified Dulbecco medium (IMDM) (Invitrogen, #12440-053) plus 2% fetal bovine serum (FBS, Hyclone, #SH30070.03). Cells then were passed thrice slowly through a 21ga needle, and 40-μm strainer. Collected cells were resuspended initially in 1 mL of phosphate-buffered saline (PBS) (Invitrogen, #14190-144), and were exposed for 2 minutes to 9 mL potassium bicarbonate–buffered 0.8% ammonium chloride, 0.1 mM Na_2 EDTA (ethylenediaminetetraacetic acid) solution, pH 7.5. 10 x PBS (1.1 mL) was then added. Cells were collected through 16mL of 50 % FBS in PBS, and washed in IMDM, 2% FBS. In expansions, cells were cultured (at $7.5x10^5$cells/mL) in StemPro-34 medium (Invitrogen) supplemented with 2.5U/mL Epo (Epoetin-alpha, Amgen), 100ng/mL mSCF (PeproTech, #250-03), 1uM dexamethasone (Sigma #D4902), 1uM beta-estradiol (Sigma, #E2758), 40ng/mL IGF-1 (PeproTech, #250-19), 75ug/mL h-transferrin (Sigma, #T0665), 0.5% BSA (Stem Cell Tech., # 9300), 0.1 mM 2- mercaptoethanol (Sigma #6250) and 1.5 mM L-glutamine (i.e., "SP34-EX" medium). At 24 hours of culture, 0.5 volumes of medium were added. At 48 hours, cells were replated at $7.5x10^5$cells/mL in 80% new media plus 20% residual conditioned media. For certain experiments, $CD71^{pos}Ter119^{neg}$ erythroblast populations were prepared (at day 3.5 of culture) via two rounds of MACS $Ter119^{pos}$ cell depletion (Miltenyi Biotech, #130-049-901) or via lineage depletion. From the cohort of day 3.5 expanded cells, Kit^{pos} and Kit^{neg} cells were isolated using CD117-microbeads (Miltenyi Biotec) by positive selection and negative selection. In differentiation experiments, expanded erythroblasts (at day-3.5 of expansion) were cultured (at

$7.5x10^5$ cells/mL) in 2.5U/mL Epo, 100ug/mL transferrin (Sigma, #T0665), 60 ng/mL insulin (Invitrogen, #41400-045), 0.5% BSA (Invitrogen, #15260-037) and 0.1mM 2-mercaptoethanol in IMDM. Differentiation was assayed based on side and forward-angle light-scatter and DRAQ5 (ALEXIS, #BOS-889-001-R200) (10 uM) staining of DNA content.

Flow cytometry and ^{3}HdT incorporation assays

Washed cells ($1x10^6$) were incubated in 0.2 mL of PBS, 1% BSA with 1μg of rat IgG (15 minutes), and with PE-Ter119 (2μg), FITC-CD71 (1μg), and/or APC-Kit (1μg) (BD Biosciences). PE/FITC-annexin V binding assays (BD Biosciences) were performed in 140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES (pH 7.4) (20 minutes). Washed cells were analyzed via flow cytometry (BD FACScalibur). In ^{3}HdT incorporation assays, expanded erythroblasts were plated in cytokine deficient SP34-EX medium ($1x10^5$ cells/mL) with Epo as indicated. At 20 hours, 1 μCi of ^{3}HdT was added, and at 25 hours, incorporation rates were determined.

Cytospins, confocal analysis and hemoglobinization assays

Cytospin analyses ($1x10^5$ cells) involved centrifugation onto slides (15 minutes, 300 rpm, Hettich Universal-16A cyto-centrifuge) followed by Dip-Stain reagent staining (Volu-Sol Inc.). Hemoglobinization was detected with Diaminoflourene (DAF) and Benizidine. For confocal staining, expanded cells (Kit^{pos} and Kit^{neg}) were stained with 1 μg of Fitc-CD71 or PE-Ter119 (BD Biosciences) and Draq-5 (10 μM, Bio-Status Limited, #BOS-889-002) or Hoechst 34580 (5 uM, Molecular Probes).

FACS and MACS Sorting

FACS of $Kit^{pos}CD71^{high}$ and $Kit^{neg}CD71^{high}$ erythroid progenitor cells involved APC-CD117 plus FITC-CD71 co-sorting (BD-FACS Vantage). Alternatively, magnetic-activated cell sorting (MACS) was performed via an initial lin^{pos} cell depletion (Stem Cell Technologies #19756) plus Kit^{pos} selection (Miltenyi Biotec #130-091-224). This generated both Kit^{pos} cells (positive selection) and Kit^{neg} cells (negative selection). Sub-population purities were $\geq$ 95%.

C) RESULTS AND DISCUSSION

Inspite of availability of various erythroid cell lines like HB60, K562, J2E, GIE2 and UT-7/Epo, they fail to simulate in vivo erythropoiesis in terms of differentiation, cell-surface marker expression, progression through precise stages of erythroblasts and finally enucleation [322-325]. A plethora of important studies elucidating the roles of various signaling proteins has been carried out in these cell-lines. However, it has always been challenging to perform these experiments in primary cells because of relative heterogeneity and insufficient number of cells. The bone-marrow expansion strategy described below is capable of generating sufficient number of homogenous erythroblasts capable of achieving full ex vivo terminal differentiation.

Bone marrow cells were isolated from 8-12 weeks old mice and expanded for 3.5 days in optimized serum-free SP34-EX media with supplements as described above. At 3.5 days of expansion, highly Epo responsive $CD71^{high}$ (~ 60 %) erythroblasts were generated (Figure 2.1). Approximately half of these were Kit^{high} and the balance of cells included Mac-1^{pos} (12%), $B220^{pos}$ (5%) and $CD41^{pos}$ (5%) cells. For further characterization, these cells were lineage depleted and KIT (CD117) selected using either MACS (magnetic activated cell sorting) or FACS (flow activated cell sorting). Purity of cells was confirmed to be more than 90 %. Morphological analyses of these cells were carried out via Wright-Giemsa plus light microscopy and CD71/DRAQ-5 confocal microscopy (Figure 2.1). $Kit^{pos}CD71^{high}$ cells, on morphological analyses, were found to resemble proerythroblasts and were large blast like cells with fine nuclear chromatin. On the other hand, $Kit^{neg}CD71^{high}$ cells resembled basophilic /polychromatophilic erythroblasts and were significantly smaller, more hemoglobinized and had compact dense nuclei (Figure 2.1).

Isolated Kit^{pos} cells were then demonstrated to achieve erythroid differentiation to the Kit^{neg} stage over a span of 72 hours (Figure 2.2). Flow cytometry demonstrates a time-dependent increase in the Kit^{neg} population with accumulation of approximately 97 % Kit^{neg} cells at 72 hours. The percentage of $Ter119^{pos}$ cells also increase over time with maximum frequency of 14 % at 72 hours (Figure 2.2). Based on flow cytometric analysis, Kit^{pos} cells were not found to co-stain for Ter119 (Figure 2.2). Kit^{pos} cells isolated in the manner described above then were tested for their Epo responsiveness (Figure 2.3). These cells exhibit a progressive increase in numbers in a Epo-dose dependent fashion with a six-fold increase in cell-count at the highest Epo dose (1.6 U/mL) (Figure 2.3). Based on 3HdT analyses, Kit^{pos} cells demonstrate increasing rates of incorporation and proliferation at higher doses of Epo. For survival assays, Fitc-annexin staining was used which demonstrated an increase in apoptosis at lower Epo doses (Figure 2.3). In the absence of Epo, approximately 80% of these cells undergo apoptosis underlying the critical nature of Epo in erythroid progenitor cell survival.

It was critical to demonstrate the functionality of erythroid progenitor cells expanded in this fashion by demonstrating effective terminal differentiation. For this, a minimal serum-free differentiation medium containing BSA, Epo (1 U/ml) and low doses of Insulin and Transferrin was developed. Assessment of terminal differentiation was carried out via flow cytometric staining of late erythroid marker Ter119, nuclear staining with cell-permeable dyes (DRAQ-5 or Hoechst) and morphological analyses (Figure 2.4). More than 80% of $Kit^{neg}CD71^{high}$ cells became low FALS (forward angle light scatter), $Ter119^{pos}$ cells (Figure 2.4). Interestingly, maximum enucleation (based on $Ter119^{high}DRAQ\text{-}5^{neg}$ frequencies) was found to be achieved

via removal of Epo from the media at a specific time-point. The frequencies of enucleated cells were found to be ~35% and 46% at 24 and 48 hours respectively (Figure 2.4). Cytospin analysis revealed these cells to be very small (7.5-8 u), highly hemoglobinized and lacking nuclei. Confocal analysis confirmed these findings (Figure 2.4). Interestingly, generation of a homogenous cohort of enucleated cells is dependent on Epo deprivation during the later stages as illustrated by flow cytometric forward scatter profiles and enucleation assay (Figure 2.5). This observation might be a result of low EpoR expression on late-stage erythroid cells in combination with a possible Epo mediated inhibitory effect on terminal enucleation. As expected, cell-counts progressively decreased with increasing culture periods of Epo deprivation. However, culture in minimal differentiation media for 18 hours followed by Epo deprivation for 30 hours resulted in a satisfactory cell-count (80-85 % compared to conditions with no Epo withdrawal) along with maximal terminal enucleation. Thus, this strategy based on Epo withdrawal at a specific time-point in culture can be employed for effective terminal enucleation of erythroid progenitor cells without compromising cell-numbers. Similar effects have been observed in the fetal liver system where erythroblasts cultured in fibronectin coated plates need to be deprived of Epo after a certain stage to achieve enucleation [326]. The development of this erythroid expansion and differentiation system allows analysis of stage-specific differences between wt-EpoR and mutant mice (e.g. EpoR-H and EpoR-HM) as well as generation of sufficient number of cells for signaling studies and western blot analysis.

Figure 2.1. Bone marrow cell expansion strategy, flow cytometric assay and morphological analysis. Illustrated here is the step-wise expansion of erythroid progenitor cells starting with bone marrow isolation and expansion in SP34-EX media for 3.5 days. This is followed by lineage depletion and either FACS or MACS selection of Kit^{pos} cells. The representative flow cytometry profiles of Kit^{high} and Kit^{low} cells are shown along with morphological analyses. Also shown are the confocal images of the two populations stained with DRAQ-5 (nuclear) and FITC-CD71.

FIGURE 2.1

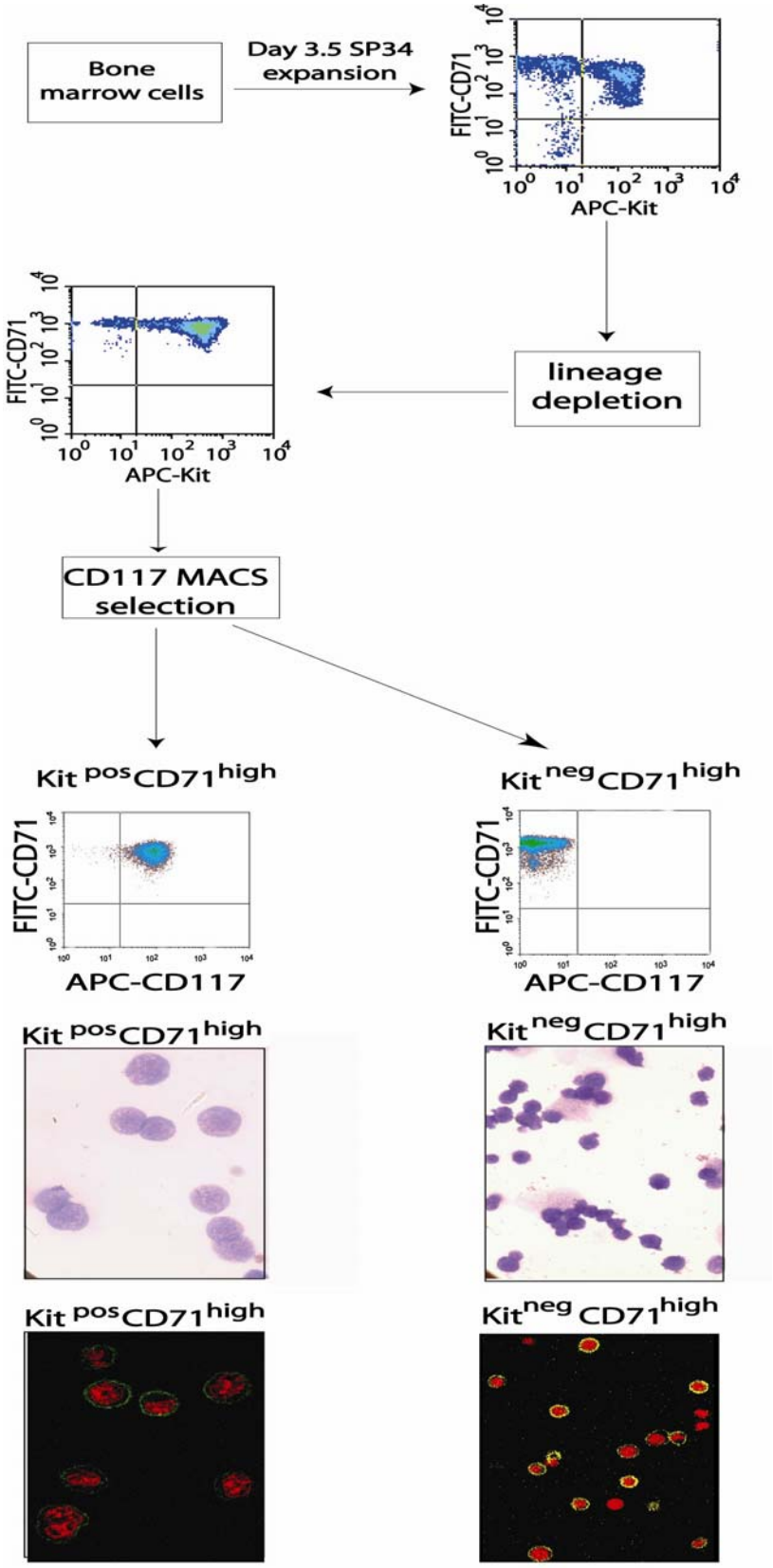

Bone marrow cells
Day 3.5 SP34 expansion
FITC-CD71
APC-Kit
lineage depletion
CD117 MACS selection
Kit pos CD71 high
Kit neg CD71 high
APC-CD117

Figure 2.2. Epo-dependent sub-stages of bone marrow erythroblast development.

$Kit^{pos}CD71^{high}$ erythroblasts were isolated from SP34-EX expansion cultures via lin^{pos}-depletion, and Kit^{pos} selection. These staged progenitor cells then were returned to culture (0 hours), and their development was observed (in flow cytometry assays) to occur stepwise over a 72 hour interval through $Kit^{pos}CD71^{high}Ter119^{neg}$, $Kit^{neg}CD71^{high}Ter119^{neg}$, and $Kit^{neg}CD71^{high}Ter119^{pos}$ compartments.

FIGURE 2.2

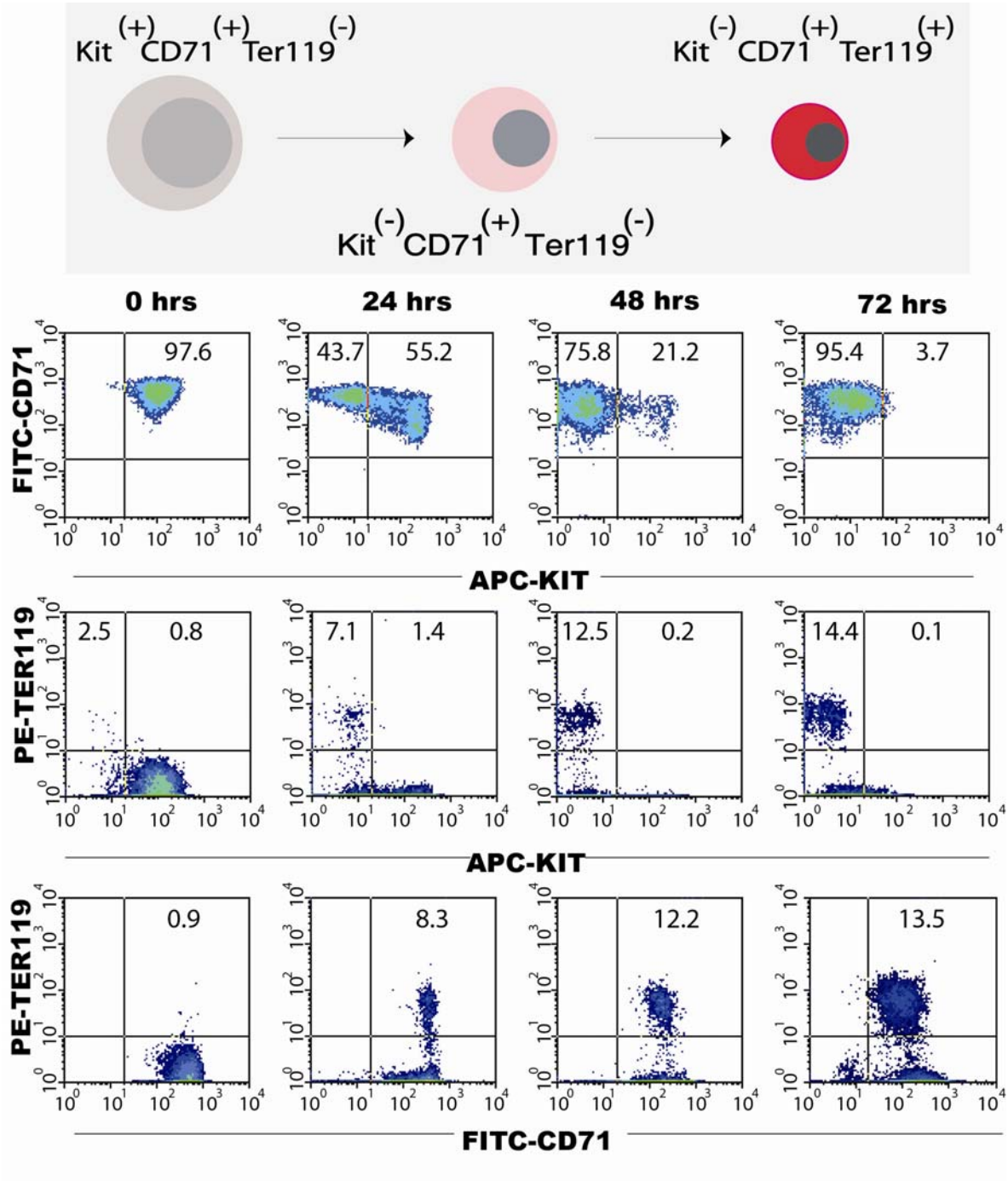

Kit(+) CD71(+) Ter119(-)
Kit(-) CD71(+) Ter119(+)
Kit(-) CD71(+) Ter119(-)
0 hrs
24 hrs
48 hrs
72 hrs
FITC-CD71
97.6
43.7
55.2
75.8
21.2
95.4
3.7
APC-KIT
PE-TER119
2.5
0.8
7.1
1.4
12.5
0.2
14.4
0.1
APC-KIT
PE-TER119
0.9
8.3
12.2
13.5
FITC-CD71

Figure 2.3. Epo dose dependent proliferation, cell growth and survival. Expanded erythroblasts were isolated and allowed to grow in SP34-EX media for 25 more hours at the indicated doses of Epo. The cell-count progressively increases with increasing Epo concentrations. Fitc-annexin positive cells indicative of apoptotic cells increase with decreasing doses of Epo. For proliferation assays, ^{3}HdT was added at 20 hours and at 25 hours, incorporation rates were determined.

FIGURE 2.3

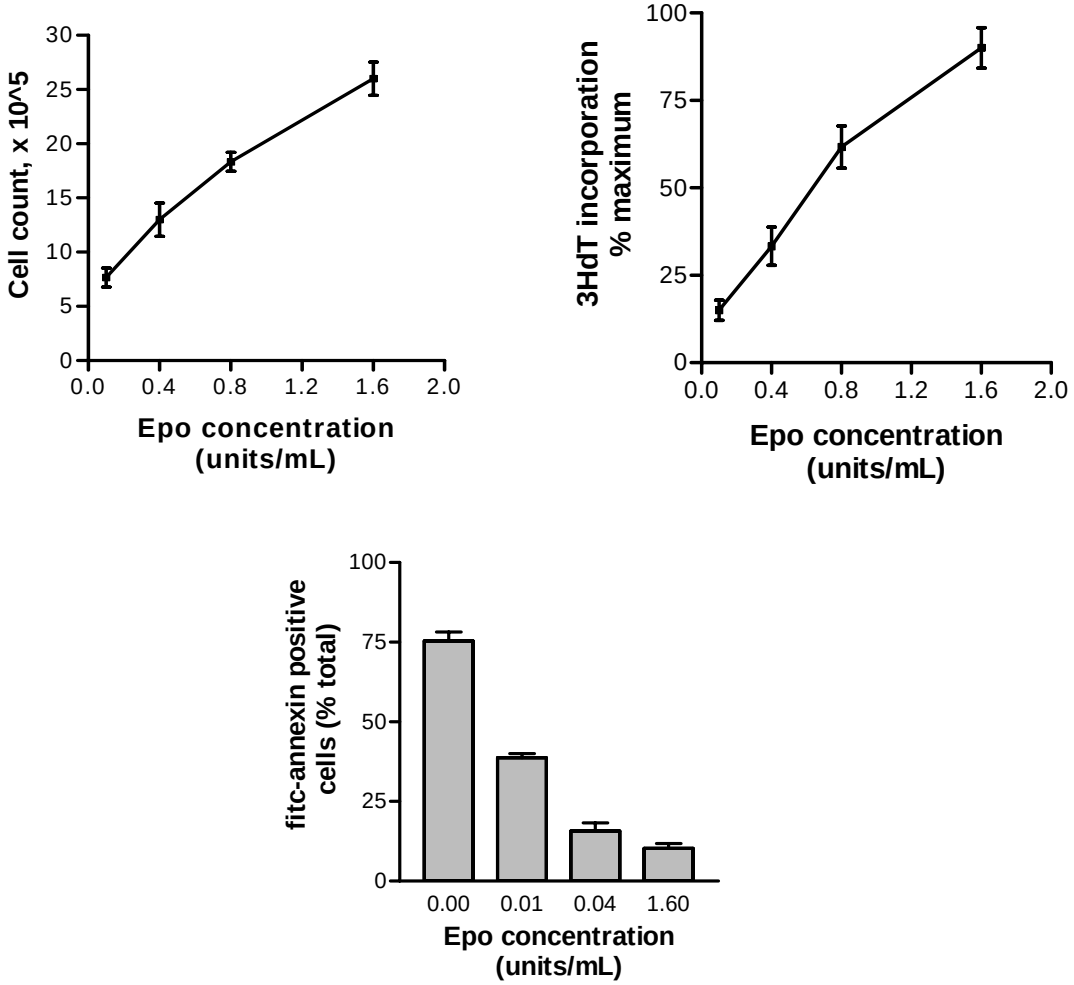

Cell count, x 10^5
30
25
20
15
10
5
0
0.0
0.4
0.8
1.2
1.6
2.0
Epo concentration
(units/mL)
3HdT incorporation
% maximum
100
75
50
25
0
0.0
0.4
0.8
1.2
1.6
2.0
Epo concentration
(units/mL)
fitc-annexin positive
cells (% total)
100
75
50
25
0
0.00
0.01
0.04
1.60
Epo concentration
(units/mL)

Figure 2.4. Effective differentiation and enucleation of Kit^{neg}CD71^{high} erythroblasts in a minimal differentiation medium. Kit^{neg}CD71^{high} cells were isolated via KIT MACS negative selection of day 3.5 expanded erythroblasts and were allowed to differentiate in a minimal serum-free differentiation medium containing Insulin, Transferrin and BSA with Epo at 1U/mL. The cells were replated in fresh medium without Epo at 14 hours and 20 hours for analyses at 24 and 48 hours respectively. Illustrated here are flow cytometric, morphological and confocal analyses of differentiated Ter119^{high}CD71^{high} cells. Percentage of enucleated cells were determined by co-staining Ter119^{pos} cells with a nuclear stain DRAQ-5. Analyses indicate a progressive increase in Ter119^{high}DRAQ-5^{neg} cells indicating enucleation. For confocal analyses, cells were either stained with PE-Ter119 or PE-Ter119 plus Fitc-CD71 and nuclear staining was achieved with Hoechst.

FIGURE 2.4

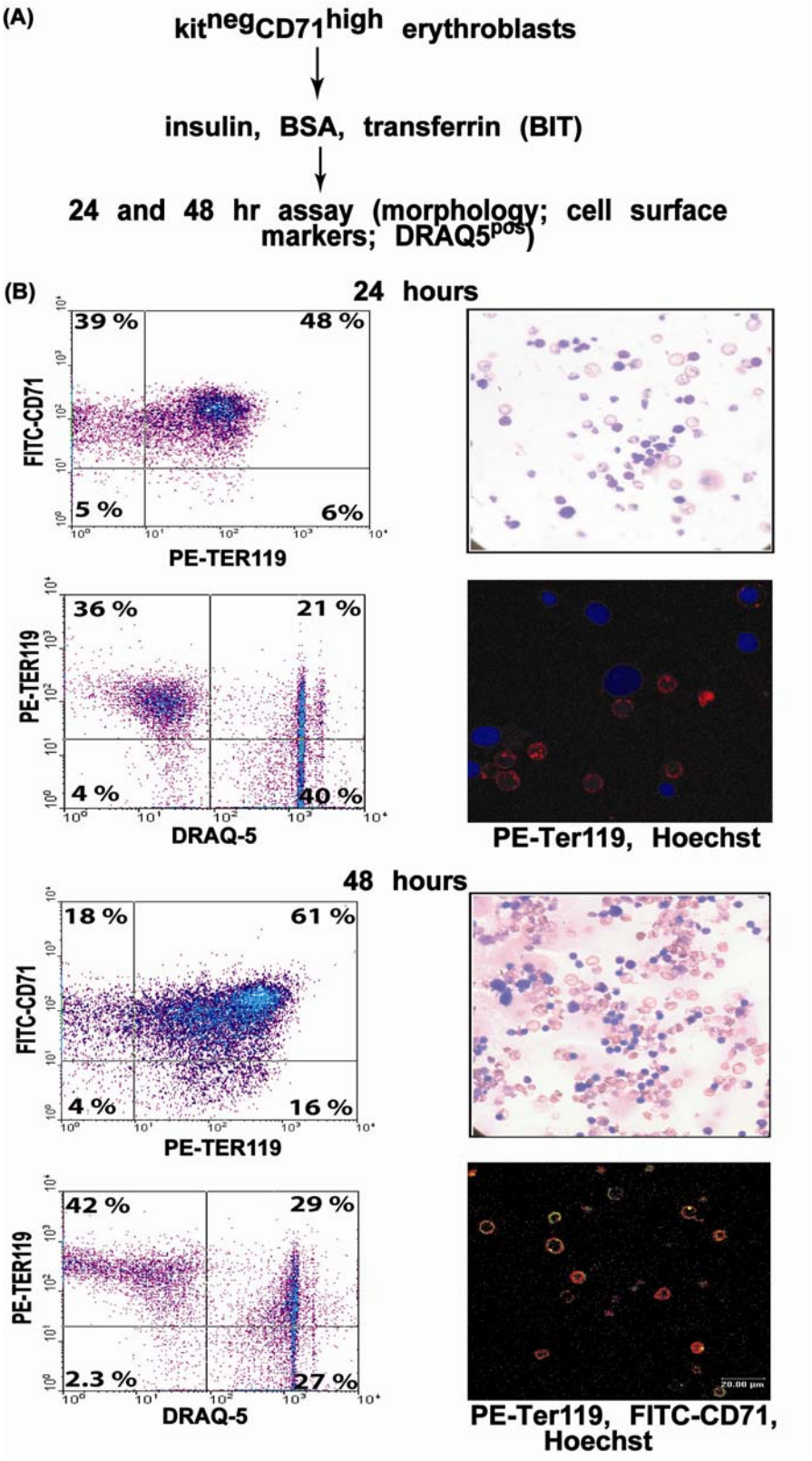
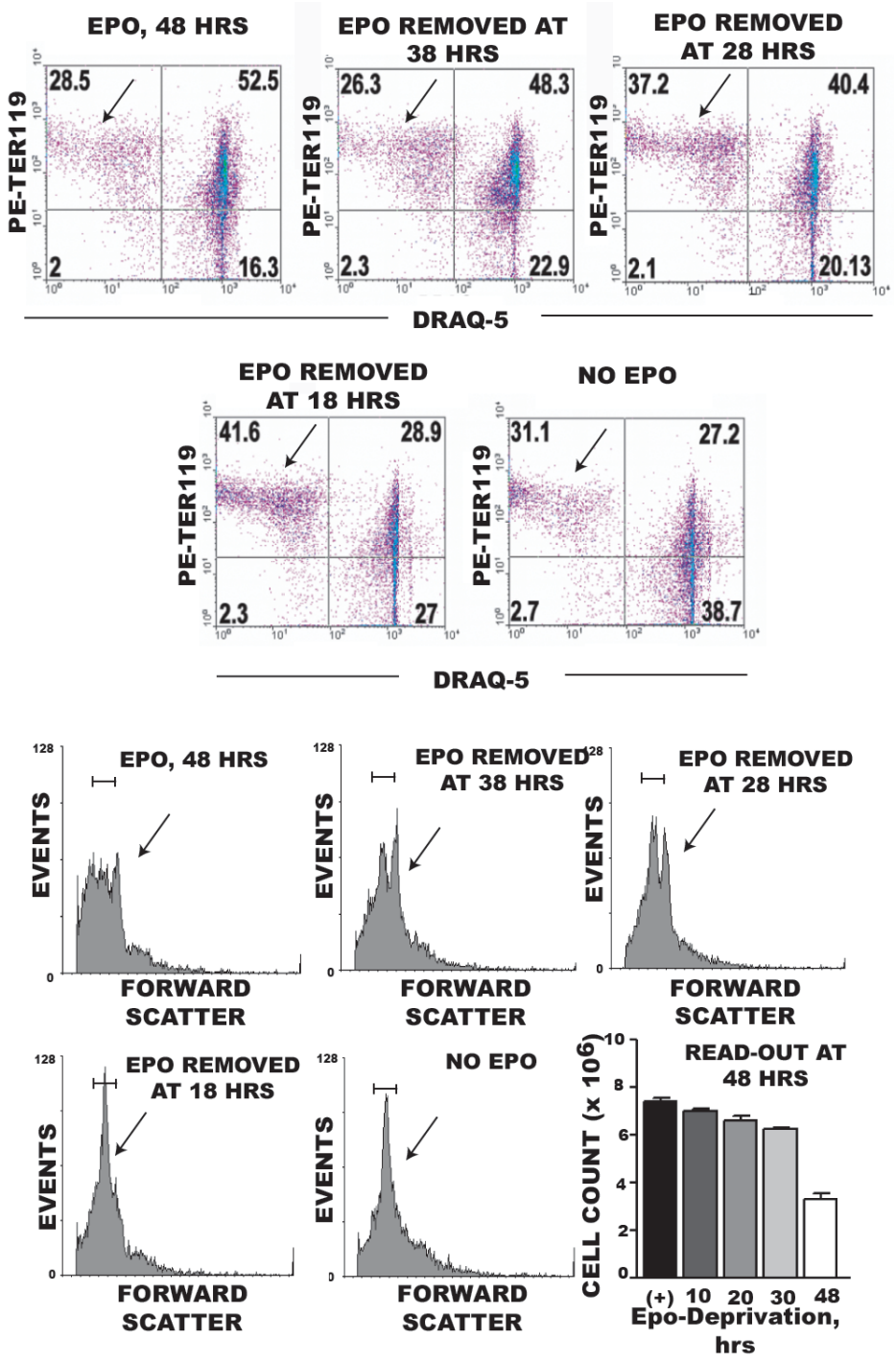

(A)
kit^neg CD71^high erythroblasts
insulin, BSA, transferrin (BIT)
24 and 48 hr assay (morphology; cell surface markers; DRAQ5^pos)
(B)
24 hours
39 %
48 %
5 %
6%
FITC-CD71
PE-TER119
36 %
21 %
4 %
40 %
PE-TER119
DRAQ-5
PE-Ter119, Hoechst
48 hours
18 %
61 %
4 %
16 %
FITC-CD71
PE-TER119
42 %
29 %
2.3 %
27 %
PE-TER119
DRAQ-5
PE-Ter119, FITC-CD71, Hoechst

Figure 2.5 Erythroblast development to enucleated Ter119^{pos} cells beyond a Kit^{neg}CD71^{high} stage can be achieved at low doses of Epo. To investigate differentiation capacities, Kit^{neg}CD71^{high}Ter119^{neg} erythroblasts were isolated from expansion cultures, and were transferred to a minimal IMDM, BSA, insulin and transferrin (BIT) medium. Possible requirements for Epo during differentiation to late-stage erythroblasts were tested by removing Epo at sequential time-points, and by the subsequent assay of Ter119^{pos} and DRAQ-5^{neg} cell formation at 48 hours (upper panels). Differentiation-associated decreases in cell-size also were assayed by forward-angle light scatter (lower panels).

FIGURE 2.5


EPO, 48 HRS
EPO REMOVED AT 38 HRS
EPO REMOVED AT 28 HRS
PE-TER119
28.5
52.5
2
16.3
26.3
48.3
2.3
22.9
37.2
40.4
2.1
20.13
DRAQ-5
EPO REMOVED AT 18 HRS
NO EPO
41.6
28.9
2.3
27
31.1
27.2
2.7
38.7
DRAQ-5
EVENTS
FORWARD SCATTER
EPO, 48 HRS
EPO REMOVED AT 38 HRS
EPO REMOVED AT 28 HRS
EPO REMOVED AT 18 HRS
NO EPO
READ-OUT AT 48 HRS
CELL COUNT (x 10^6)
(+) 10 20 30 48
Epo-Deprivation, hrs

CHAPTER 3

STAGE-SPECIFIC DIFFERENTIATION AND SIGNALING PROPERTIES OF WT-EPOR (WILD TYPE), EPOR-HM (PY-NULL) AND EPOR-H (PY-343 ONLY) FORMS AS STUDIED IN MURINE BONE-MARROW DERIVED ERYTHROID PROGENITOR CELLS (EPCs)

This research was originally published in *BLOOD*. "Menon MP, Fang J, Wojchowski DM. Core erythropoietin receptor signals for late erythroblast development. Blood. 2006;107:2662-2672". (As per American Society of Hematology copyright requirement)

A) ABSTRACT

Essential signals for erythroblast development are conducted by activation of JAK2 and subsequently the tyrosine residues in the erythropoietin receptor (EpoR). However, steady-state erythropoiesis seems to be maintained effectively by EpoR alleles that are either completely lacking, or minimal in terms of cytoplasmic phosphotyrosine sites. In order to better define the core downstream erythropoietin receptor mechanisms, signaling properties of minimal PY-null (EpoR-HM) and PY343-retaining (EpoR-H) alleles were studied in murine bone marrow–derived erythroid progenitor cells. As an initial step, uniformity of JAK2 signaling was confirmed amongst the three alleles. STAT-5 was found to be activated via EpoR-H and wt-EpoR but not via EpoR-HM. STAT-1 activation was nominal for all EpoR forms. For both EpoR-HM and EpoR-H, AKT and p70S6-kinase activation was decreased significantly. For Map kinases, JNK activation was minimal in the truncated alleles. However, ERK was hyperactivated uniquely via EpoR-HM allele. Epo expression in the kidney was slightly elevated in EpoR-HM as compared to wt-EpoR and EpoR-H. However, Epo induced reticulocyte formation was attenuated in EpoR-HM mice. In *in vitro* studies, EpoR-HM erythroblast differentiation was diminished (based on

enucleation analysis, forward-angle light scatter profiles, and hemoglobinization). At least, in vitro, These EpoR-HM–specific differentiation defects were corrected not only in the PY-343 restored EpoR-H allele, but also upon MEK1,2 inhibition. Therefore, EpoR-HM allele functionality seems to involve ERK (albeit, in a deregulated fashion) but is independent of STAT-5, STAT-1, p70S6-kinase, and JNK. Conversely, Wt-EpoR and EpoR-H functions seem to predominantly involve the PY343-STAT-5 axis.

B) INTRODUCTION

Epo and EpoR mediated signals

Signals provided by Epo and its single transmembrane receptor (EpoR) are essential for erythroblast formation [152]. Biochemical and structural studies have revealed unique mechanisms for Epo binding, and conformation-dependent activation of EpoR-JAK2 kinase complexes [134,164]. Epo-activated signaling pathways also are well-studied in various cell-line and primary cell systems (fetal liver) [90], yet discrepancies exist regarding the role of EpoR cytoplasmic phospho-tyrosines and key Epo regulated signal transduction pathways. It is relevant to address this basic issue not only in the light of erythropoiesis but also due to apparent EpoR mediated cytoprotection of injured myocardial, neuronal, endothelial and renal cells, and its association with angiogenesis, VHL carcinomas, melanoma, and myoma formation [121]. Epo binding occurs via high-affinity A, B, D helix site-1, and low-affinity A, C helix site-2 interactions of the Epo molecule with bipartite seven beta-strand ligand-binding sites in appositioned EpoR dimers [134,164]. Via a cytoplasmic juxtamembrane box-1 domain, the EpoR also preassembles with JAK2 kinase. Epo-EpoR interactions stimulate JAK2 phosphorylation at Y1007/Y1008 sites [166], and JAK2 (potentially in concert with Src, Btk, STK and/or Kit tyrosine kinases) then mediates the phosphorylation of multiple EpoR cytoplasmic tyrosine motifs. In the EpoR, eight distal phosphotyrosine motifs are conserved that possess established binding specificities for SH2-domain encoding effectors. These include: PY343 binding of STAT-5 [284]; PY401 binding of cytokine-inducible SH2-domain containing protein Cis-1, SH2 inositol 5-phosphatase, SHIP-1, Gab-2, SOCS-3 and/or Syp/SH2-PTP2 [183,205,220,259,327]; PY429 and PY431 binding of SOCS-3 and/or SHP-1 [184,248]; PY460 binding of CrkL [328] and regulation of intracellular calcium flux [239]; PY464 and/or PY479 binding of Lyn [188]; and PY479 binding of alpha-p85/PI3 kinase [199]. In the

human EpoR, an additional membrane proximal cytoplasmic PY285 site has been demonstrated in 32D cells to modulate STAT-5 and STAT-1 activation [329].

Based on the evolutionary conservation of these EpoR PY sites and their demonstrated role as an assembling scaffold for the above effectors [90], EpoR phosphotyrosine motifs are predicted to be important for Epo's actions. The extent to which these EpoR phosphotyrosines and their linked pathways act in an essential context or perhaps only in a modulatory fashion is not clear. This point was highlighted by the ability of PY-deficient EpoR alleles to support steady-state erythropoiesis *in vivo* [300]. Specifically, steady-state erythropoiesis in mice expressing a knocked-in PY-null EpoR-HM allele is affected to the extent that hematocrits are decreased ~8-points on average, and RBC counts are decreased ~ 15% [300]. This suggests that signals provided by JAK2 (in the absence of EpoR PY- signals) efficiently support Epo-dependent erythroblast formation (at least in the context of steady state erythropoiesis). These findings raise basic questions concerning the nature of essential signaling pathways that are utilized by these minimal EpoR alleles (and the wt-EpoR). However, because of the challenges involved in isolating the low frequency erythroid progenitor cells for protein analysis, signaling studies in these EpoR forms in bone marrow-derived erythroblasts were limited to single-point EMSA analyses of STAT-5 activity [300].

Experimental approach

To address the above basic problems in Epo signaling, primary culture systems (as explained in chapter 2) were implemented to investigate Epo-activated signals in erythroblasts derived from adult bone marrow of mice expressing a PY-null EpoR-HM allele, or a related knocked-in EpoR-H allele in which a single PY343 STAT-5 binding site is selectively restored. Specifically, these

studies involved ex-vivo expansion of bone marrow derived cells in erythroid expansion specific media (SP34-EX) for 3 days. Subsequently, either these cells per se or Ter119 depleted $CD71^{pos}$ cells were cytokine-deprived for 5.5 hours and stimulated with Epo (2.5 U/mL) to generate whole cell lysates for western blotting experiments. Cell differentiation and enucleation was assayed using a differentiation promoting media (BIT) followed by flow cytometric and hemoglobinization assays. These analyses provide several new lines of insight into signals that are relayed via EpoR-HM/JAK2 and EpoR-H/JAK2/STAT-5 axes.

Introduction to data

PY independent (EpoR-HM) EpoR- plus-JAK2-activated erythropoietic signals appear to be relayed primarily via STAT-5,-1, JNK- and p70S6K-independent, but ERK 1,2-dependent routes. Also, EpoR-HM derived erythroblasts exhibit distinct stage specific differentiation and survival disadvantages. These defects in EpoR-HM erythroblasts are also underlined by a decrease in the protein expression of Bcl-xl (anti-apoptotic protein) and an increase in DAPK-2 (pro-apoptotic protein). EpoR-PY343 (EpoR-H) signals, by comparison, restore erythropoietic capacities and most but not all of the wild-type capacities via suggested actions of key STAT-5-target genes. Also, differentiation capabilities of EpoR-HM erythroblasts seems to be restored to wt-EpoR levels upon MEK-1/2 inhibition via U0126.

C) METHODS

Mice and primary erythroid cell culture

EpoR-HM and EpoR-H mice were obtained from Dr. Jim Ihle's lab [300] and were generated as follows. The distal 108 amino acids in the cytoplasmic tail of EpoR was deleted to generate mice which either retain a single phosphotyrosine site in the EpoR cytoplasmic tail (PY-343) or which have the Y-343 mutated to F-343 (phosphotyrosine-null EpoR-HM mice). To isolate bone marrow, Femurs and tibiae were collected, and marrow cells were gently flushed from cavities using 21ga (femur) or 23ga (tibia) needles and 10 mL of Iscove's Modified Dulbecco medium (IMDM) (Invitrogen, #12440-053) plus 2% fetal bovine serum (FBS, Hyclone, #SH30070.03). Cells then were passed thrice slowly through a 21ga needle, and 40-μm strainer. Collected cells were resuspended initially in 1 mL of phosphate-buffered saline (PBS) (Invitrogen, #14190-144), and were exposed for 2 minutes to 9 mL potassium bicarbonate–buffered 0.8% ammonium chloride, 0.1 mM Na_2 EDTA (ethylenediaminetetraacetic acid) solution, pH 7.5. 10 x PBS (1.1 mL) was then added. Cells were collected through 16mL of 50 % FBS in PBS, and washed in IMDM, 2% FBS. In expansions, cells were cultured (at $7.5x10^5$cells/mL) in StemPro-34 medium (Invitrogen) supplemented with 2.5U/mL Epo (Epoetin-alpha, Amgen), 100ng/mL mSCF (PeproTech, #250-03), 1uM dexamethasone (Sigma #D4902), 1uM beta-estradiol (Sigma, #E2758), 40ng/mL IGF-1 (PeproTech, #250-19), 75ug/mL h-transferrin (Sigma, #T0665), 0.5% BSA (Stem Cell Tech., # 9300), 0.1 mM 2- mercaptoethanol (Sigma #6250) and 1.5 mM L-glutamine (i.e., "SP34-EX" medium). At 24 hours of culture, 0.5 volumes of medium were added. At 48 hours, cells were replated at $7.5x10^5$cells/mL in 80% new media plus 20% residual conditioned media. For certain experiments, $CD71^{pos}Ter119^{neg}$ erythroblast populations were prepared (at day 3.5 of culture) via two rounds of MACS $Ter119^{pos}$ cell depletion (Miltenyi

Biotech, #130-049-901). In differentiation experiments, expanded erythroblasts (at day-3 of expansion) were cultured (at $7.5x10^5$ cells/mL) in 2.5U/mL Epo, 150ug/mL transferrin (Sigma, #T0665), 10ug/mL insulin (Invitrogen, #41400-045), 0.5% BSA (Invitrogen, #15260-037), 0.1mM 2-mercaptoethanol, 10% FBS (Hyclone, #SH30070.03) in IMDM. Differentiation was assayed based on side and forward-angle light-scatter, DRAQ5 (ALEXIS, #BOS-889-001-R200) (10 uM) staining of DNA content.

Erythropoietin injections

At 0 and 24 hours, EpoR-HM, EpoR-H and wt-EpoR mice were injected intraperitoneally with Epo (Epoietin-alpha, Amgen) (2.5U per g-mouse). On day 5, reticulocyte levels were determined via thiazole orange staining (Retic-count reagent, BD Biosciences 349204,) and flow cytometry (BD Facscalibur).

Flow cytometry and cytospin

Cells ($1x10^6$) were incubated in 0.2 mL of PBS, 1% BSA with 1μg of rat IgG (15 minutes), and with PE-Ter119 (2μg), FITC-CD71 (1μg), and/or APC-Kit (1μg) (BD Biosciences). PE/FITC-annexin V binding assays (BD Biosciences) were performed in 140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES (pH 7.4) (20 minutes). Washed cells were analyzed via flow cytometry (BD FACScalibur). In all experiments, equivalent numbers of gated events were analyzed. Flow activated cell sorting was performed with a BD Vantage-SE system. Cytospin analyses ($1x10^5$ cells) involved centrifugation onto slides (15 minutes, 300 rpm, Hettich Universal-16A cyto-centrifuge) followed by Dip-Stain reagent staining (Volu-Sol Inc.).

Quantitative RT-PCR and primers

RNA was purified using Trizol reagent (Invitrogen). RT was with Superscript III (Invitrogen). Q-PCR (I-Cycler) utilized Sybr-green reagents (BioRad, Hercules CA) and the following oligo pairs: *Pim-1* - 5'-TTC-TGG-ACT-GGT-TCG-AGA-GG-3' and 5'-GCT-CCT-CGT-TCG- GTGATA-AA-3'; *Oncostatin-M* - 5'-AAC-TGA-GCA-AGC-CTC-ACT-TCC-3' and 5'-ATG-CCG- AGGATA-TTG-TGC-CG-3'; *SOCS-3* - 5'-CCG-CTT-CGA-CTG-TGT-ACT-CAA-G-3' and 5'-TCT-TCTCGC-CCC-CAG-AAT-AGA-T-3'; *Cis-1* - 5'-CCA-CTG-GCT-TTG-TCA-AGA-AGG-3' and 5'-AGGCCA-CAT-AGT-GCT-GCA-CAA-3'; *Bcl-x* - 5'-ACT-GTG-CGT-GGA-AAG-CGT-AGA-3' and 5'-TGC-TGC-ATT-GTT-CCC-GTA-GAG-3'; *Epo* - 5'-AGA-ATG-GAG-GTG-GAA-GAA-CAG-G-3' and 5'-CTG-GTG-GCT-GGG-AGG-AAT-TG-3'; *Actin* - 5'-CGT-GCG-TGA-CAT-TAA-AGA-GAAG-3' and 5'-TGG-ATG-CCA-CAG-GAT-TCC-ATA-3'.

Protein extraction and western blotting

For lysates, expanded erythroblasts ($1x10^7$) were washed, incubated for 6 hours in 0.5% BSA, 10ug/mL transferrin, 10ng/mL insulin, 0.1mM 2-mercaptoethanol in IMDM, and then exposed to Epo at the indicated doses. Upon washing in three volumes of 2^0C PBS, cells were lysed in 0.2 mL of 1% Igepal, 150mM NaCl, 50mM NaF, 2mM Na_2EDTA, 0.1mM $NaVO_3$, 1mM dithiothreitol, 10mM sodium pyruvate, 25mM beta-glycerol phosphate, 10% glycerol, 50mM HEPES (pH 7.5) plus 0.25 mg/mL phenylmethylsulfonylfluoride, 1x protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, #P8340, #P5726). 1% Triton-X-100, 0.5% sodium deoxycholate, 0.1% SDS, 112.5mM NaCl, 37.5mM Tris-HCL (pH 7.4) was then added (0.2 mL) and cleared extracts (25μg) were denatured, electrophoresed, and transferred to PVDF

membranes. Blocked membranes (0.05% Tween-20, 3% fat-free milk, 1% BSA, 0.15M NaCl, 20mM Tris, pH 7.4) were incubated with antibodies to AKT (SantaCruz Biotechnology, Santa Cruz, CA #sc-1618), p60Src (SantaCruz Biotechnology #sc-8056), p38 MAPK (SantaCruz Biotechnology, #535), Bax (Chemicon, #AB2915), DAPK-2 (Chemicon, Temecula, CA #3606), Bcl-x (BD Biosciences, San Diego, CA #610211), and the following antibodies from Cell Signaling, Beverly, MA: STAT-5 (#9352), PY-STAT-5 (#9351), PS-AKT (#9271), PY-p60Src (#2101), PY/T-p38MAPK (#9211), ERK1,2 (#9102), PY/T ERK1,2 (#4375), SAPK/JNK (#9252), PY/T-SAPK/JNK (#9251), PT/S p70S6-kinase (#9204) and p70S6-kinase (#9202). For JAK2, Ipegal lysates (without SDS, Triton-X-100 or deoxycholate) were incubated with anti-JAK2 antibodies (Upstate #06-255) and protein-A magnetic microbeads (Miltenyi), and immunoprecipitates were isolated. Phospho-JAK2 was detected with 4G10 (Upstate, Waltham, MA). Chemiluminescence utilized HRP-conjugated secondary antibodies (Jackson Immunoresearch, Westgrove, PA) and Dura reagent (Pierce #34076, Rockford, IL). Band signal intensities were analyzed with ImageQuant-TL (Amersham Biosciences, Piscataway, NJ).

D) RESULTS

JAK2 and Stat activation through the Epo receptor

To enable quantitative analyses of EpoR allele signaling in primary bone marrow-derived erythroblasts, a system for the efficient *in vitro* expansion of erythroid progenitor cells was implemented. This involved gentle disaggregation of marrow, limited exposure to NH_4Cl, and culture in serum-free SP34 media with optimized supplements. Supplement sources were important, as were subculture details. At day 3.5 of culture, $2x10^7$ cells were propagated on-average per mouse – and 45% to 50% of these cells reproducibly were highly Epo-responsive $CD71^{high}Ter119^{low}$ erythroblasts. The balance of cells included Mac-1^{pos} (12%), $B220^{pos}$ (5%) and $CD41^{pos}$ (5%) cells. This was the case for marrow cells from wt-EpoR mice, as well as mice expressing the minimal EpoR alleles EpoR-HM (PY-null form) and EpoR-H (PY343- retaining form) (Figure 3.1A and 3.1B).

In expanded wt-EpoR, EpoR-HM and EpoR-H erythroblast preparations, Epo-induced JAK2 activation first was analyzed (Figure 3.2A). Hematopoietic cytokines were withdrawn (for 6 hours), and erythroblasts then were exposed to Epo (2.5U/mL) for the indicated intervals. Via each EpoR allele, JAK2 activation was rapid (>50% maximum activation by 3 minutes) and progressed over highly similar time-courses. To confirm this result, and to account for possible variable responsiveness among $Ter119^{pos}$ subpopulations, analyses were repeated using Ter119-depleted preparations (and extended time-courses) (Figure 3.2A, lower panel). JAK2 again was activated by the wt-EpoR, EpoR-HM and EpoR-H at similar levels, and rates. Differential JAK2 activation therefore does not appear to underlie differences in EpoR allele biosignaling capacities.

Stat activation via EpoR alleles next was studied. STAT-5 is most frequently linked to Epo signaling [284,296,330] and was first analyzed. In time course experiments, EpoR-H activation of STAT-5 paralleled that of the wt-EpoR. STAT-5, however, was not detectably activated via EpoR-HM (Figure 3.2B). As above, analyses of STAT-5 activation were repeated in independent erythroblast preparations following Ter119^{pos} cell depletion. Essentially equivalent results were obtained (3.2B, lower panel). In addition, the abilities of EpoR-HM, EpoR-H and wt-EpoR alleles to support Epo-induction of five genes which have been indicated in cell line studies to comprise Epo and STAT-5- response genes were studied – *Pim1*, *oncostatin-M*, *SOCS-3*, *Cis-1* and *Bcl-x*. This involved cytokine-withdrawal, exposure to Epo (2.5U/mL, 90 minutes), RNA isolation and quantitative RT-PCR. Pilot experiments for *Cis-1* indicated maximal Epo-induction at 60 minutes (see Figure 3.3). *Pim1*, *oncostatin-M*, *SOCS-3* and *Cis-1* each were induced via the wt-EpoR and EpoR-H between 5 to 35-fold (Table 3.1) but not via EpoR-HM. Somewhat unexpectedly, *Bcl-x* was not significantly induced by any EpoR alleles. Together, these results further discount EpoR PY-independent mechanisms for STAT-5 activation via EpoR-HM. In cell line models, Epo-activation of STAT-1 and -3 has been reported [302]. In addition, STAT-1-deficient mice exhibit decreased erythroid progenitor cell levels [303], and *STAT-1*$^{-/-}$ BFU-E show decreased Epo-responsiveness [303]. Activation of these Stats via EpoR alleles in primary marrow-derived erythroblasts therefore was analyzed. STAT-3 activation was undetectable. STAT-1 activation was detected, but only at the limits of optimized ECL sensitivity (Figure 3.4). This suggests limited contributions of these STATS to Epo bioactivities in this primary erythroblast system.

Figure 3.1. wt-Epor, EpoR-HM and EpoR-H alleles and flow cytometric profiles of expanded erythroblasts. **A]** *Minimal EpoR alleles* - Diagrammed are knocked-in PY-null EpoR-HM and PY343- encoding EpoR-H alleles, together with the wt-EpoR. **B]** Erythroid progenitor cells from wt-EpoR, EpoR-HM and EpoR-H mice were expanded to yield (on day-3) 45 to 50% frequencies of $CD71^{high}$ erythroblasts. In all expansion experiments, CD71 and Ter119 marker expression was assessed, and representative distributions are shown. Also shown are flow cytometric profiles from MACS Ter119 depleted day 3.5 $CD71^{high}Ter119^{neg}$ samples.

FIGURE 3.1

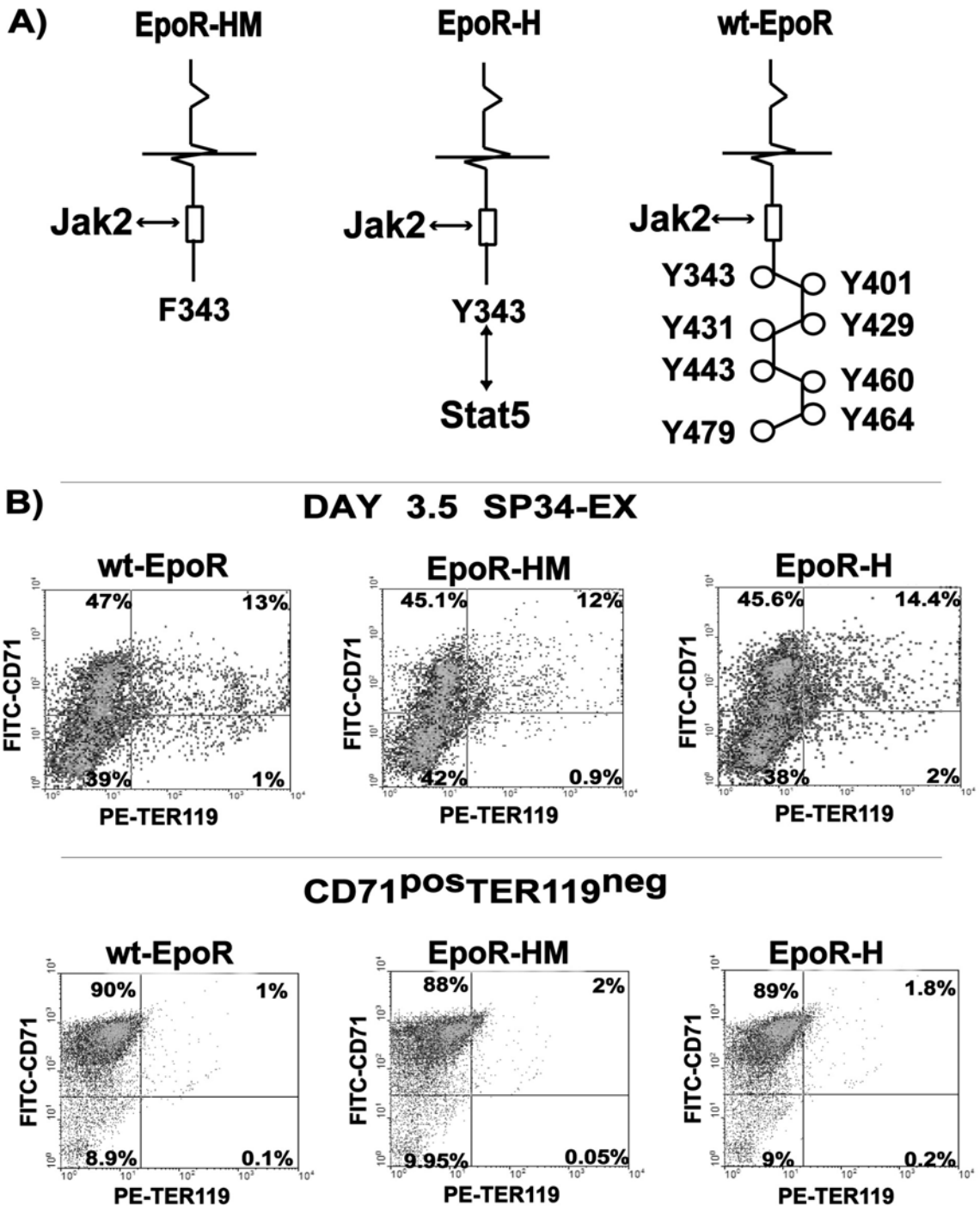

A)
EpoR-HM
Jak2
F343
EpoR-H
Jak2
Y343
Stat5
wt-EpoR
Jak2
Y343
Y401
Y431
Y429
Y443
Y460
Y479
Y464
B)
DAY 3.5 SP34-EX
wt-EpoR
47%
13%
39%
1%
FITC-CD71
PE-TER119
EpoR-HM
45.1%
12%
42%
0.9%
FITC-CD71
PE-TER119
EpoR-H
45.6%
14.4%
38%
2%
FITC-CD71
PE-TER119
CD71posTER119neg
wt-EpoR
90%
1%
8.9%
0.1%
FITC-CD71
PE-TER119
EpoR-HM
88%
2%
9.95%
0.05%
FITC-CD71
PE-TER119
EpoR-H
89%
1.8%
9%
0.2%
FITC-CD71
PE-TER119

Figure 3.2. JAK2 and STAT-5 activation via EpoR-HM and EpoR-H alleles in primary bone marrow-derived erythroblasts. A] *JAK2 activation profiles via minimal EpoR alleles* – Erythroid progenitor cells from wt-EpoR, EpoR-HM and EpoR-H mice were expanded and washed cells were cultured for 6 hours in the absence of hematopoietic cytokines (10ug/mL transferrin, 10ng/mL insulin, 0.5% BSA in IMDM). Cells then were exposed to Epo (2.5U/mL) and at the indicated intervals, lysates were prepared for western blot analyses. For phospho-JAK2, note the fairly uniform activation profiles supported via EpoR-H, EpoR-HM and wt-EpoR erythroblasts. JAK2 activation was also analyzed as above, for erythroblast preparations from which $Ter119^{pos}$ cells were depleted. **B]** *STAT-5 activation via EpoR-H, but not EpoR-HM alleles* - In parallel analyses, EpoR allele- mediated activation of STAT-5 was assessed both for expansion cultures and for Ter119-depleted cultures ($CD71^{pos}Ter119^{neg}$ populations).

FIGURE 3.2

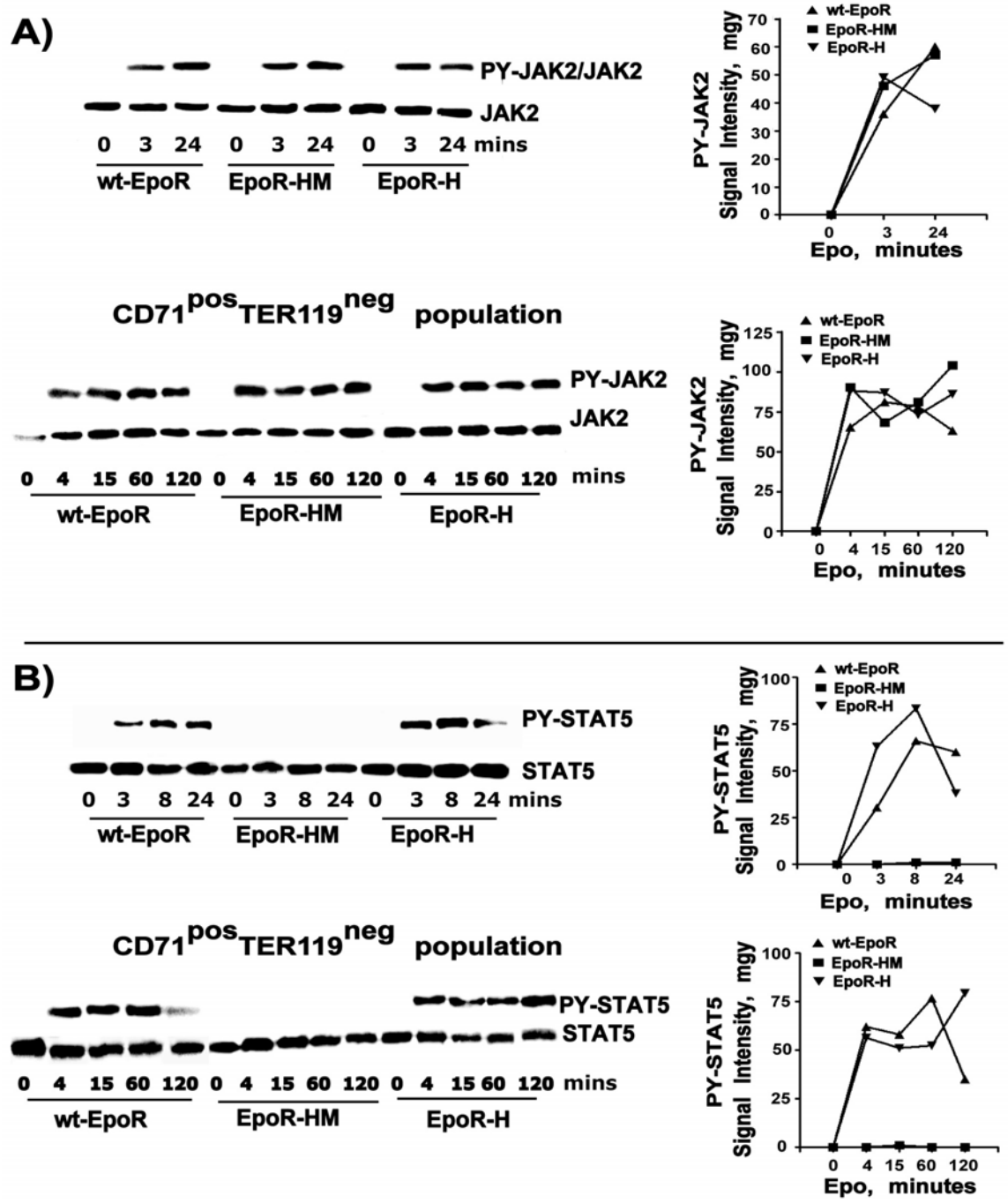

A)
PY-JAK2/JAK2
JAK2
0 3 24 0 3 24 0 3 24 mins
wt-EpoR
EpoR-HM
EpoR-H
wt-EpoR
EpoR-HM
EpoR-H
PY-JAK2 Signal Intensity, mgy
Epo, minutes
CD71pos TER119neg population
PY-JAK2
JAK2
0 4 15 60 120 0 4 15 60 120 0 4 15 60 120 mins
wt-EpoR
EpoR-HM
EpoR-H
wt-EpoR
EpoR-HM
EpoR-H
PY-JAK2 Signal Intensity, mgy
Epo, minutes
B)
PY-STAT5
STAT5
0 3 8 24 0 3 8 24 0 3 8 24 mins
wt-EpoR
EpoR-HM
EpoR-H
wt-EpoR
EpoR-HM
EpoR-H
PY-STAT5 Signal Intensity, mgy
Epo, minutes
CD71pos TER119neg population
PY-STAT5
STAT5
0 4 15 60 120 0 4 15 60 120 0 4 15 60 120 mins
wt-EpoR
EpoR-HM
EpoR-H
wt-EpoR
EpoR-HM
EpoR-H
PY-STAT5 Signal Intensity, mgy
Epo, minutes

TABLE 3.1. INDUCTION OF CANDIDATE STAT-5-RESPONSE GENES IN WT-EPOR, EPOR-HM AND EPOR-H ERYTHROBLASTS

	Pim-1	***onco-M***	***SOCS-3***	***Cis-1***	***Bcl-x***
wt-EpoR	6.3 +/- 0.8	10.6 +/-1.2	16 +/- 1.1	37.3 +/- 1.7	1.8 +/- 0.5
EpoR-HM	1.9 +/- 0.5	1.4 +/-0.3	1.8 +/- 0.5	1.0 +/- 0.4	0.9 +/- 0.4
EpoR-H	8.1 +/- 0.4	15.6 +/-1.2	19.7 +/- 0.8	31.3 +/- 5.0	1.1 +/- 0.2

[Values are mean levels, +/- SE, of transcript induction over baseline at 90 minutes of Epo exposure, 2.5 U/mL]

FIGURE 3.3

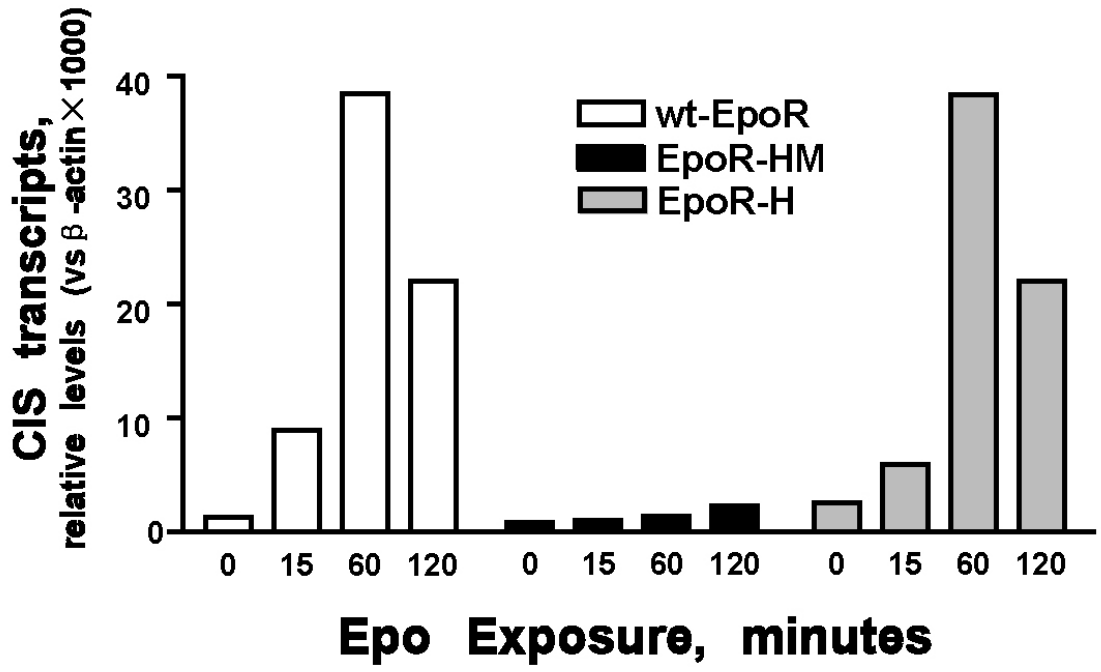


Figure 3.3. Epo induction of *Cis-1* transcript expression in wt-EpoR and EpoR-H, but not EpoR-HM erythroblasts. Erythroblasts were prepared from wild-type EpoR, EpoR-H, and EpoR-HM bone marrow. Expanded cells were washed, incubated for 6 hours in the absence of hematopoietic cytokines and stimulated with Epo for the indicated intervals. Cells then were lysed in Trizol reagent and total RNA was isolated. *Cis-1* transcript levels were analyzed by quantitative RT-PCR

FIGURE 3.4

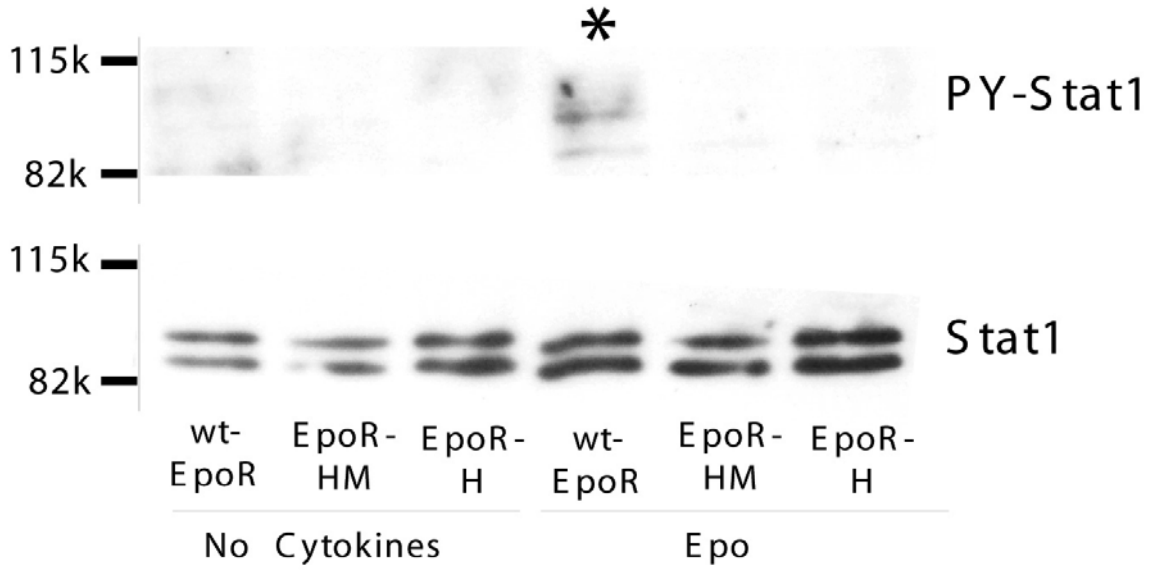


Figure 3.4. Epo activation of STAT-1 in wild-type-EpoR erythroblasts. Erythroblasts were expanded from wt-EpoR, EpoR-HM and EpoR-H bone marrow preparations. Isolated $CD71^{high}Ter119^{neg}$ cells then were washed, incubated for 6 hours in the absence of hematopoietic cytokines, and stimulated with Epo for 10 minutes. Lysates then were prepared directly, and were analyzed via western blotting for levels of activated PY-STAT-1 and total STAT-1. In wt-EpoR erythroblasts, Epo-activation of STAT-1 was detected, but only upon prolonged ECL exposure.

Epo receptor activation of AKT and p70s6 kinase

In cell lines and in fetal liver cells, AKT and p70S6K each have been shown to be activated by Epo [197,199,211]. These response pathways are linked in that PI3-kinase stimulates AKT, AKT activates mTOR, and mTOR comprises a major p70S6K regulator [331,332]. Each response also can affect progenitor cell survival [332]. p70S6K and AKT activation therefore was examined in primary EpoR-HM and EpoR-H erythroblasts. p70S6K was activated efficiently via the wt-EpoR, but was not significantly stimulated via EpoR-H or -HM alleles (Figure 3.5A). This outcome is consistent with an indicated role for an EpoR PY479 site recruitment of PI3-kinase upstream of p70S6K activation [199]. For AKT, activation via EpoR-H and -HM alleles was diminished markedly, but each nonetheless activated AKT at residual levels (~ 20% of wt-EpoR levels) (Figure 3.5B). The extent to which this limited AKT activation may affect EpoR-H and EpoR-HM bioactivity is unclear.

p60-Src also has been demonstrated to interact with the EpoR, and to affect EpoR phosphorylation [193]. Epo-induced activation of p60-Src in EpoR-HM, EpoR-H and wt-EpoR erythroblasts therefore was analyzed. Via the wt-EpoR, p60-Src was activated several-fold, and maximally so at 8 minutes of Epo stimulation (Figure 3.6A). In cytokine-deprived wt-EpoR cells, background levels of activated p60-Src, however, were sustained (as contrasted with JAK2 and STAT-5, for example). In EpoR-HM erythroblasts, Epo activated p60-Src although in a deficient fashion. Levels of PY-p60-Src overall were also decreased several-fold as compared directly to wt-EpoR or EpoR-H cells (and to an extent this involved an apparent decrease in EpoR-HM cells of total p60-Src levels). Restoration of PY343 in EpoR-H cells rescued essentially wild-type levels of Epo-induced (and background) p60-Src activation, and expression.

These results indicate a previously unappreciated role for EpoR PY343 signals in up-modulating p60-Src, possibly via STAT-5. Possible differential effects of PP2 (a SFK inhibitor) on the *in vitro* expansion of wt-EpoR, EpoR-HM and EpoR-H erythroblasts also were assessed (Figure 3.6A). PP2 inhibited the expansion of each, but was significantly less effective against EpoR-HM expansion. PP3 (as an inactive orthologue) exerted only nominal overall effects. In pilot experiments, PP2 doses above 15μM incurred toxicity, while lower doses did not efficiently inhibit p60-Src activation. Overall outcomes indicate only limited utilization of p60-Src by EpoR-HM, and are consistent with Src contributions to EpoR-H and wt-EpoR activities.

Figure 3.5. EpoR-HM, EpoR-H and wt-EpoR modulation of p70S6-kinase and AKT. A] *p70S6-kinase activation via the wt-EpoR, but not EpoR-H or EpoR–HM* **–** Erythroblasts from wt-EpoR, EpoR-HM and EpoR-H mice were expanded, washed, cultured for 6 hours in the absence of cytokines, and then stimulated for the indicated intervals with Epo (2.5U/mL). Lysates then were prepared and levels of phosphorylated and total p70S6-kinase were assayed by western blotting. **B]** *Deficient AKT activation via EpoR-HM and EpoR-H alleles* - Erythroblasts were prepared as above, exposed to Epo, and analyzed for AKT-activation. Note the multi-fold deficit activation of AKT via EpoR-HM and -H alleles.

FIGURE 3.5

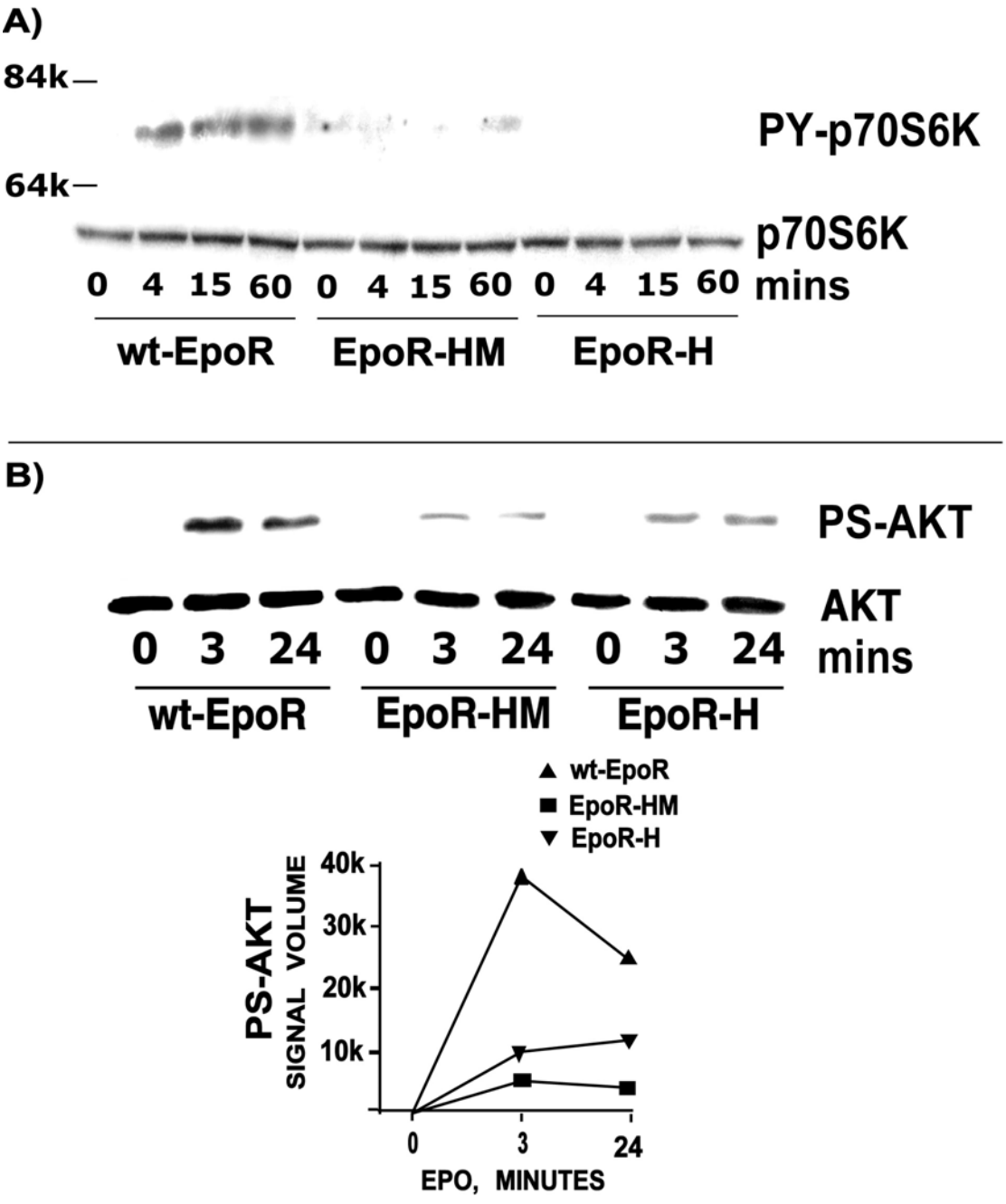

A)
84k—
PY-p70S6K
64k—
p70S6K
0 4 15 60 0 4 15 60 0 4 15 60 mins
wt-EpoR
EpoR-HM
EpoR-H
B)
PS-AKT
AKT
0 3 24 0 3 24 0 3 24 mins
wt-EpoR
EpoR-HM
EpoR-H
wt-EpoR
EpoR-HM
EpoR-H
PS-AKT SIGNAL VOLUME
40k
30k
20k
10k
0
3
24
EPO, MINUTES

FIGURE 3.6

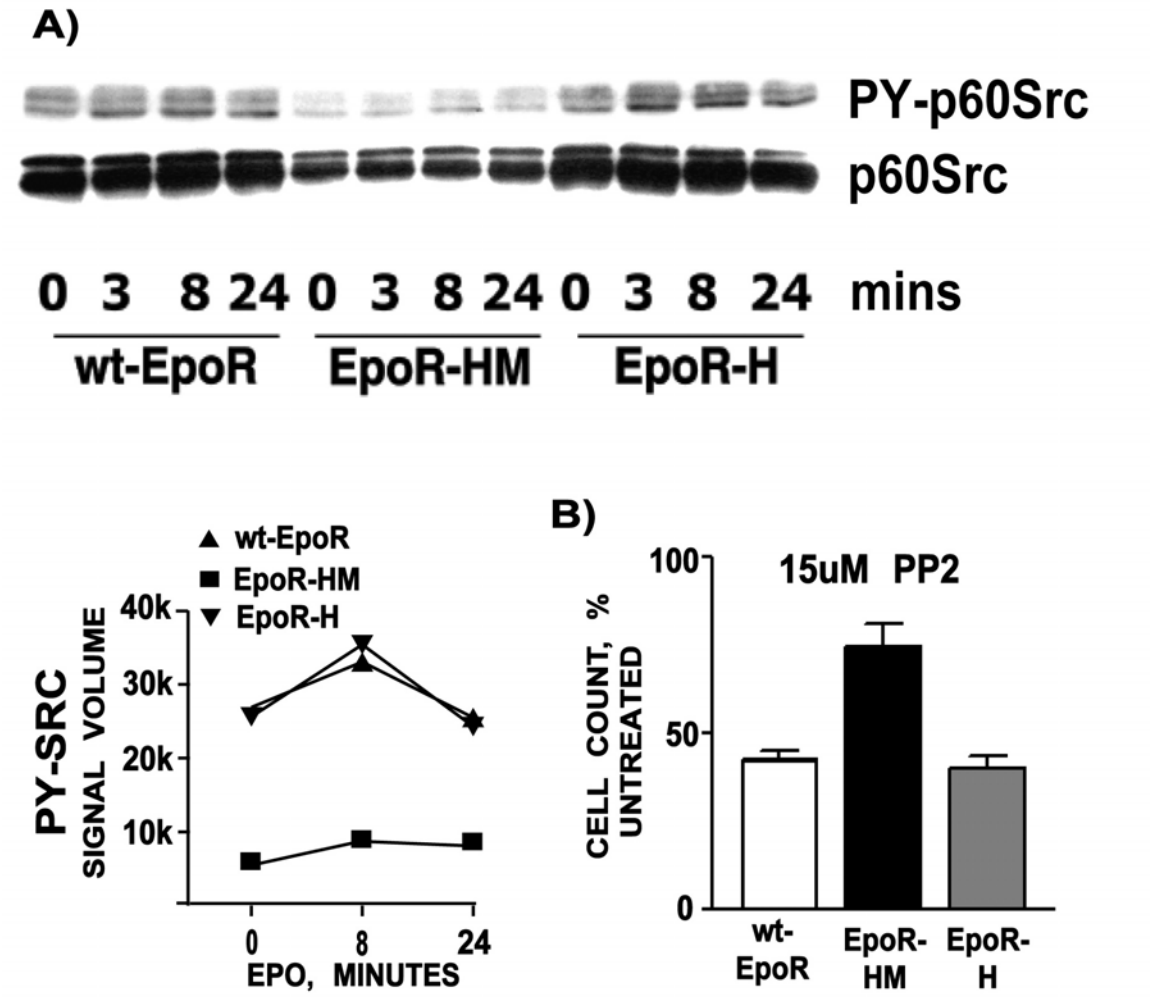


Figure 3.6. EpoR-HM, EpoR-H and wt-EpoR modulation of p60-Src. A] *Deficient PY-416 p60-Src expression via EpoR-HM –* In the above cells and samples, levels of phospho-p60-Src (and p60-Src) were assayed by western blotting (and digital densitometry imaging). **B]** *Differential PP2 inhibition of EpoR-HM, EpoR-H and wt-EpoR erythroblast expansion –* During *in vitro* expansions, wt-EpoR, EpoR-H, and EpoR-HM erythroblasts were exposed to 15uM PP2. Effects on erythroblast formation were assessed by direct cumulative cell counts at day-3 of expansion, and are normalized to numbers for parallel DMSO-exposed control cultures.

Regulation of MAPK through the Epo receptor

With regards to MAPKs, p38, JNKs and ERKs have each been demonstrated in cell lines and/or primary erythroid cell preparations to be activated by Epo. Regulation of each in wt-EpoR, EpoR-H and EpoR-HM in bone marrow derived erythroblasts therefore was analyzed. For p38, activation was supported by each EpoR allele, but fold-induction over background was limited (Figure 3.7). For JNKs, two isoforms were rapidly stimulated by Epo via the wt-EpoR (Figure 3.8A). Little to no JNK activation was detected however via EpoR-HM or EpoR-H alleles (Figure 3.8B). ERKs, in contrast, proved to be induced via each Epo receptor allele, but interestingly were discovered to be selectively hyper-activated via the PY-null EpoR-HM allele (Figure 3.9A). This latter finding was also examined further, and was confirmed, in Ter119^{pos} cell-depleted erythroblast preparations (Figure 3.9B).

FIGURE 3.7

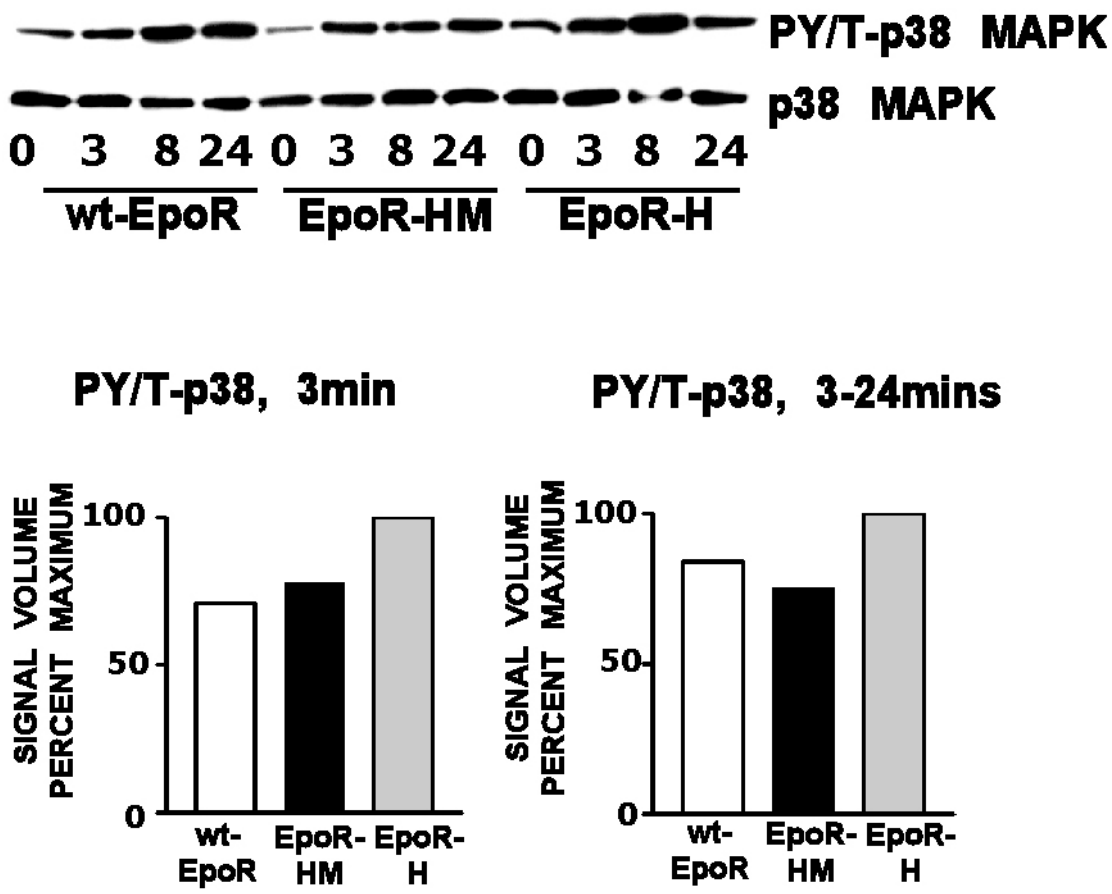


Figure 3.7. p38 MAPK activation via minimal Epo receptor alleles. wt-Epo-R, EpoR-HM, and EpoR-H erythroblasts were expanded for 3 days. $CD71^{high}Ter119^{neg}$ cells were then isolated, washed, and incubated for 6 hours in the absence of hematopoietic cytokines. At the subsequent indicated intervals of Epo-exposure, lysates were prepared and analyzed by western blotting for levels of activated and total p38-MAPK.

Figure 3.8. Epo receptor allele activation of JNKs A] *Efficient JNK activation via the wt-EpoR* - Erythroblasts expanded from wt-EpoR, EpoR-HM and EpoR-H bone marrow preparations were washed, cultured for 6 hours in the absence of cytokines, and stimulated with Epo (2.5U/mL) for the indicated intervals. Levels of phospho-JNKs (and total JNKs) were assayed by western blotting, and digital densitometry imaging. This illustrates results for the wt-EpoR, and includes co-analyzed positive controls (Cell Signaling #9253). **B]** *EpoR-H and EpoR-H alleles are deficient in JNK activation as compared to wt-EpoR allele* - In the lower panel, note the nominal activation of JNKs via EpoR-HM and -H alleles as compared to wt-EpoR allele.

FIGURE 3.8

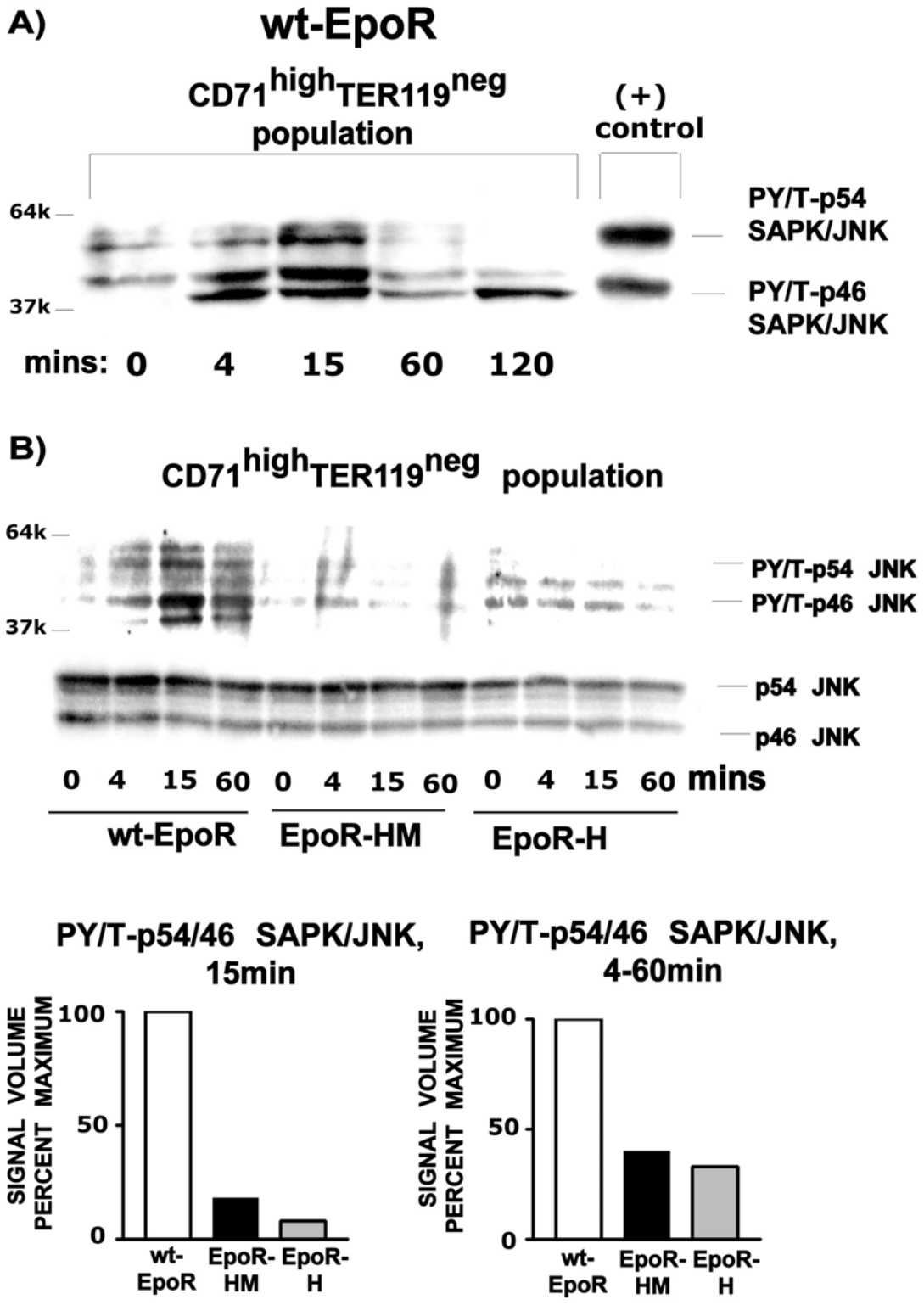
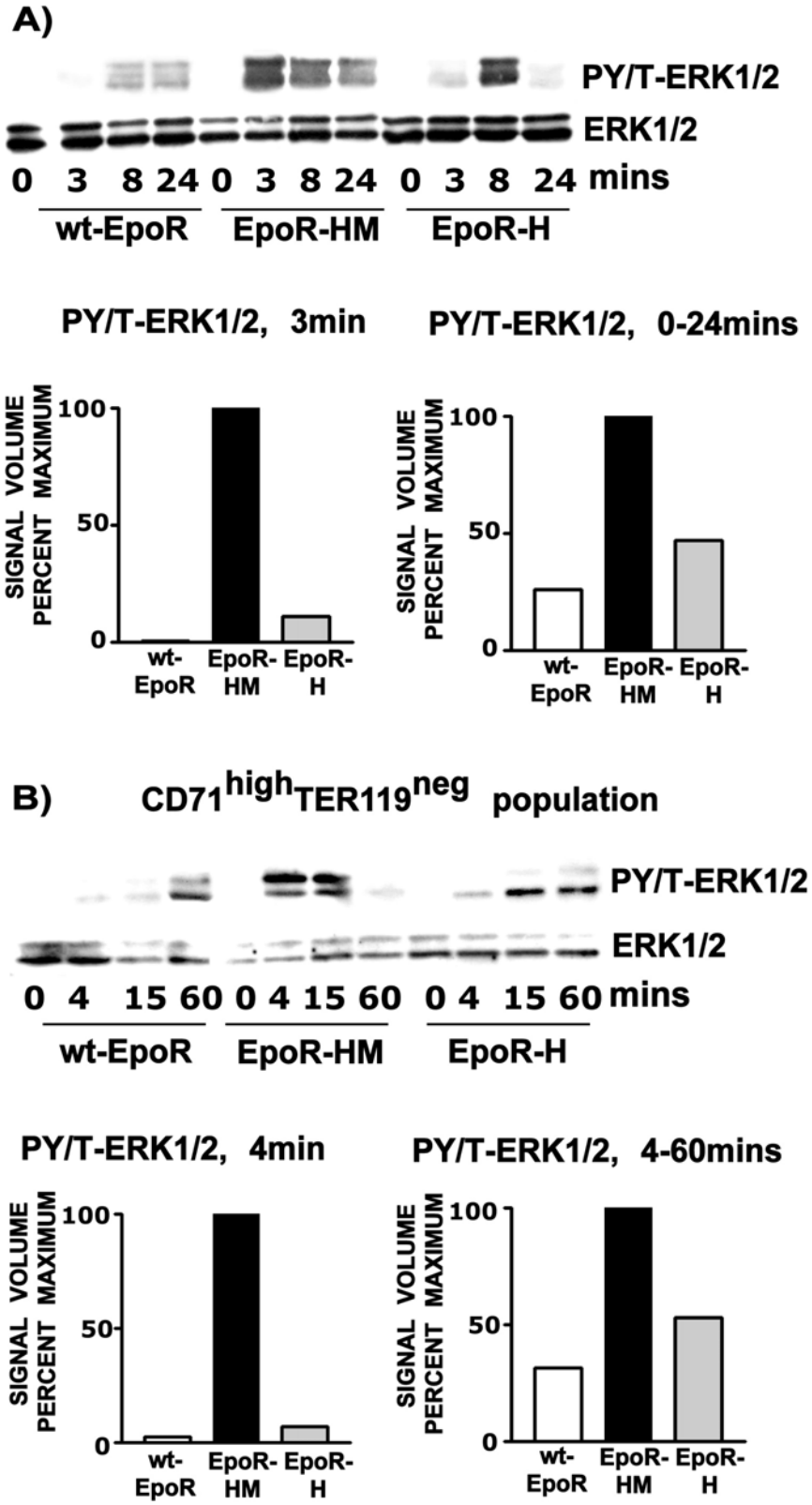

A)
wt-EpoR
CD71high TER119neg population
(+) control
64k
37k
PY/T-p54 SAPK/JNK
PY/T-p46 SAPK/JNK
mins: 0 4 15 60 120
B)
CD71high TER119neg population
64k
37k
PY/T-p54 JNK
PY/T-p46 JNK
p54 JNK
p46 JNK
0 4 15 60 0 4 15 60 0 4 15 60 mins
wt-EpoR
EpoR-HM
EpoR-H
PY/T-p54/46 SAPK/JNK, 15min
SIGNAL VOLUME PERCENT MAXIMUM
100
50
0
wt-EpoR
EpoR-HM
EpoR-H
PY/T-p54/46 SAPK/JNK, 4-60min
SIGNAL VOLUME PERCENT MAXIMUM
100
50
0
wt-EpoR
EpoR-HM
EpoR-H

Figure 3.9. Epo receptor allele activation of ERKs. **A]** *ERK hyper-activation via EpoR-HM* – **A]** Erythroblasts expanded from wt-EpoR, EpoR-HM and EpoR-H bone marrow preparations were washed, cultured for 6 hours in the absence of cytokines, and stimulated with Epo (2.5U/mL) for the indicated intervals. Levels of phospho-ERK1,2 (and total ERK1,2) were assayed by western blotting, and digital densitometry imaging. **B]** Parallel analyses of EpoR allele activation of ERKs were performed using expanded, Ter119-depleted wt-EpoR, EpoR-HM and EpoR-H CD71^{high} erythroblasts (and Epo exposure was extended to 60 minutes).

FIGURE 3.9


A)
PY/T-ERK1/2
ERK1/2
0 3 8 24 0 3 8 24 0 3 8 24 mins
wt-EpoR
EpoR-HM
EpoR-H
PY/T-ERK1/2, 3min
PY/T-ERK1/2, 0-24mins
SIGNAL VOLUME PERCENT MAXIMUM
100
50
0
wt-EpoR
EpoR-HM
EpoR-H
B)
CD71high TER119neg population
PY/T-ERK1/2
ERK1/2
0 4 15 60 0 4 15 60 0 4 15 60 mins
wt-EpoR
EpoR-HM
EpoR-H
PY/T-ERK1/2, 4min
PY/T-ERK1/2, 4-60mins

Ex-vivo differentiation defects in EpoR-HM derived murine bone- marrow EPCs, which are reversed by either restoration of PY-343 site or inhibition of MEK1/2

The limited signal transduction capacities observed for EpoR-HM in primary erythroblasts prompted additional follow-up biofunctional investigations. Compromised erythropoiesis often induces elevated Epo production. Epo levels in EpoR-HM, EpoR-H and wt-EpoR mice therefore were first assessed. RT-PCR analysis of renal *Epo* transcript levels provided high sensitivity and reproducibility, and was employed. In EpoR-HM mice, *Epo* levels were elevated on-average to 1.9-fold above wt-EpoR controls. By direct comparison, levels in EpoR-H mice were decreased to approximately 60 % of wild-type. These differences were uniformly observed in all mice assayed (n=3 per group) and were significant at a level of $p<0.01$ (Table 3.2). Second, Epo-induced reticulocyte production in response to Epo dosing was assayed. Mice were injected twice with Epo (2.5U/g), and reticulocytes were assayed on day 5. Levels in EpoR-HM mice were diminished several-fold as compared to wild-type congenic controls, and to EpoR-H mice (Figure 3.10A). Third, late-stage development of EpoR-HM and EpoR-H erythroblasts was analyzed *in vitro*. In brief, this involved expansion for 3 days, followed by culture in a differentiation medium containing transferrin, insulin and Epo. At 40 hours of culture, maturation was assessed quantitatively based on CD71 and Ter119 marker expression, plus side and forward-angle light scatter. Interestingly (and despite the inclusion of Epo at a non-limiting 2.5U/mL concentration), EpoR-HM cells faltered in their maturation. Defects in late-stage differentiation first were observed in analyses of $CD71^{high}Ter119^{pos}$ cell formation (Figure 3.10B1, left panels) in parallel with a $\geq$ 2.5-fold disadvantage in the formation of low forward-angle light scatter late-stage erythroblasts (Figures 3.10-B1 and 3.10-B2). For EpoR-H erythroblasts, a detectable attenuation of Ter119 marker expression also was observed.

Progression to low FALS populations, however, was essentially normal. Apparent defects in EpoR-HM erythroblast differentiation were characterized further based on DRAQ5 staining, hemoglobinization, and cyto-morphology (Figure 3.11). For differentiating EpoR-HM erythroblasts, frequencies of immature DRAQ5^{pos} cells were increased over EpoR-H and wt-EpoR erythroblasts, while EpoR-HM Ter119^{pos}DRAQ5^{neg} enucleated cells were correspondingly decreased (Figure 3.11A). In addition, direct benzidine staining of differentiating erythroblasts as well as diaminofluorene assays of lysed cells revealed $\geq$ 2.4-fold defects in EpoR-HM hemoglobinization, and attenuated differentiation of EpoR-HM erythroblasts was visually obvious in pelleted cells, and cytospin preparations (Figure 3.11B and C).

Based on the above-observed hyperactivation of ERKs in EpoR-HM erythroblasts, experiments also were performed to test the extent to which U0126 inhibition of MEK1,2 [333,334] might impact on Epo receptor-dependent erythroblast differentiation (especially as supported by EpoR-HM). In initial experiments, U0126 at 10μM was observed to have little effect on expansion (but at 20μM detectably affected viability). EpoR-HM, EpoR-H and wt-EpoR erythroblasts therefore were expanded in the presence of 10μM U0126 and then shifted to differentiation medium. Upon differentiation, U0126 proved to essentially correct dysregulated EpoR-HM erythroblast maturation as revealed first by clear decreases in forward-angle light scatter (Figure 3.12A). As assayed based on Ter119 and CD71 marker expression, U0126 also promoted the maturation of a sub-population of EpoR-HM Ter119^{pos}CD71^{low} erythroblasts (Figure 3.12B). In contrast, U0126 at this dose had no significant effects on the maturation of wt-EpoR erythroblasts (or on EpoR-H erythroblasts). Dose-dependency of this U0126 effect on EpoR-HM erythroblasts also is illustrated (Figure 3.13A). The capacity of U0126 to inhibit EpoR-mediated ERK activation also

was assessed directly in expanded wt-EpoR, EpoR-HM and EpoR-H erythroblasts. At the 10 μM dosage used in bioresponse assays, U0126 proved to effectively inhibit the ability of each EpoR form to activate ERKs as analyzed in cytokine-withdrawal and Epo-stimulate format (Figure 3.13B). For comparison, possible effects of the MAPK-p38 inhibitor SB202190 and JNK-inhibitor SB600125 on wt-EpoR, EpoR-HM and EpoR-H differentiation were tested. At concentrations of 0.25, 1 and 4μM (SB202190) and 1 and 15μM (SB600125), no significant effects were observed.

Figure 3.10. Faltered Epo-induced reticulocyte formation in EpoR-HM mice, and attenuated maturation of EpoR-HM erythroblasts *in vitro*. A] *Deficient reticulocyte production in Epo-treated EpoR-HM mice* - At 1 and 24 hours, Epo was administered to wt-EpoR, EpoR-HM and EpoR-H mice (2.5U/g). On day 5, induced levels of reticulocytes were assayed. Illustrated are representative flow cytometric profiles of thiazole orange staining, together with mean reticulocyte values (+/- SE) (n=4 wt-EpoR, EpoR-HM and EpoR-H mice per group). **B]** *Attenuated formation of low FALS CD71^{pos} Ter119^{pos} EpoR-HM erythroblasts in vitro* - Bone marrow-derived erythroid progenitor cells were expanded for 3 days in SP34-EX medium, and were then transferred to differentiation medium (containing Epo, insulin and transferrin). At 40 hours of culture, frequencies of maturing CD71^{high}Ter119^{pos} erythroblasts were analyzed by flow cytometry (B-1, left panels). Maturation also was assessed based on transitions to low side- and forward-angle light scatter populations (B-1, right panels). In panel B-2, defects in this transition for EpoR-HM erythroblasts are graphically summarized. The red circle indicates a Ter119^{neg} CD71^{neg} high FALS early erythroid population, which is represented at higher frequencies in EpoR-HM derived EPCs indicating a block in differentiation.

FIGURE 3.10

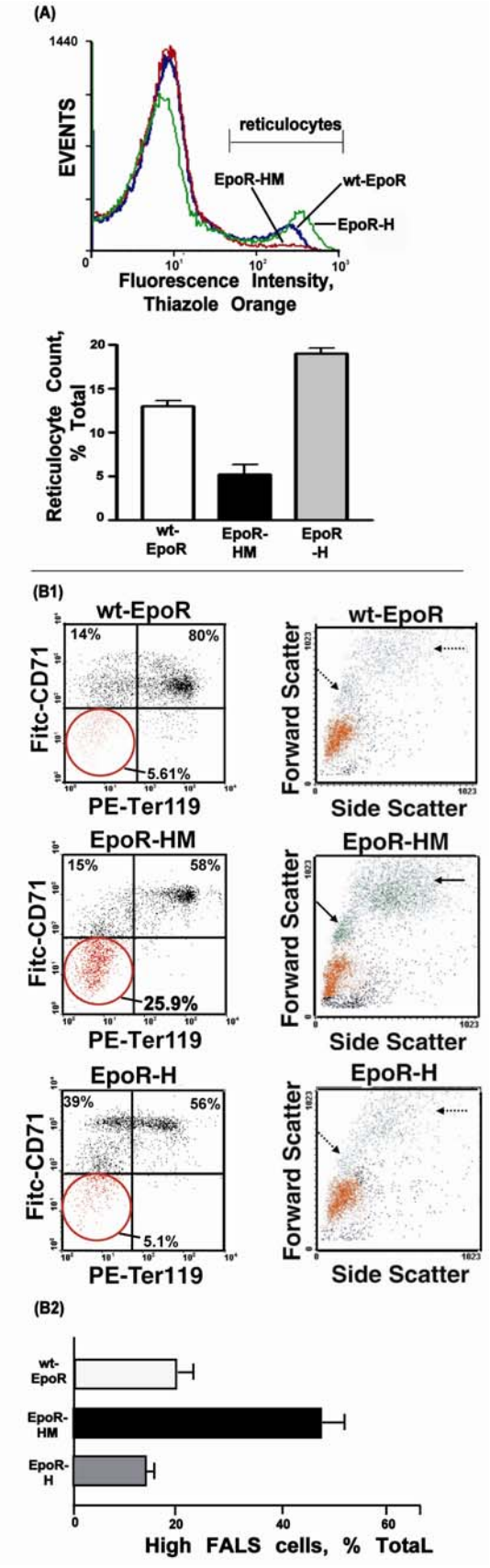

(A)
1440
EVENTS
reticulocytes
EpoR-HM
wt-EpoR
EpoR-H
Fluorescence Intensity, Thiazole Orange
Reticulocyte Count, % Total
wt-EpoR
EpoR-HM
EpoR-H
(B1)
wt-EpoR
14%
80%
5.61%
Fitc-CD71
PE-Ter119
Forward Scatter
Side Scatter
EpoR-HM
15%
58%
25.9%
EpoR-H
39%
56%
5.1%
(B2)
High FALS cells, % TotaL

Figure 3.11. Sustained DRAQ5-positivity, decreased hemoglobinization and altered cytomorphology of maturing EpoR-HM erythroblasts. A] Bone marrow-derived erythroid progenitor cells were expanded in SP34-EX medium, and subsequently were cultured in differentiation medium for 40 hours. Frequencies of $DRAQ5^{neg}Ter119^{pos}$ erythroblasts then were determined. **B]** In parallel, cultures were analyzed for hemoglobinization (benzidine-positive colonies). **C]** Hemoglobin levels in maturing wt-EpoR, EpoR-HM and EpoR-H erythroblasts also were assayed using diaminofluorene, and by visualization of pelleted cells. For EpoR-HM erythroblasts, apparently immature morphologies were observed in cytospin preparations (right panel).

FIGURE 3.11

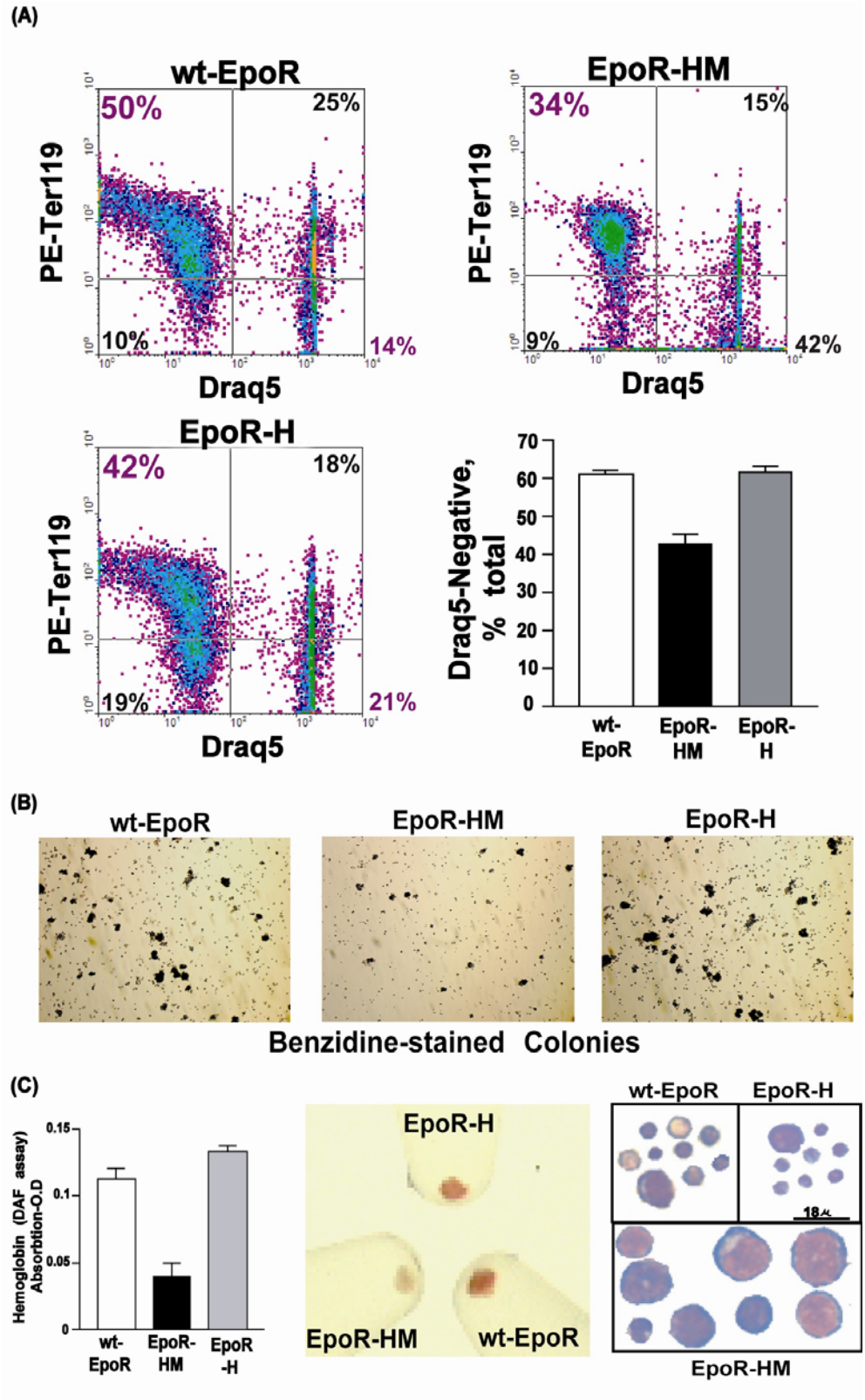

(A)
wt-EpoR
50%
25%
10%
14%
PE-Ter119
Draq5
EpoR-HM
34%
15%
9%
42%
EpoR-H
42%
18%
19%
21%
Draq5-Negative, % total
70
60
50
40
30
20
10
0
wt-EpoR
EpoR-HM
EpoR-H
(B)
wt-EpoR
EpoR-HM
EpoR-H
Benzidine-stained Colonies
(C)
Hemoglobin (DAF assay) Absorbtion-O.D
0.15
0.1
0.05
0
wt-EpoR
EpoR-HM
EpoR-H
EpoR-H
EpoR-HM
wt-EpoR
wt-EpoR
EpoR-H
EpoR-HM

Figure 3.12. Mek 1,2 inhibition reverses EpoR-HM erythroblast stage-specific differentiation defects. A] Bone marrow derived wt-EpoR, EpoR-HM and EpoR-H erythroid progenitor cells were cultured for 72 hours in SP34-EX medium containing U0126 (+/- 10μM). Expanded erythroblasts then were differentiated (in transferrin, BSA and insulin-containing medium) with Epo at 2.5U/mL and U0126 (+/-10μM). At 40 hours of culture, frequencies of high forward-angle light scatter erythroblasts were assayed. Note the reversal of differentiation defects in EpoR-HM erythroblasts as illustrated by U0126-induced decreases in forward scatter (cell size). **B]** U0126 reversal of EpoR-HM erythroblast differentiation defects as analyzed by Ter119 and CD71 marker expression. At 40 hours of differentiation, EpoR-HM erythroblasts also exhibited significantly decreased frequencies of $Ter119^{pos}$ erythroblasts specifically within a sub-population of maturing cells with decreased CD71 expression. U0126 reversed this defect (but had no significant effects on control wt-EpoR cells).

FIGURE 3.12

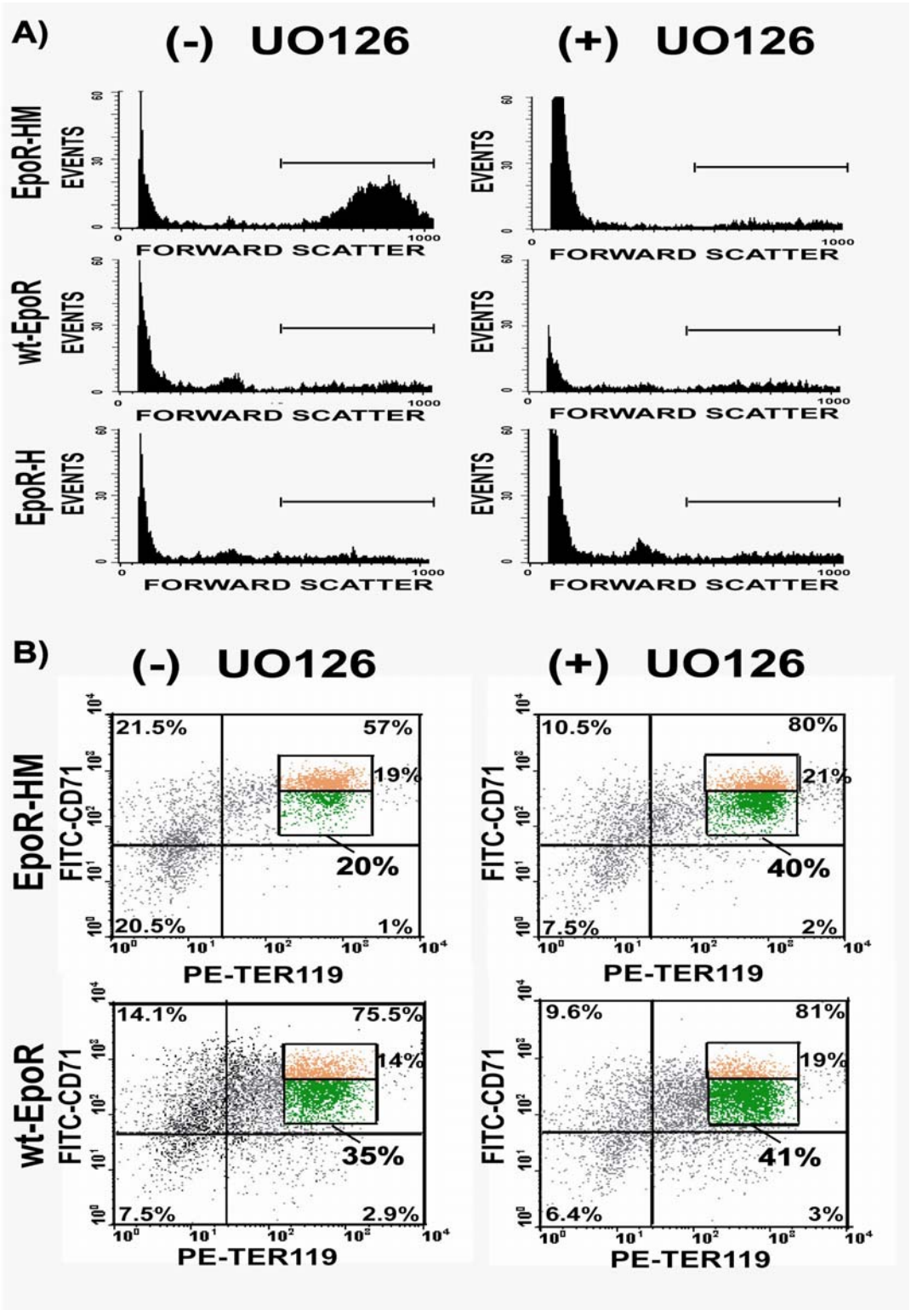

A)
(-) UO126
(+) UO126
EpoR-HM
wt-EpoR
EpoR-H
EVENTS
FORWARD SCATTER
B)
(-) UO126
(+) UO126
EpoR-HM
wt-EpoR
FITC-CD71
PE-TER119
21.5%
57%
19%
20%
20.5%
1%
10.5%
80%
21%
40%
7.5%
2%
14.1%
75.5%
14%
35%
7.5%
2.9%
9.6%
81%
19%
41%
6.4%
3%

Figure 3.13. Mek 1,2 inhibition dependent increase in Ter119 positive cells and UO128 dependent downstream ERK inhibition. A] U0126 dose-dependent reversal of EpoR-HM erythroblast differentiation defects also was observed based on U0126-dependent increases in frequencies of $Ter119^{pos}$ erythroblasts. **B]** U0126 inhibition of ERK1,2 activation in primary wt-EpoR, EpoR-HM and EpoR-H erythroblasts – The capacity of U0126 to inhibit the Epo-stimulated activation of ERKs was confirmed directly by exposing expanded, Ter119-depleted erythroblast preparations to +/- 10μM U0126.

FIGURE 3.13

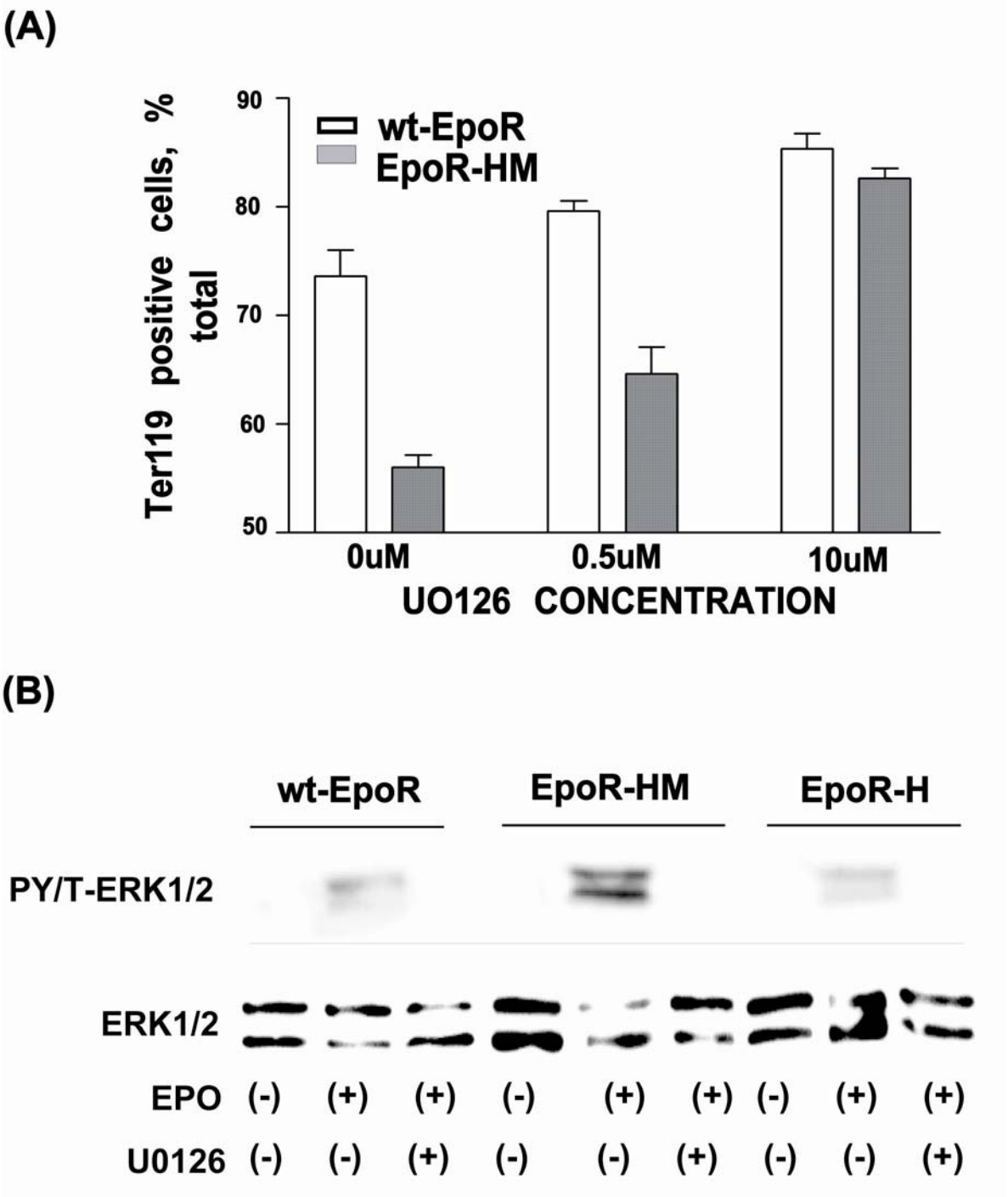

(A)
Ter119 positive cells, % total
90
80
70
60
50
wt-EpoR
EpoR-HM
0uM
0.5uM
10uM
UO126 CONCENTRATION
(B)
wt-EpoR
EpoR-HM
EpoR-H
PY/T-ERK1/2
ERK1/2
EPO (-) (+) (+) (-) (+) (+) (-) (+) (+)
U0126 (-) (-) (+) (-) (-) (+) (-) (-) (+)

TABLE 3.2. STEADY-STATE RENAL *EPO* TRANSCRIPT LEVELS IN EPOR-HM, EPOR-H AND WT-EPOR MICE.

	EpoR-HM	**EpoR-H**	**wt-EpoR**
Epo transcript levels, * % wt-EpoR	186 +/- 8 %	55 +/- 2 %	100 %

*****[Mean values +/- SE, n=3]

Regulation of pro and anti-apoptotic molecules by Epo (bcl-xl, bax and dapk2)

Based on Epo's primary role as an anti-apoptotic factor [335], possible differences among EpoR-HM, EpoR-H and wt-EpoR erythroblast survival, and Bcl-xl, Bax and DAPK-2 expression were analyzed (Figure 3.14 and 3.15). In expanded EpoR-HM erythroblasts, Bcl-xl levels proved to be decreased several-fold (Figure 3.14A and B). Bax expression levels, by comparison, were similar for each EpoR allele. In repeated analyses in Ter119-depleted $CD71^{high}$ wt-EpoR, EpoR-HM and EpoR-H populations, findings were similar (Figure 3.14A and B). The extent to which altered EpoR-HM signaling capacities might correlate with compromised erythroblast survival potential also was assessed. In these experiments, Kit^{pos} cells first were isolated (by MACS) from bone marrow preparations prior to expansion in SP34-EX medium. This provided improved flow cytometric discrimination of stepwise development. Co-staining with annexin-V revealed increased stage-specific staining of relatively late-stage EpoR-HM $CD71^{high}Kit^{neg}$ as well as differentiated $Ter119^{pos}$ erythroblasts as compared directly to wt-EpoR and EpoR-H cells (Figure 3.15A), and this was despite sustained exposure to high-dose Epo (2.5 U/mL). In part via gene-profiling analyses of purified developmentally-staged erythroblasts, we recently reported on predominant late-stage erythroid expression of the proapoptotic death-associated protein kinase-2 (DAPK2) [336]. In expanded wt-EpoR, EpoR-HM and/or EpoR-H progenitors, possible differential expression of DAPK-2 was assessed. Interestingly, DAPK-2 levels proved to be significantly elevated selectively in EpoR-HM erythroblasts (Figure 3.15B). This observation is consistent with the decreased survival potential of EpoR-HM cells (and with possible roles for EpoR PY343, and STAT-5, in down-modulating DAPK-2).

Figure 3.14. Altered Bcl-xl expression in EpoR-HM erythroblasts. A] Levels of Bcl-xl in expanded wt-EpoR, EpoR-HM and EpoR-H erythroblasts were assayed (by western blotting) at two time points – Directly following cytokine withdrawal, and at 30 minutes of Epo exposure. Note the decreased Bcl-xl levels in EpoR-HM erythroblasts. For comparison, Bax levels also were analyzed. **B]** Bcl-xl expression in wt-EpoR, EpoR-HM and EpoR-H erythroblasts also was analyzed in Ter119-depleted, $CD71^{high}$ erythroblasts.

FIGURE 3.14

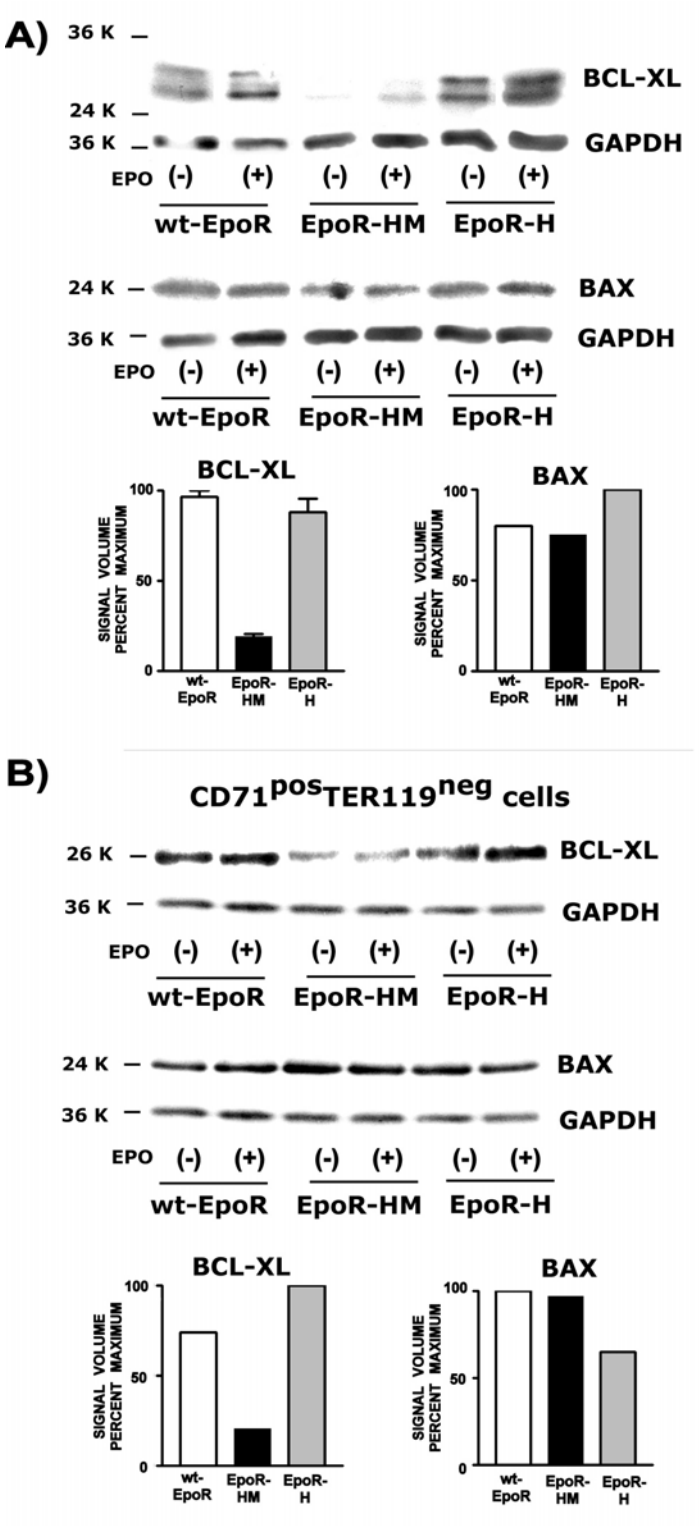
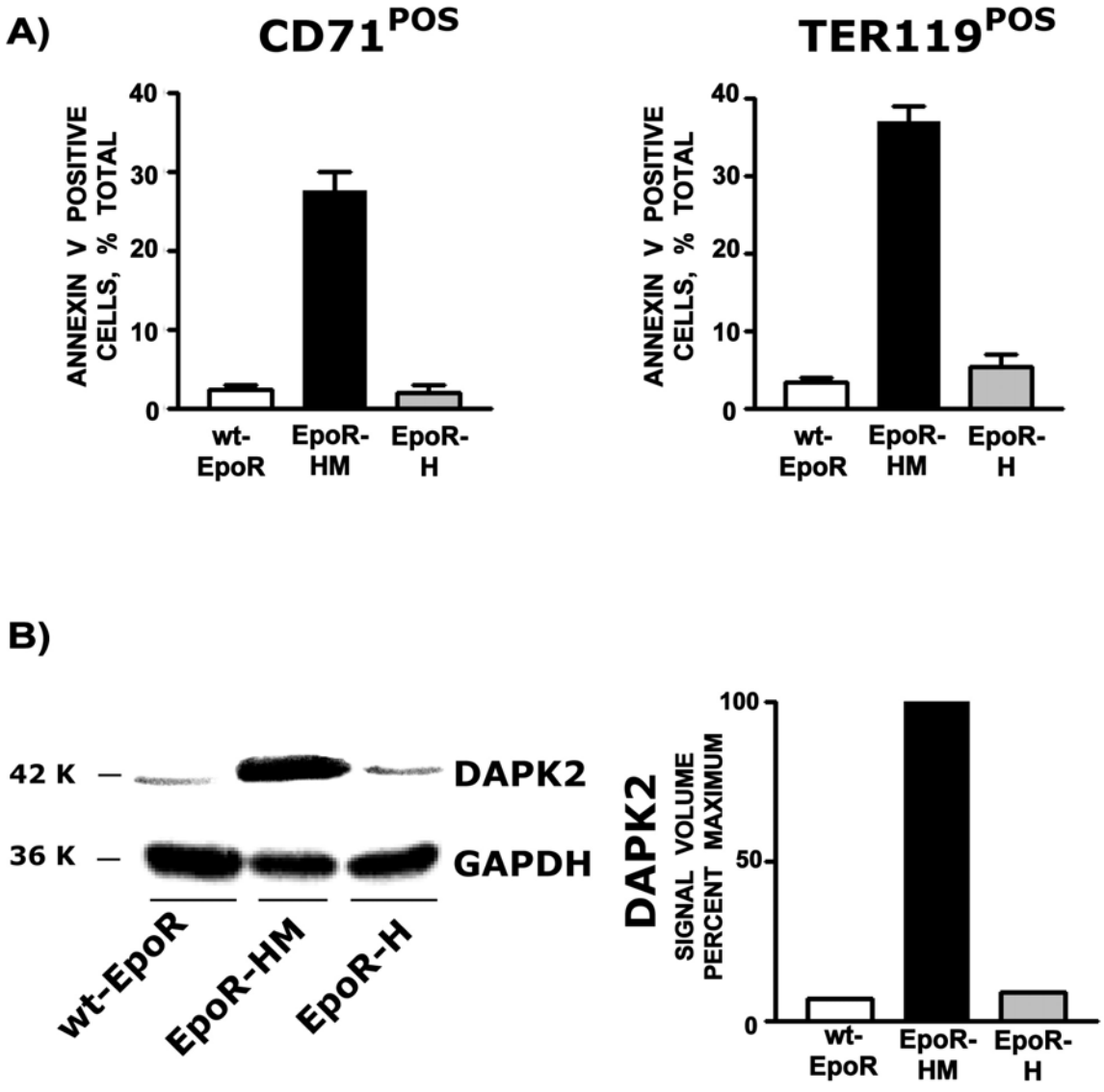

A)
36 K
BCL-XL
24 K
36 K
GAPDH
EPO (-) (+) (-) (+) (-) (+)
wt-EpoR EpoR-HM EpoR-H
24 K
BAX
36 K
GAPDH
EPO (-) (+) (-) (+) (-) (+)
wt-EpoR EpoR-HM EpoR-H
BCL-XL
SIGNAL VOLUME PERCENT MAXIMUM
100
50
0
wt-EpoR
EpoR-HM
EpoR-H
BAX
SIGNAL VOLUME PERCENT MAXIMUM
100
50
0
wt-EpoR
EpoR-HM
EpoR-H
B)
CD71posTER119neg cells
26 K
BCL-XL
36 K
GAPDH
EPO (-) (+) (-) (+) (-) (+)
wt-EpoR EpoR-HM EpoR-H
24 K
BAX
36 K
GAPDH
EPO (-) (+) (-) (+) (-) (+)
wt-EpoR EpoR-HM EpoR-H
BCL-XL
SIGNAL VOLUME PERCENT MAXIMUM
100
50
0
wt-EpoR
EpoR-HM
EpoR-H
BAX
SIGNAL VOLUME PERCENT MAXIMUM
100
50
0
wt-EpoR
EpoR-HM
EpoR-H

Figure 3.15. Defective survival and enhanced DAPK-2 expression in EpoR-HM erythroblasts. A] *Defective survival of EpoR-HM CD71^{high}Kit^{neg} erythroblasts, and rescue of survival potential by PY343 in EpoR-H –* Kit^{pos} progenitor cells were prepared from wt-EpoR, EpoR-HM and EpoR-H bone marrow, and were expanded in SP34-EX media. At day-3 of culture, CD71 and Ter119 marker expression was assayed, and cells were co-stained with Annexin-V. Relative frequencies of Annexin V-positive cells among CD71^{pos} subpopulations of EpoR-HM, EpoR-H, and wt-EpoR erythroblasts are graphed. Expanded cells also were shifted to differentiation medium, and frequencies of Annexin-V and Ter119 co-positive cells were analyzed. **B]** *Elevated DAPK-2 expression in EpoR-HM erythroblasts –* Death-associated protein kinase-2 (DAPK-2) expression in wt-EpoR, EpoR-HM and EpoR-H erythroblasts was assayed by western blotting (and digital densitometry). Note the several-fold increase in DAPK-2 levels in EpoR-HM erythroblasts (representative of three independent experiments).

FIGURE 3.15


A)
CD71POS
TER119POS
ANNEXIN V POSITIVE CELLS, % TOTAL
40
30
20
10
0
wt-EpoR
EpoR-HM
EpoR-H
B)
42 K
36 K
DAPK2
GAPDH
wt-EpoR
EpoR-HM
EpoR-H
DAPK2
SIGNAL VOLUME PERCENT MAXIMUM
100
50
0

E) DISCUSSION

EpoR mediated Jak-Stat activation

As revealed through transgenic mouse models, EpoR is essential for erythroblast formation [154], and its conditional hematopoietic expression is sufficient for normal overall development [154]. Crystallographic and structural analyses have provided detailed insight into Epo-mediated dimerization of its receptor [134,164], and conformation-dependent mechanisms of JAK2 activation [161]. Epo signal transduction studies also have gone far to define a broad, yet select set of factors and associated pathways that mediate and/or modulate Epo's actions as a clinically important anti-anemia agent [337,338]. The present work uses mice with knocked-in minimal EpoR alleles and primary marrow-derived erythroblasts to further define core signal transduction events which are integral to Epo receptor function.

A minimal model for EpoR action is one whereby JAK2 fulfills a central role, and supports Epo action independently from EpoR PY modulating effects. This model is predicted by the *in vivo* erythropoietic capacity of EpoR-HM, and has previously been framed in cell line models. Specifically, EpoR-HM has been reported in 32D cells to induce *Bcl-x* and *c-Myc* expression [304,339], and to support ERK activation at wild-type levels [340]. In the present investigations EpoR-HM's capacity in primary erythroblasts to activate JAK2, STAT-5, STAT-5-target genes, *Bcl-x* and *Myc* was analysed (see below). JAK2 activation profiles were essentially normal and equivalent. In the absence of the distal cytoplasmic domains, aberrations in protein folding and surface expression of truncated EpoR receptors were a distinct possibility. Therefore, equality of JAK2 activation amongst the three alleles was an essential first step to confirm in order to

interpret EpoR mediated downstream signaling. This essentially ruled in similar EpoR expression profiles and Epo binding amongst wt-EpoR, EpoR-HM and EpoR-H alleles. Also, in the absence of PY343, no significant induction of STAT-5, or STAT-5-target genes was detected

Deregulation of MAPK pathway in the absence of PY-343

Essential mechanisms of EpoR-mediated ERK activation are incompletely defined, but previous cell line studies have outlined several EpoR PY-dependent routes. This includes a PI3-kinase dependent pathway [341] ; a recently described PY479 plus PLC-gamma dependent route [222]; a Shc plus Grb2 pathway to mSos and Ras [217]; a CrkL plus C3G- coupled route [342]; as well as a SOCS-3 mediated mechanism involving PY-SOCS box sequestration of GAP [343]. In contrast, the presently observed hyperactivation of ERKs via EpoR-HM in primary erythroblasts suggests that PY sites may be non-essential for ERK activation. Mechanisms in EpoR-HM leading to ERK activation are presently undefined, but G-proteins as well as PKCs stimulate MAPK modules [224,344] and might be possibly involved as potential Epo-regulated MAPK activators. Based on the relatively normal steady state erythropoiesis exhibited by EpoR-HM, PY-independent mechanisms leading to this process need to be defined. In addition, a specific loss of PY343 signaling is associated with EpoR-HM mediated ERK hyper-activation. The mechanisms involved in possible PY-343 mediated negative regulation of MAPK is presently unclear. One possible candidate is MAPK phosphatase. MKP-7 haploinsufficiency, for example, has been associated with BCR-ABL induced proliferation [345], while increased MKP-1 activity is associated with pre-adipocyte differentiation [346] .

Possible cell-fate determination roles by Epo-regulated ERKs also merit consideration. In a cell specific context, ERK signaling can affect differentiation, survival and proliferation (reviewed

Roux et al. [347] and Wada et al. [348]). However, ERKs are most commonly activated via growth factor receptors [347] and have been shown in primary erythroid cells to be the prime effector of H-Ras induced transformation [225]. Also, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been proposed to mediate its antidifferentiative effects in erythroid cells through ERK activation [226]. Therefore, ERK hyperactivation in EpoR-HM erythroblasts may inappropriately enforce proliferation at the expense of differentiation. This theory is supported by the restoration of wild-type differentiation profiles for EpoR-HM erythroblasts upon exposure to the MEK1,2 inhibitor U0126 . In some instances, though, chronic activation of ERKs also can induce apoptosis [349], and hyperactivation of ERKs in EpoR-HM might be another explanation for the observed increase in apoptosis.

PI3K-AKT regulation through EpoR

As compared to the wt-EpoR, PY-deficient EpoR-HM and EpoR-H alleles faltered in their abilities to support Epo-induction of AKT and its downstream target p70S6-kinase. For AKT and p70S6-kinase (and as discussed above) this likely reflects uncoupling from PI3-kinase [197,211]. In the absence of PY479 site (which binds and activates PI3-kinase) in both EpoR-H and EpoR-HM alleles, Epo induced AKT activation would be predicted to be absent. Therefore, low-level activation of AKT in EpoR-H and EpoR-HM erythroblasts is intriguing and might be a result of PY479 independent activation of PI3-kinase. In other systems (HCD57 and BAF-3 cell lines) it has been previously reported that adaptor molecules like GAB and IRS-2 which are activated by Epo, can bind and activate PI3-kinase [205,207]. Also, activation of IRS-2 in response to Epo was reported to be independent of PY-sites in the Erythropoietin receptor [207]. Further mechanisms

involving PY-independent activation of IRS-2 (including possible JAK2 and GAB, IRS-2 interactions) need to be investigated.

Bcl-xl expression in wt-EpoR, EpoR-HM and EpoR-H derived erythroblasts

The role of Bcl-xl as an essential anti-apoptotic factor in the process of erythropoiesis is clearly established. Bcl-xl expression is highly upregulated in an Epo-dependent fashion during the process of erythroid maturation [261]. Bcl-xl $^{-/-}$ mice are embryonic lethal because of massive apoptosis of immature erythroid progenitor cells [264]. Conditional Bcl-xl $^{-/-}$ mice exhibit severe hemolytic anemia, hyperplasia of immature erythroid progenitor cells and splenomegaly [262]. Less clear, is the role of STAT-5 in regulating Bcl-xl. Previous studies have implicated Bcl-xl to be a downstream target gene of STAT-5 and a STAT-5 consensus binding sites in the Bcl-xl gene regulatory region (within first intron) have been described [296]. Splenocytes from STAT-5 $^{-/-}$ have a deficient Bcl-xl expression at the protein level which is coupled with embryonic anemia, increased apoptosis in fetal liver EPCs and deficient splenic erythropoiesis [296,299]. However, similar studies conducted by Zang et al. in these STAT-5 $^{-/-}$ mice did not observe the previously reported deficiency in Bcl-xl protein expression [300]. Also, recent studies by Dolznig et al. have proposed that the anti-apoptotic action of Epo is not mediated through Bcl-xl and that, Bcl-xl mediated survival is restricted to late-stage hemoglobinized erythroblasts [266]. Present studies fail to observe Epo dependent induction of Bcl-xl transcript in wt-EpoR, EpoR-H and EpoR-HM alleles. This, however, is in contrast to an observed decrease in Bcl-xl at the protein level in EpoR-HM erythroblasts. Three possible explanations exist for this discrepancy; first, Bcl-xl might be regulated at the post-translational level by factors specific to the Epo-PY3434-STAT-5 pathway. Second, Epo induction of Bcl-xl may be a highly dose, time and cell-stage dependent

phenomenon. Third and lastly, the observed deficiency in Bcl-xl protein expression in EpoR-HM erythroblasts might be a simple reflection of increased basal apoptosis. In summary, these studies reveal the critical signal transduced through a JAK-2 only PY-null form that seems to be sufficient to support steady state erythropoiesis.

CHAPTER 4

DEFECTIVE STRESS ERTHROPOIESIS AND EPO-SCF SYNERGY IN PY-NULL EPOR-HM MICE THAT ARE REVERSED IN PY343-ONLY EPOR-H MICE.

This work was originally published in Journal of Clinical Investigation, "Menon MP, Karur V, Bogacheva O, Bogachev O, Cuetara B, Wojchowski DM. Signals for stress erythropoiesis are integrated via an erythropoietin receptor-phosphotyrosine-343-STAT-5 axis. J Clin Invest. 2006;116:683-694". (As per American Society of Clinical Investigation copyright requirements)

ABSTRACT

Anemia due to chronic disease or chemotherapy often is ameliorated by erythropoietin (Epo). Present studies reveal that unlike steady-state erythropoiesis, erythropoiesis during anemia depends sharply upon an Epo receptor-PY343-STAT-5 signaling axis. In mice expressing a phosphotyrosine-null Epo receptor allele (EpoR-HM), severe and persistent anemia was induced by hemolysis, or 5-fluorouracil. In short-term transplantation experiments, donor EpoR-HM bone marrow cells also failed to efficiently repopulate the erythroid compartment. In each context, stress erythropoiesis was rescued to wild-type levels upon the selective restoration of EpoR PY343 STAT-5 binding site (EpoR-H allele). As studied using a unique primary culture system, EpoR-HM erythroblasts exhibited marked stage-specific losses in Epo-dependent growth and survival. EpoR-H PY343 signals restored efficient erythroblast expansion, and the selective Epo-induction of the STAT-5 target genes *Pim-1* and *oncostatin-M*. *Bcl-x*, in contrast, was not significantly induced via wt-EpoR, EpoR-HM or EpoR-H alleles. In $Kit^{pos}CD71^{pos}$ erythroblasts, EpoR-PY343 signals furthermore enhanced stem cell factor (SCF) growth effects, and SCF modulation of Pim-1 kinase and *oncostatin-M* expression. In maturing $Kit^{neg}CD71^{pos}$ erythroblasts, oncostatin-M exerted anti-apoptotic effects, which likewise depended upon EpoR-

PY343- mediated events. Stress erythropoiesis therefore requires stage-specific EpoR-PY343-STAT-5 signals, certain of which selectively bolster SCF and oncostatin-M action.

INTRODUCTION

Erythropoietin receptor signaling requirements; Steady-state erythropoiesis

As a central hormonal regulator of red cell production, erythropoietin (Epo) is required for development beyond the CFUe stage, and functions primarily as an erythroblast survival factor [152]. Via hypoxia inducible factor 1-3 alpha/beta-regulated mechanisms [118], Epo is expressed in the adult kidney and is secreted as a complex sialoglycoprotein [90]. As discussed in previous chapters, its actions on erythroid progenitor cells then depend on Epo binding to pre-formed erythropoietin receptor (EpoR) dimers [164]. Extensive studies of Epo and EpoR interactions have assisted the development of highly active recombinant erythropoietins as important anti-anemia agents [337]. Nonetheless, resistance to Epo during chronic anemia and myelodysplasia [350,351], Epo hyper-responsiveness in congenital and familial polycythemias [352], and efforts to develop novel Epo orthologues [134,337] continue to raise important questions concerning Epo action mechanisms. Impetus for extended studies of action mechanisms also is provided by reported Epo cytoprotection of retinal, glial, cardiomyocyte, endothelial and renal tubular epithelial cells [121] and by roles for Epo during angiogenesis [121] and possibly tumorigenesis [353].

Epo receptor signals for erythroblast formation involve first the activation of JAK2, an essential upstream Janus kinase which pre-assembles at a conserved EpoR box-1 domain [90]. JAK2 then mediates the phosphorylation of eight conserved EpoR cytoplasmic tyrosine (PY) motifs [90]. These EpoR PY sites function as a scaffold for the binding of a complex, yet fairly well defined set of Src homology-2 and phosphotyrosine binding protein domain signal transduction factors. These factors have been discussed in detail in the previous sections (Chapter 1 and 3).

Epo's predominating positive signals are linked to a distinct subset of EpoR PY sites, and coupled effectors [90]. PI3-kinase binding at EpoR PY479 leads to Akt, mTOR and p70S6K activation [199,211]. Grb2-Shc binding at PY464 (together with Syp phosphatase binding at PY425) [220] have been linked to mSos/Ras/Raf/MEK regulation, while PLCgamma-1 activation and TRPC2-mediated calcium flux appear to couple to PY460 [242]. EpoR PY- site dependent signals, in addition, have been implicated in Gab docking protein [206] and NF-kB modulation [354]. Finally, STAT-5 activation occurs predominantly via PY343 [284] and may promote Bcl-xl expression [296].

Despite this conserved evolution of EpoR PY signaling scaffolds [174], steady-state erythropoiesis unexpectedly has been shown to be supported by a minimal PY-null EpoR allele (EpoR-HM) as expressed from the endogenous murine *EpoR* locus [300]. Specifically, EpoR-HM mice maintain hematocrits within 8-points of normal, RBC counts at ~80% of normal, and essentially wild-type levels of burst forming unit-erythroid (BFUe) and colony forming unit-erythroid (CFUe). Core EpoR-JAK2- dependent, EpoR PY site- independent signals therefore appear to be sufficient for steady-state erythropoiesis.

Experimental approach

The present studies focus on the concept that one (or more) positively- acting EpoR PY sites might be selectively required for erythropoiesis during anemia. In the following experiments, mice expressing knocked-in PY-mutated EpoR alleles have been used to discover, and characterize Epo receptor-associated events that selectively affect stress erythropoiesis. Stress erythropoiesis is an accelerated erythropoietic response exerted especially in extramedullary organs like spleen to assist recovery from anemia induced by hemolysis or tissue hypoxia. Two

reasons exist for evaluating stress erythropoiesis in mice with the truncated EpoR alleles (EpoR-HM and EpoR-H). First, pilot *in vitro* experiments with bone marrow derived EPCs from EpoR-HM mice revealed deficiencies in erythropoiesis at physiological Epo doses [281]. Based on this, lack of an overt in vivo defect in EpoR-HM mice steady-state erythropoiesis might depend on one or more compensatory factors. Such compensatory factors, however, might not be sufficient to support the accelerated splenic erythropoiesis that is engaged during hematopoeitic stress. Second, recent advances have characterized several molecules that are differentially engaged between steady-state and stress erythropoiesis. These include bone morphogenic protein-4 (BMP4)/Madh5 regulation of splenic erythroid progenitor cell expansion [355]; enhancement of erythropoiesis during chronic anemia by Gas6 (a Tyro3/Axl/Mer RTK ligand) [356]; and differential effects of oncostatin-M on extramedullary *vs.* bone marrow erythropoiesis [357]. In animal models, anemia can be induced by irradiation, hemolytic agents e.g. phenylhydrazine (PHZ) or anti-neoplastic agents e.g. 5-flourouracil (5-FU). Phenylhydrazine induces hemolytic anemia by peroxidation of membrane lipids in erythrocytes and this effect is a direct result of interaction of oxygen radicals with membrane lipids [358]. 5-FU is a pyrimidine antagonist that inhibits DNA synthesis in progenitor cells [359]. Recovery from either phenylhydrazine or 5-FU induced anemia is dependent on erythropoiesis in extramedullary erythropoietic organs (predominantly spleen). Hence, the ability of EpoR-HM mice to recover from anemia induced via these agents was directly tested. In a bone marrow transplantation context, it has been demonstrated previously that short-term radioprotection post irradiation is provided predominantly by myelo-erythroid progenitors in the donor cells [360]. Therefore, the comparative ability of bone-marrow derived EPCs from EpoR-HM to rescue irradiated recipients was tested in a short-term bone marrow transplantation model.

Introduction to data

Investigations reveal that EpoR PY-independent, JAK2-dependent cell signals fail to efficiently support erythropoiesis during hemolytic anemia, 5-fluorouracil suppression of progenitor cell renewal, or marrow transplantation. Efficient stress erythropoiesis, however, is rescued by an EpoR-H allele in which a PY343 STAT-5 binding site is selectively restored. In a unique ex vivo system, EpoR-PY343-STAT-5 signals furthermore are shown to be important for Epo-dependent $Kit^{pos}CD71^{pos}$ erythroblast growth and survival, and for the selective induction of proviral integration site-1 *Pim-1 kinase* and *oncostatin-M* expression. Finally, EpoR-PY343-STAT-5 signals are also revealed to enhance both SCF and oncostatin-M effects on primary erythroblast development.

METHODS

Mice and anemia models

Mice with knocked-in EpoR-HM and EpoR-H alleles (and wild type controls, Jackson Labs stock #1009003) were as described in the previous chapter [300,361]. Each was maintained as a C57BL/6-129 line, and were used at 8-12 weeks. Phenylhydrazine (60mg/kg) was administered subcutaneously at 1 and 24 hours. 5-Fluorouracil was administered intraperitoneally at a dose of 150 mg/kg. For bone marrow transplantation experiments, bone marrow cells were depleted of T-cells (CD90.2 negative-selection) and injected ($1x10^6$ cells) into lethally irradiated Ly5.1 B6.SJL recipients (Jackson Labs stock #002014). The Ly marker allowed distinction between donor cells (Ly5.2) and recipient cells (Ly5.1) based on flow cytometric assays. Hematocrits were assayed by capillary centrifugation (Autocrit Ultra3). Reticulocyte levels were assayed by Thiazole orange staining (Retic-count reagent, BD Biosciences 349204) and flow cytometry (BD Facscalibur). Spleens were fixed in paraformaldehyde and later embedded in paraffin. Subsequently, 4μ sections were generated and stained with hematoxylin-eosin.

Erythroid progenitor cell preparation and culture

Splenocytes from phenylhydrazine-treated mice were disrupted, passed through 40μ mesh, exposed to NH_4Cl (Stem Cell Technologies), and collected through 50% FBS in PBS (Invitrogen #14190-144). For BFUe and CFUe assays, splenocytes were plated in triplicate ($1.2x10^5$ and $3.5x10^5$ cells/mL). Subsequently, hemoglobinized colonies were benzidine-stained, and scored using grid-plates at day 2 (CFU-E) or day 7 (BFU-E). Bone marrow cells were expanded in SP34-EX media till day 3.5 as described in the previous chapter. They were cultured at a concentration of $7.5x10^5$ cells/mL in StemPro-34 medium (Invitrogen) supplemented with

2.5U/mL Epo, 100ng/mL mSCF (Peprotech), 1uM dexamethasone, 1uM beta-estradiol, 75ug/mL h-transferrin, 1% BSA (Stem Cell Technologies), and 0.1mM 2-mercaptoethanol ("SP34-EX" medium). At 24 hours, 0.6 volume of SP34-EX was added. At 48 hours, cells were replated ($7.5x10^5$ cells/mL) in 0.8 volumes of new, and 0.2 volumes of residual conditioned media. From the cohort of day 3.5 expanded cells, Kit^{pos} cells were isolated using CD117-microbeads (Miltenyi Biotec) by positive selection. In differentiation experiments, expanded erythroblasts were transferred to IMDM (Invitrogen #12440-053) containing Epo (2.5U/mL), transferrin (150ug/mL), insulin (10ug/mL), 0.1mM 2-mercaptoethanol and 10% FBS. Slide-centrifugation ($1x10^5$ cells) was for 15 minutes (300 rpm, Hettich AG). Staining for the cytospins was with Dip-Stain reagent (Volu-Sol Inc.).

FACS and MACS Sorting

FACS of $Kit^{pos}CD71^{high}$ and $Kit^{neg}CD71^{high}$ erythroid progenitor cells involved APC-CD117 plus FITC-CD71 co-sorting (BD-FACS Vantage). Alternatively, magnetic-activated cell sorting (MACS) was performed via an initial lin^{pos} cell depletion (Stem Cell Technologies #19756) plus Kit^{pos} selection (Miltenyi Biotec #130-091-224). This generated both Kit^{pos} cells (positive selection) and Kit^{neg} cells (negative selection). Sub-population purities were $\geq$ 95%.

Flow cytometry, apoptosis assays and ^{3}HdT incorporation assays

In flow cytometry (BD FACScalibur), washed cells ($1x10^6$ per 0.2mL PBS, 0.1% BSA) were incubated with rat IgG (1ug), and stained with 1 ug of APC-CD117 plus PE-Ter119 and/or FITC-CD71, FITC-Mac1 or FITC-B220 (BD Biosciences). APC-Ly5.2 and 5.1 antibodies were from Ebioscience. Annexin-V binding (BD Biosciences) was in 140mM NaCl, 2.5mM $CaCl_2$,

10mM HEPES, pH 7.4. In ^{3}HdT-incorporation assays, expanded erythroblasts were plated in SP34-EX medium ($1x10^{5}$ cells/mL) with the indicated cytokines. Splenic erythroid progenitor cells were plated at $5x10^{6}$ cells/mL. At 20 hours, 1μCi of ^{3}HdT was added [361] and incorporation rates (per 5 hours) were determined.

RT-PCR

RNA was prepared using Trizol reagent (Invitrogen). cDNA was prepared with Superscript III (Invitrogen). Q-PCR (I-Cycler) utilized Sybr-green reagents (BioRad Laboratories). Primer pairs are as follows;

Epo, 5'- AGAATGGAGGTGGAAGAACAGG-3' and 5'-CTGGTGGCTGGGAGGAATTG-3';

Pim-1, 5'-TTCTGGACTGGTTCGAGAGG-3' and 5'-GCTCCTCGTTCGGTGATAAA-3';

Onco-M, 5'-AACTGAGCAAGCCTCACTTCC-3' and 5'-ATGCCGAGGATATTGTGCCG-3';

Bcl-x 5'-ACTGTGCGTGGAAAGCGTAGA-3' and 5'-TGCTGCATTGTTCCCGTAGAG-3';

SOCS3 5'-CCGCTTCGACTGTGTACTCAAG-3' and 5'-TCTTCTCGCCCCCAGAATAGAT-3' *Gas-6*, 5'-GCCATCCAGCAGACAGTCAAG-3' and 5'-TGGTTTCCGTGCCGACATC-3';

Pim-2, 5'-CCAGAACCTCTGGTCCCTAA-3' and 5'-CTAAAGAGCTGCTGGGGATG-3';

Onco-MRβ, 5'-CCAGCCCTCAGCACAAACC-3' and 5'- GGACCATCAAGGACTCAGGAG-3'; *Actin*, 5'-CGTGCGTGACATTAAAGAGAAG-3' and 5'-TGGATGCCACAGGATTCCATA-3'; *Cis-1* - 5'-CCA-CTG-GCT-TTG-TCA-AGA-AGG-3' and 5'-AGG-CCA-CAT-AGT-GCT-GCA-CAA-3'.

Protein extraction and western blotting

For western blotting, erythroblasts were incubated for 6 hours in 0.5% BSA, 10ug/mL transferrin, 10ng/mL insulin, 0.1mM 2-mercaptoethanol in IMDM ($8x10^5$ cells/mL) and exposed to Epo, SCF or oncostatin-M as indicated. Cells then were combined with four volumes of 2^oC PBS, collected, and lysed in 1% Igepal, 150mM NaCl, 50mM NaF, 2mM Na_2EDTA, 10mM sodium pyruvate, 25mM beta-glycerol phosphate, 10% glycerol, 1mM dithiothreitol, 50mM HEPES (pH 7.5) supplemented with 0.25mg/mL phenylmethylsulfonylfluoride, 1x protease inhibitor and 1x phosphatase inhibitor cocktails (Sigma-Aldrich Inc. #P8340, #P5726) (0.15mL lysis solution per $1x10^7$ cells). An equal volume of 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 112.5mM NaCl, 37.5mM Tris-HCL (pH 7.4) then was added and cleared extracts were denatured, electrophoresed (25ug), and transferred to PVDF membranes. The membranes were then incubated overnight with primary antibodies at 4^0C. Primary antibodies used were from Cell Signaling Technology (CS), Abcam Ltd. (AC), or Upstate Cell signaling Solutions (UPS) as designated: Pim-1, CS # 4722; phospho-STAT-5, CS #9351; phospho-STAT-3, CS #9131; phospho-STAT-1, CS #9171, STAT-5, CS #9352; STAT-3, CS #9132; STAT-1, CS #9172; phospho-ERK, CS #9101; ERK, CS #9102; JAK2, UPS #06-255; phospho-JAK2, UPS #07-123; GAPDH, AC #ab9485. For chemiluminescence, HRP-secondary antibodies (Jackson Immunoresearch Laboratories) and Super-Signal West-Dura reagent (Pierce Biotechnology) were used.

RESULTS

Defective recovery from phenylhydrazine and 5-Fluorouracil induced anemia in EpoR-HM as compared to wt-EpoR and EpoR-H mice

As described in previous sections, the minimal EpoR allele, EpoR-HM, retains a JAK2 binding membrane proximal domain but otherwise lacks all additional known cytoplasmic signaling motifs [300,361]. Unexpectedly, mice expressing the truncated HM allele effectively support steady-state erythropoiesis in terms of near-normal hematocrits and comparable survival rates [300]. Previous studies in STAT-5$^{-/-}$ mice have reported deficient stress erythopoiesis. In order to test whether PY-343-STAT-5 deficient EpoR-HM mice exhibit similar deficiencies in stress erythropoiesis, EpoR-HM along with mice expressing an EpoR-H allele with singularly restored PY343 STAT-5 binding site [300] were treated with either phenylhydrazine or 5-fluorouracil, and the resulting anemia was characterized over 30-day time-courses (Figure 4.1A). In a model simulating bone-marrow suppression, 5-fluorouracil suppression of proliferating erythroid progenitor cells resulted in a sharp and persistent anemia as compared to wt-EpoR and EpoR-H mice. In response to a different kind of anemia (phenylhydrazine induced hemolytic anemia), a similar defect in erythropoiesis was observed in EpoR-HM mice in terms of sustained low hematocrits. In contrast, no such defect was observed in EpoR-H mice, and PY343-mediated signals proved to restore stress erythropoiesis to wild-type levels.

Factors underlying defective erythropoiesis in EpoR-HM mice during hemolytic anemia were considered further based on spleen histomorphologies, and erythroid progenitor cell formation (Figure 4.1B). As compared to wild type controls, spleen architectures in phenylhydrazine-treated EpoR-HM mice were aberrant. This included small pockets of apparently rapidly

maturing eosin-positive erythroblasts, and abnormal retention of white pulp (Figure 4.1B). In contrast, phenylhydrazine treated EpoR-H spleens were comparable to wild-type spleen architecture. Also, in response to phenylhydrazine, EpoR-HM mice had deficient splenomegaly as compared to wt-EpoR and EpoR-H. This is evident both in terms of gross appearance (Figure 4.1B bottom panel) as well as spleen/body weight ratio (Figure 4.1B). Flow cytometric analyses of EpoR-HM erythroid progenitor cells further revealed decreased representation of $Ter119^{pos}$ cells (Figure 4.2A). In contrast, $Ter119^{pos}$ cells in EpoR-H spleens were comparable to that of wt-EpoR mice. Also, proliferation assays of Epo-dependent ^{3}HdT incorporation revealed deficiencies in EpoR-HM derived splenic EPCs as compared to wt-EpoR and EpoR-H. (Figure 4.2B). Furthermore, progenitor pools (as assayed by CFUe and BFUe assays) were markedly decreased in EpoR-HM spleens (~ 4-5 fold less), while frequencies in EpoR-H spleens closely approximated wt-EpoR controls (Figure 4.2C). Thus, signals emanating from JAK2 only PY-null EpoR-HM receptor fails at a relatively early progenitor cell level to effectively support extramedullary erythropoiesis in reponse to anemia. These defects, however, are reversed in EpoR-H mice and EpoR-H PY343 mediated signals seem to be sufficient to sustain stress erythropoiesis.

Figure 4.1. Efficient stress erythropoiesis in response to 5-fluorouracil or phenylhydrazine is not supported by a minimal PY-null EpoR-HM allele, but is rescued by EpoR-H/PY343/STAT-5 signals. A] Mice expressing the diagrammed EpoR alleles (n=8 mice per allele) were treated with 5-fluorouracil (5-FU) or phenylhydrazine (PHZ). Induced anemia, and recovery, were then monitored over a 30-day time-course based on hematocrits. Note the sustained anemia incurred in EpoR-HM mice in each model. Hematocrits are means (+/- SE) for n=4 mice per time-point. **B]** *Aberrant splenic architecture and deficient splenomegaly in response to hemolytic anemia in phenylhydrazine-treated EpoR-HM mice, and rescue of extramedullary erythropoiesis by EpoR-H***.** At day 4 post phenylhydrazine treatment, spleens from EpoR-HM, EpoR-H and wt-EpoR mice were fixed, sectioned, stained (hematoxylin-eosin) and examined for white and red pulp architecture. Histomorphologies are representative of four mice per group (and 20 sections per mouse) and are presented at 40X magnification. Also illustrated are EpoR-HM specific deficiencies in splenomegaly. Representative whole spleen photographs from wt-EpoR, EpoR-HM and EpoR-H mice post phenylhydrazine injection on day 4 are shown.

FIGURE 4.1

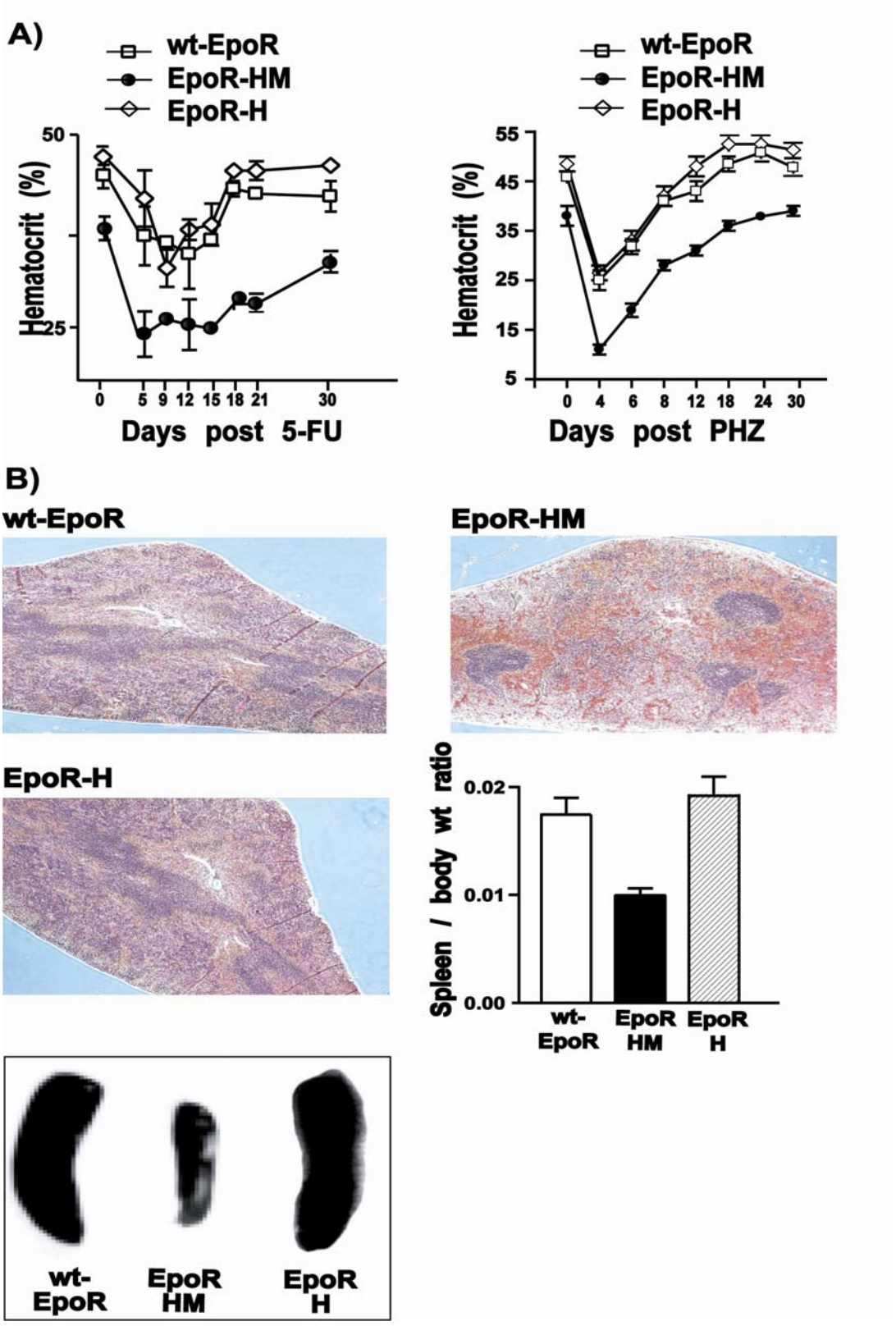
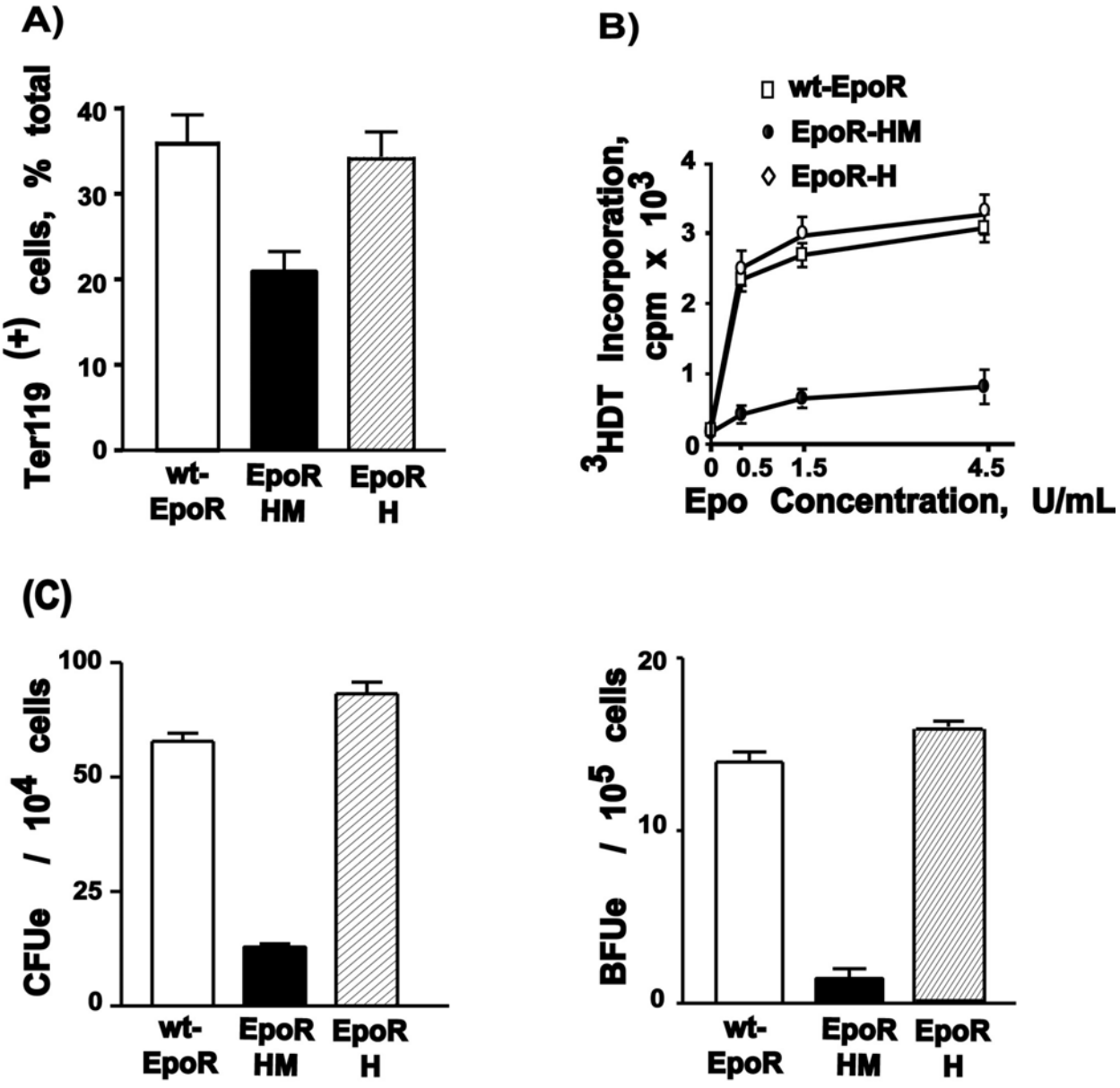

A)
wt-EpoR
EpoR-HM
EpoR-H
Hematocrit (%)
50
25
0 5 9 12 15 18 21 30
Days post 5-FU
wt-EpoR
EpoR-HM
EpoR-H
Hematocrit (%)
55
45
35
25
15
5
0 4 6 8 12 18 24 30
Days post PHZ
B)
wt-EpoR
EpoR-HM
EpoR-H
Spleen / body wt ratio
0.02
0.01
0.00
wt-EpoR
EpoR HM
EpoR H
wt-EpoR
EpoR HM
EpoR H

Figure 4.2. Deficient Ter119^{pos} cell, CFUe and BFUe formation in phenylhydrazine-treated EpoR-HM mice spleens, and rescue of extramedullary erythropoiesis by EpoR-H. **A]** *EpoR-HM specific defects in splenic Ter119^{pos} erythroblast formation***.** Frequencies of Ter119^{pos} erythroblasts in day 4 splenocyte preparations from PHZ-treated wt-EpoR, EpoR-HM and EpoR-H mice are shown. Note the decreased frequencies of Ter119^{pos} cells in EpoR-HM spleens. **B]** *Epo-dependent proliferation, and rescue by EpoR-H*. Rates of Epo-induced ^{3}HdT incorporation for splenic erythroid progenitor cells (prepared at day 3) are graphed. EpoR-HM derived splenocytes are deficient in terms of ^{3}HdT incorporation. **C]** *Decreased CFUe and BFUe frequencies in EpoR-HM spleens as compared to that of wt-EpoR and EpoR-H*. At day-2 post phenylhydrazine treatment, frequencies of splenic CFUe and BFUe were determined. For each analysis, mean values (+/- SE) are illustrated (n=3 mice per group). Results are representative of three independent experiments.

FIGURE 4.2


A)
Ter119 (+) cells, % total
40
30
20
10
0
wt-EpoR
EpoR HM
EpoR H
B)
wt-EpoR
EpoR-HM
EpoR-H
^{3}HDT Incorporation, cpm x 10^3
4
3
2
1
0
0 0.5 1.5 4.5
Epo Concentration, U/mL
(C)
CFUe / 10^4 cells
100
50
25
0
wt-EpoR
EpoR HM
EpoR H
BFUe / 10^5 cells
20
10
0
wt-EpoR
EpoR HM
EpoR H

Defective short-term transplantation capabilities of EpoR-HM derived bone-marrow cells as compared to wt-EpoR and EpoR-H mice

Based on the expression of Epo receptor in non-erythropoetic organs (vascular, cardiac and renal cells [121]), possibility exists that the action of the PY-null allele in these tissues might impact on stress erythropoiesis in an indirect manner. To evaluate this possibility, and to test the function of the truncated EpoR alleles in a clinically relevant stress model, short-term repopulating capacities of bone-marrow derived hematopoietic progenitor cells from EpoR-HM and EpoR-H mice were assessed. The distinction between donor and recipient cells was made on the basis of different Ly marker (Ly5.2 in donor cells vs. Ly5.1 in recipient tissue). In wild-type recipient spleens, on gross inspection, transplanted EpoR-HM donor cells gave rise to visibly smaller colonies of engrafted cells as compared to that of wt-EpoR donor controls (Figure 4.3A). Also, reticulocyte levels in mice transplanted with EpoR-HM cells were decreased >four-fold and hematocrits were approximately two-fold below wt-EpoR controls. Furthermore, the representation of early erythroid progenitor cells ($CD71^{pos}Ter119^{pos}$ erythroblasts) was six-fold lower in EpoR-HM transplanted recipient spleens as compared to wt-EpoR controls (Figure 4.3A). Monocytes ($MAC1^{pos}$) and B-lymphocytes ($B220^{pos}$), in comparison, were represented at approximately wild-type frequencies. For transplanted EpoR-H cells, the above mentioned defects in EpoR-HM transplanted mice were reversed to that of wt-EpoR capacities (Figures 4.3A and B).

In earlier studies conducted by our lab, we had observed a significantly higher proliferative response of an erythroid cell line (GIE2) in response to EpoR-HM serum as compared to that of wt-EpoR [281]. This suggests a compensatory increase of one or more hematopoietic growth factors

in EpoR-HM serum. One factor that might contribute in a compensatory fashion to rescue EpoR-HM steady state erythropoiesis is Epo. Indeed, clinical situations resulting in tissue hypoxia including anemia, lung disease, or cyanotic heart disease, lead to increased levels of serum Epo (100-1000 fold) [362]. Specifically, possible compensatory Epo expression in steady-state EpoR-HM mice might result in reaching of upper limits of Epo production. This might effectively blunt the required Epo response (and accelerated stress-erythropoiesis) to phenylhydrazine and/or 5-fluorouracil induced anemia. In *STAT-5a,b*$^{-/-}$ mice, for example, previous reports have indicated more than 100-fold spontaneous increases in Epo production (in combination with splenomegaly) [299]. In contrast, *Epo* transcript levels in untreated EpoR-HM mice, however, were not markedly elevated (Figure 4.4). Also, splenomegaly was not observed in steady-state untreated EpoR-HM mice. Upon Phenylhydrazine treatment, though, renal *Epo* transcript expression in EpoR-HM mice was induced at levels several fold higher than in EpoR-H mice or wt-EpoR controls. Overall means differed significantly from wt-EpoR values (and from one another, $p<0.01$), and outcomes are representative of three independent experiments. Therefore, for EpoR-HM, the possibility of a reduced ability to mount an Epo response (during requirements of accelerated erythropoiesis) leading to deficient stress erythropoiesis is discounted. Interestingly, for EpoR-H, the renal *Epo* transcript levels were somewhat lower (~1.5 fold) as compared to wt-EpoR levels. This phenomenon is consistent with suggested overall negative roles for distal EpoR cytoplasmic domains and the possibility of EpoR-H being a hyper-allele [363].

FIGURE 4.3

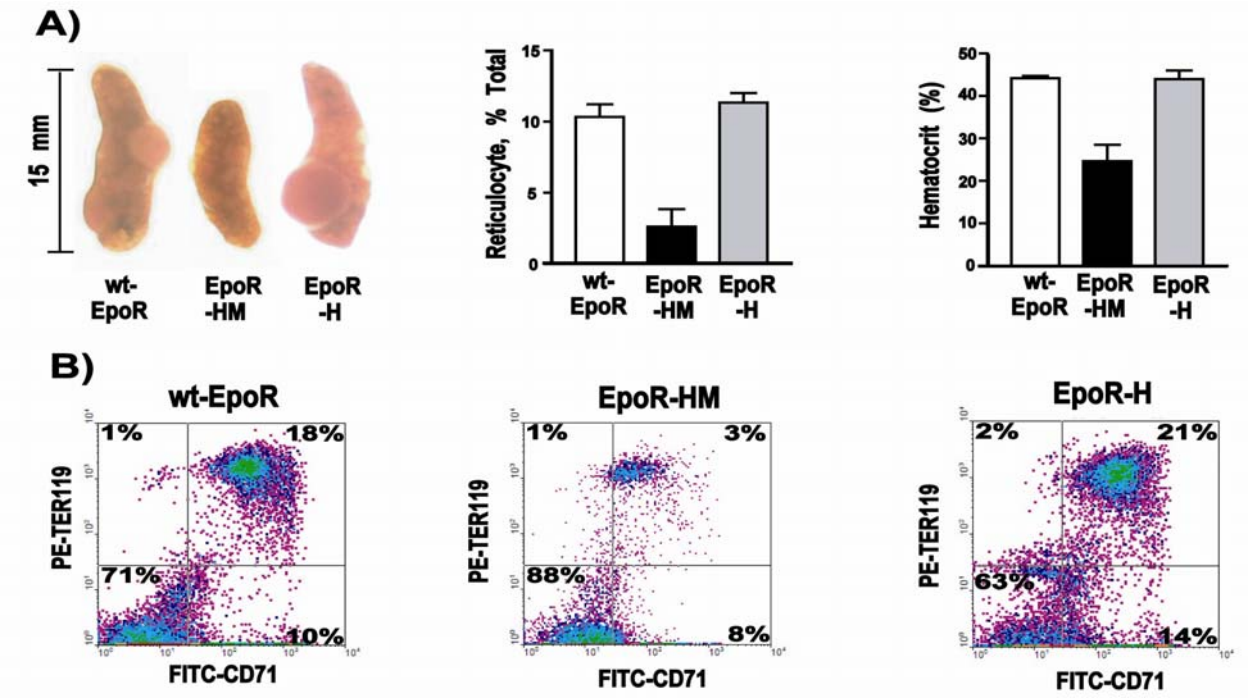
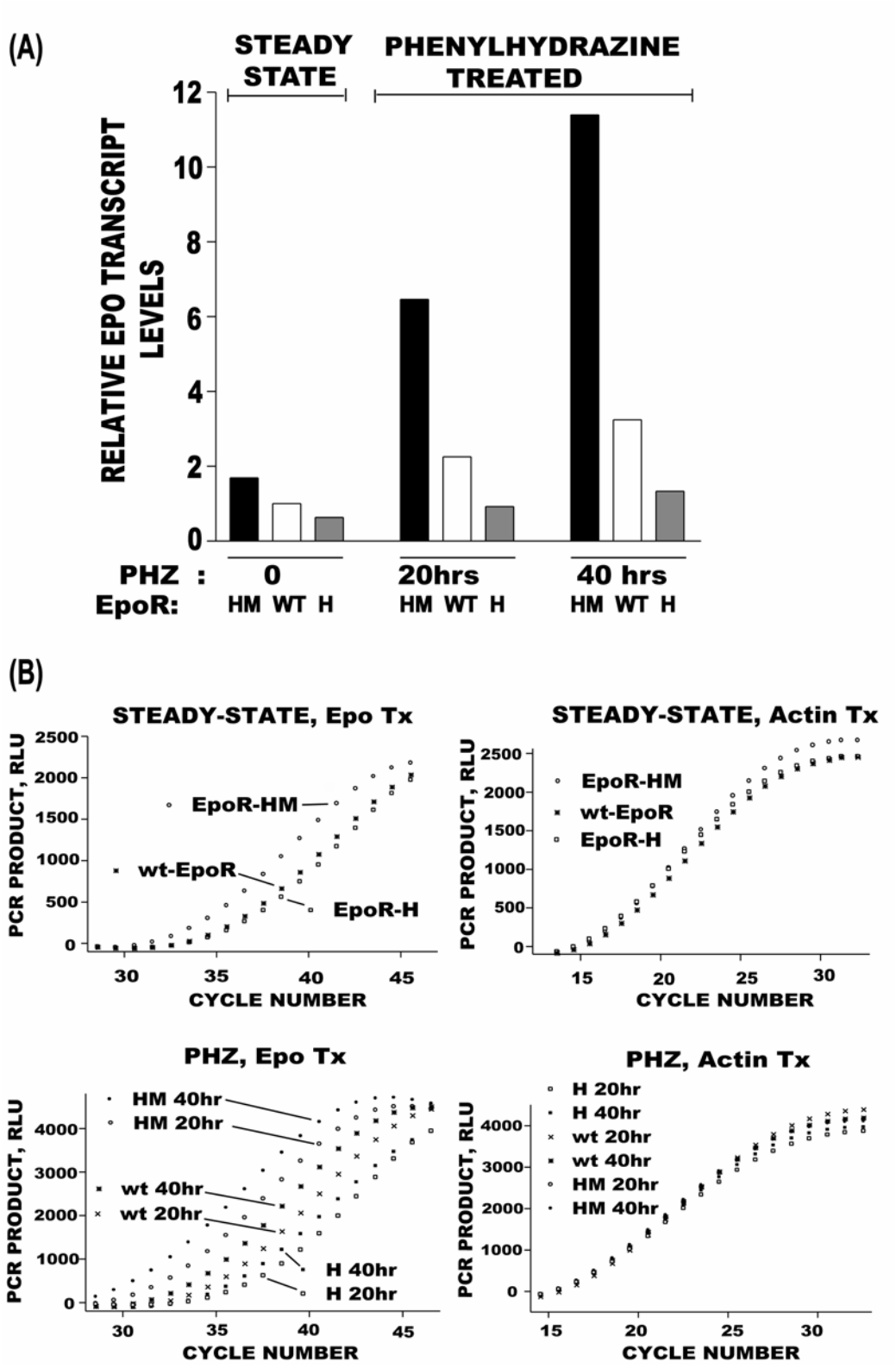


Figure 4.3. EpoR-HM derived bone marrow EPCs are deficient in terms of their short term repopulating abilities A] *Decreased spleen colonies, hematocrits and reticulocytes in recipient mice transplanted with EpoR-HM derived bone-marrow EPCs*. In transplantation experiments, bone-marrow preparations (Ly5.2) from wt-EpoR, EpoR-HM and EpoR-H mice were transplanted into lethally irradiated Ly5.1-marked recipients. For mice with $\geq$ 95 % Ly5.2 donor cell contributions, day-15 reticulocyte levels and hematocrits are graphed and representative spleens were photographed. **B]** *Decreased frequencies of early erythroid progenitor cells in recipient spleens of mice transplanted with EpoR-HM EPCs*. Frequencies of $CD71^{high}Ter119^{pos}$ erythroblasts at day 15 in spleens of recipient mice transplanted with wt-EpoR, EpoR-HM and EpoR-HM derived EPCs are graphed.

Figure 4.4. Epo expression in EpoR-HM mice is not substantially elevated during steady-state erythropoiesis, but is hyperactivated in response to anemia. A] EpoR-HM, wt-EpoR and EpoR-H mice were treated with phenylhydrazine (PHZ, +/- 100 mg/kg dose). Baseline (steady-state) and phenylhydrazine-induced levels of renal *Epo* transcripts (at 0, 20 and 40 hours post-PHZ dosing) then were determined by quantitative RT-PCR (and are normalized for co-assayed levels of *actin* transcripts). **B]** Representative primary PCR data also are shown (Tx, transcript).

FIGURE 4.4


(A)
STEADY STATE
PHENYLHYDRAZINE TREATED
RELATIVE EPO TRANSCRIPT LEVELS
12
10
8
6
4
2
0
PHZ : 0 20hrs 40 hrs
EpoR: HM WT H HM WT H HM WT H
(B)
STEADY-STATE, Epo Tx
PCR PRODUCT, RLU
EpoR-HM
wt-EpoR
EpoR-H
CYCLE NUMBER
STEADY-STATE, Actin Tx
EpoR-HM
wt-EpoR
EpoR-H
PCR PRODUCT, RLU
CYCLE NUMBER
PHZ, Epo Tx
HM 40hr
HM 20hr
wt 40hr
wt 20hr
H 40hr
H 20hr
PCR PRODUCT, RLU
CYCLE NUMBER
PHZ, Actin Tx
H 20hr
H 40hr
wt 20hr
wt 40hr
HM 20hr
HM 40hr
PCR PRODUCT, RLU
CYCLE NUMBER

Defects in bone marrow-derived EpoR-HM erythroblast development

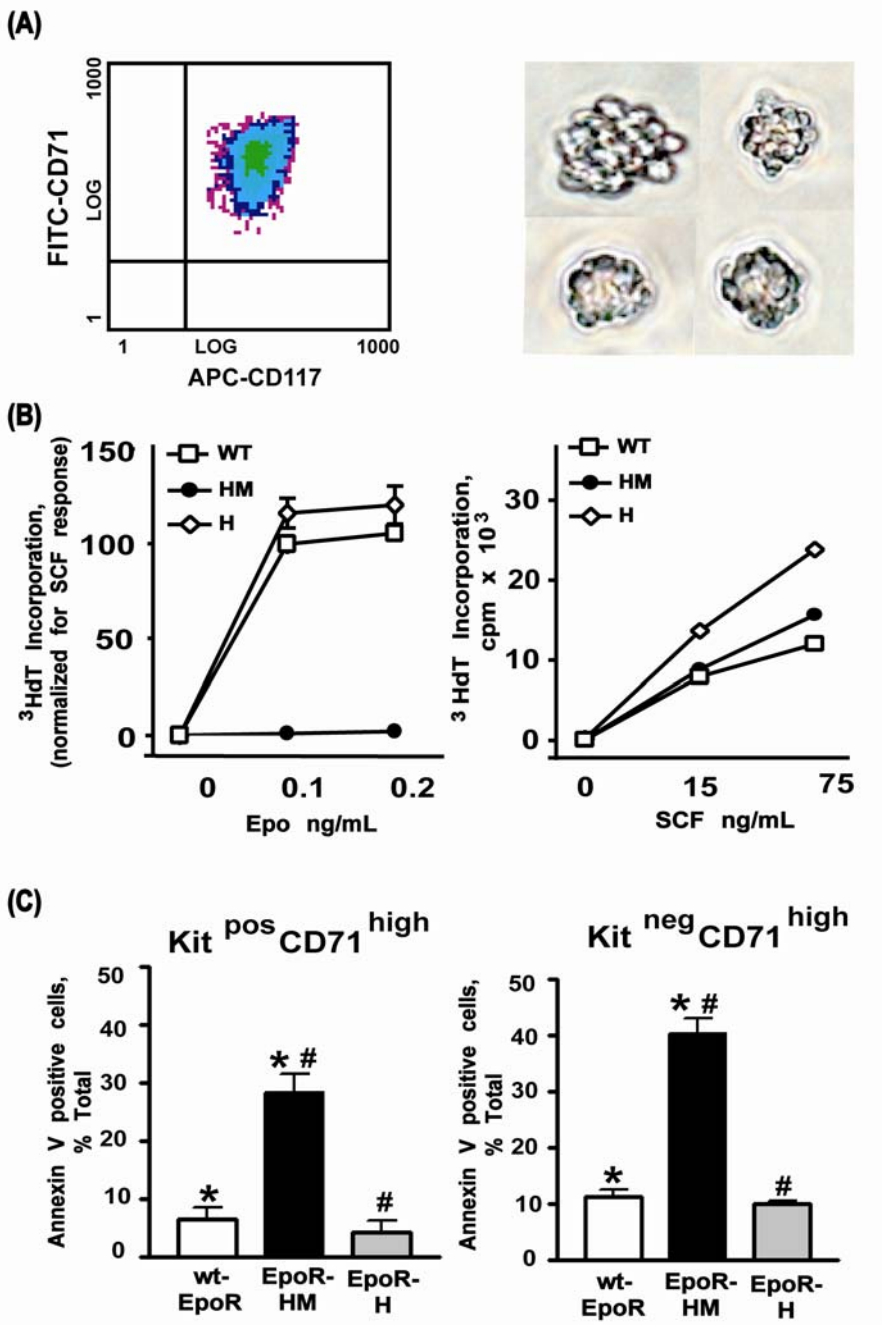
The above studies defined in vivo defects in stress erythropoiesis as supported by EpoR-HM (PY-null allele), especially for splenic erythropoiesis. Several factors that differentially affect extramedullary *vs.* bone marrow erythropoiesis recently have been described [355-357]. In addition, spleen has been proposed to contain a discrete progenitor pool that rapidly expands during stress erythropoiesis [355]. Therefore, studies were conducted in an ex-vivo environment on EpoR-HM and EpoR-H action within adult bone marrow-derived erythroblasts. Specifically, a unique in vitro expansion system was established that supports the step-wise development of primary marrow-derived proerythroblasts (Chapter 2). This involved Kit^{pos} progenitor cell isolation and expansion in "SP34-EX", a SP34 serum-free medium supplemented with optimized levels of SCF, Epo, dexamethasone, beta-estradiol, transferrin and BSA. In this medium, erythroid progenitor cells developed over a 3-day course from a $Kit^{pos}CD71^{low}$ cohort, to a $Kit^{pos}CD71^{high}$ population, and further to $Kit^{neg}CD71^{high}$ late-stage erythroblasts (Chapter 2, Figure 2.1).

For ex vivo analyses, EpoR-HM, EpoR-H and wt-EpoR erythroblasts were expanded and were isolated as $Kit^{pos}CD71^{high}$ populations either by fluorescence activated cell sorting (FACS), or by lineage depletion and Kit^{pos} magnetic-activated cell selection (MACS). These $Kit^{pos}CD71^{high}$ cells were observed to form colonies with CFUe-like morphologies (Figure 4.5A). Previously, a case that such $CD71^{high}$ cohorts correspond to CFUe-like progenitors has been provided via analyses of erythroid cells from *G1-HRD-GFP* mice [18]. In EpoR allele biosignaling analyses, Epo-dependent ^{3}HdT-incorporation responses first were assessed (Figure 4.5B). wt-EpoR and EpoR-H $Kit^{pos}CD71^{high}$ erythroblasts proved to be highly Epo-responsive. EpoR-HM cells, in contrast, exhibited a $\geq$30-fold defect in Epo-induced ^{3}HdT incorporation rates. Response profiles

are representative of three independent experiments, and significance testing of mean response values at 0.1U/mL confirmed significant differences between EpoR-HM *vs.* wt-EpoR or EpoR-H erythroblasts ($p<0.001$) (as well as between wt-EpoR *vs*. EpoR-H erythroblasts, $p<0.05$). Interestingly, higher proliferation rates were observed in EpoR-H as compared to wt-EpoR, suggesting its functioning as a hyper-allele. In these erythroblast populations, and in $Kit^{neg}CD71^{high}$ erythroblasts, apoptosis also was analyzed. Annexin-V- staining revealed $\geq$ 3-fold increases in apoptosis for EpoR-HM erythroblasts in each population as compared directly to wt-EpoR and EpoR-H erythroblasts (Figure 4.5C).

Figure 4.5. At a Kit^{pos} CD71^{high} stage, EpoR-HM erythroblasts exhibit proliferation and survival defects which are corrected by EpoR-H PY343 signals. A] Erythroid progenitor cells from wt-EpoR mice were expanded in SP34-EX medium. At 72 hours of culture, Kit^{pos}CD71^{high} erythroblasts were isolated by FACS and plated in methylcellulose. Colonies with CFUe morphologies uniformly formed as shown in right panels (and as confirmed by benzidine staining, not shown). **B]** Isolated Kit^{pos}CD71^{high} wt-EpoR, EpoR-HM and EpoR-H erythroblasts were cultured in SP34-EX medium in the presence of Epo or SCF at the concentrations indicated. At 20 hours, cytokine-induced ^{3}HdT incorporation rates were determined. For Epo, mean ^{3}HdT incorporation rates (+/- SD, n=3) are graphed, and are normalized for SCF-responsiveness. Results are representative of two independent experiments (and n=3 wt-EpoR, EpoR-HM and EpoR-H mice per experiment). **C]** For Kit^{pos}CD71^{high} and Kit^{neg}CD71^{high} wt-Epo, EpoR-HM and EpoR-H erythroblasts, frequencies of Annexin-V- positive cells were assayed by flow cytometry. Values are means +/-SD of triplicate analyses (* and #, $p<0.01$).

FIGURE 4.5


(A)
FITC-CD71
1000
LOG
1
1
LOG
1000
APC-CD117
(B)
3HdT Incorporation, (normalized for SCF response)
150
100
50
0
WT
HM
H
0
0.1
0.2
Epo ng/mL
3 HdT Incorporation, cpm x 10 3
30
20
10
0
WT
HM
H
0
15
75
SCF ng/mL
(C)
Kit pos CD71 high
Annexin V positive cells, % Total
50
40
30
20
10
0
* #
*
#
wt-EpoR
EpoR-HM
EpoR-H
Kit neg CD71 high
Annexin V positive cells, % Total
50
40
30
20
10
0
* #
*
#
wt-EpoR
EpoR-HM
EpoR-H

Study of STAT-5 response genes in wt-EpoR, EpoR-HM and EpoR-H derived erythroid progenitor cells

As an extension of the earlier study on STAT-5 response genes (chapter 3), the ability of EpoR-H and EpoR-HM $Kit^{pos}CD71^{high}$ erythroblasts to support Epo-induction of candidate Epo and STAT-5- response genes was studied in a time-course format. These genes included included *Pim-1*, *oncostatin-M, SOCS-3* and *Bcl-x*. For comparison, recently described genes involved in erythropoiesis; *Pim-2, Gas6* [356] and *EDR* (erythroid differentiation regulator) [364] also were analyzed. For *Pim-1*, *oncostatin-M* and *SOCS-3,* expression was induced 5- to 14-fold via the wt-EpoR and 8- to 20-fold via EpoR-H, but was not substantially induced via EpoR-HM (Figure 4.6). For each, differences among mean maximal induction levels for wt-EpoR, EpoR-HM and EpoR-H alleles were significant at an F-distribution level of $p< 0.01$. For all transcript analyses shown, results are representative of three independent experiments. This observed Epo-induction of *Pim-1*, *oncostatin-M* and *SOCS-3* in primary bone marrow-derived erythroblasts is novel, and each of these factors has the potential to support EpoR- mediated erythroblast growth and survival [293,343,357]. The enhanced responses for EpoR-H may reflect an absence of inhibitory motifs within EpoR distal cytoplasmic domains [363]. Even at a later time-point, *Bcl-x* expression was not significantly induced via wt-EpoR, EpoR-H or EpoR-HM alleles.

For oncostatin-M, further consideration was given to its expression by erythroid progenitor cells in vivo. This involved analyses of transcript expression in marrow and spleens of wt-EpoR, EpoR-HM and EpoR-H mice during phenylhydrazine induced anemia. If erythroid cells contribute in meaningful ways to oncostatin-M production, then overall levels of *oncostatin-M* transcripts should be decreased selectively in EpoR-HM mice. This proved to be the case, and

oncostatin-M expression levels in EpoR-HM mice were decreased 4- and 1.8-fold in spleen and bone marrow, respectively (Table 4.1). During anemia, erythroid progenitor cells therefore appear to be a significant source of *oncostatin-M*.

With further regards to Pim-1, Pim kinases recently have been shown to modulate eIF4E, and to regulate hematopoietic cell survival [365-367]. Possible contributions of Pim-1 to $Kit^{pos}CD71^{high}$ erythroblast survival therefore were tested indirectly, but functionally, based on possible differential sensitivity of EpoR-H *vs.* EpoR-HM erythroblasts to rapamycin (mTOR inhibitor). Based on annexin-V staining, EpoR-HM cells reproducibly exhibited >eight-fold increased rapamycin-sensitivity (Table 4.2). This difference may reflect increased dependence of EpoR-HM on mTOR activation in the absence of Pim-1's effects on eIF4E [365-367].

Figure 4.6. EpoR-PY343 signals support Epo-induction of *Pim-1, oncostatin-M* and *SOCS-3*, but not *Bcl-x*. $Kit^{pos}CD71^{high}$ erythroblasts were isolated from expanded wt-EpoR, EpoR-HM and EpoR-H marrow preparations, and cultured for six hours in 0.5% BSA, 10ng/mL insulin, 0.1mM 2-ME, IMDM. At 0, 30, 90 and 270 minutes of subsequent Epo-exposure (1U/mL), cells were lysed in Trizol reagent. Quantitative RT-PCR then was used to determine Epo-induced levels of *Pim-1*, *oncostatin-M*, *SOCS-3*, *Bcl-x*, *Gas6*, *Pim-2*, and *EDR* transcripts (graphed as fold-increases over time-zero baselines). In all RT-PCR reactions, *actin* was used as an internal control.

FIGURE 4.6

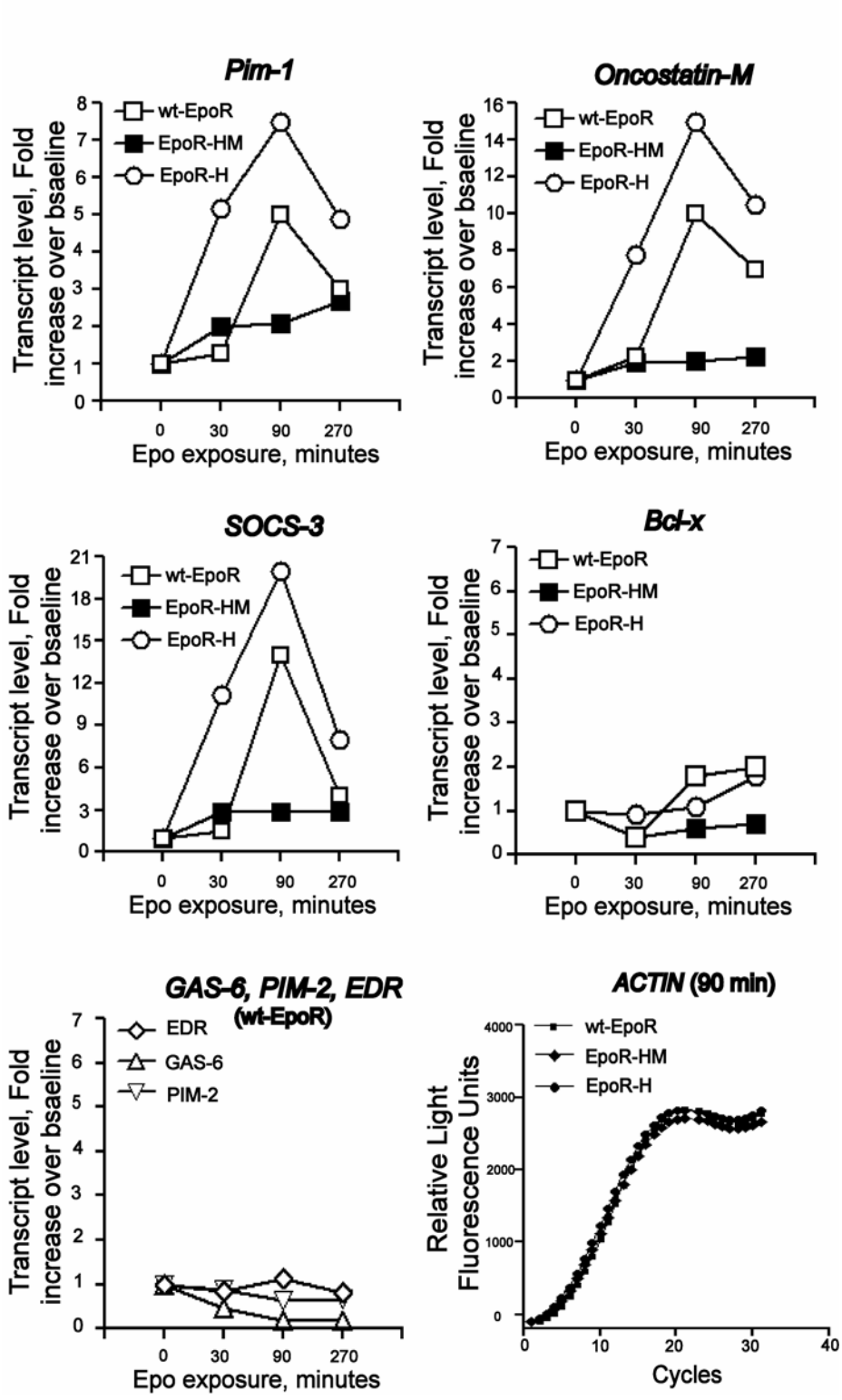

Pim-1
Transcript level, Fold increase over bsaeline
wt-EpoR
EpoR-HM
EpoR-H
0 30 90 270
Epo exposure, minutes
Oncostatin-M
Transcript level, Fold increase over bsaeline
wt-EpoR
EpoR-HM
EpoR-H
0 30 90 270
Epo exposure, minutes
SOCS-3
Transcript level, Fold increase over bsaeline
wt-EpoR
EpoR-HM
EpoR-H
0 30 90 270
Epo exposure, minutes
Bcl-x
Transcript level, Fold increase over bsaeline
wt-EpoR
EpoR-HM
EpoR-H
0 30 90 270
Epo exposure, minutes
GAS-6, PIM-2, EDR
(wt-EpoR)
Transcript level, Fold increase over bsaeline
EDR
GAS-6
PIM-2
0 30 90 270
Epo exposure, minutes
ACTIN (90 min)
Relative Light Fluorescence Units
wt-EpoR
EpoR-HM
EpoR-H
0 10 20 30 40
Cycles

TABLE 4.1. EXPRESSION OF ONCOSTATIN-M IS DECREASED IN BONE MARROW AND SPLEEN OF PHENYLHYDRAZINE-TREATED EpoR-HM MICE.

	***Oncostatin-M* transcript levels**	
	Bone Marrow	**Spleen**
wt-EpoR	100%	100%
EpoR-HM	56%	25%
EpoR-H	117%	102%

(Values are mean levels of *oncostatin-M* transcripts as assayed by quantitative RT-PCR, and normalized to levels in wt-EpoR controls).

TABLE 4.2. EpoR-HM ERYTHROBLASTS EXHIBIT INCREASED SENSITIVITY TO RAPAMYCIN

Rapamycin induced Annexin-V positivity, (% over background)

	$Kit^{neg}CD71^{high}$	**$Kit^{pos}CD71^{high}$**
wt-EpoR	3 +/- 0.1%	2 +/- 0.15%
EpoR-HM	26 +/- 5%	20 +/- 2%
EpoR-H	2 +/- 0.2%	1.5 +/- 0.3%

(Values are mean frequencies of annexin V-positive erythroblasts corrected for background levels in the absence of Rapamycin).

Integration of Kit and EpoR-PY343 signals during early erythroblast formation

Kit supports stress erythropoiesis, and can act in synergy with the EpoR [170,368]. Recent cell line studies of PY-mutated hEGFR/mEpoR chimeras have further suggested that Kit's signals might selectively integrate with EpoR PY343 signals [281]. This concept was tested in primary erythroblasts expanded from EpoR-HM, EpoR-H and wt-EpoR mice initially by decreasing SCF levels to physiologic concentrations, and assessing associated effects on $Kit^{pos}CD71^{high}$ and $Kit^{neg}CD71^{high}$ erythroblast formation. Interestingly, and specifically for EpoR-HM progenitor cells, this resulted in deficient progression of Kit^{pos} cells to a $Kit^{neg}CD71^{high}$ stage (Figure 4.7A). In particular, when SCF dosing was decreased from 50 to 5ng/mL, wt-EpoR and EpoR-H erythroblasts continued to progress from a $Kit^{pos}CD71^{high}$ stage to a $Kit^{neg}CD71^{high}$ stage (59 to 70%, and 57 to 80% progression, respectively). For EpoR-HM erythroblasts, this event faltered, and frequencies of $Kit^{neg}CD71^{high}$ erythroblasts decreased when SCF was limited (i.e., from 22% to 10%). Results (representative of three independent experiments) therefore indicate important roles for EpoR-PY343 (and STAT-5) signals in Kit-mediated proerythroblast expansion, especially beyond a $Kit^{pos}CD71^{high}$ stage. Direct cell counts further revealed defects in the capacities of EpoR-HM progenitor cells to expand in a SCF dose-dependent context (Figure 4.7B). SCF- and Epo- activation of Stats also was analyzed. In cell lines, STAT-5, -3 and -1 activation by SCF, and by Epo has been reported [302,369]. In bone marrow-derived primary erythroblasts, Epo efficiently activated STAT-5 but did not detectably activate STAT-1 or STAT-3 (Figure 4.7C). In contrast, SCF efficiently activated STAT-1 but did not detectably activate STAT-5 or STAT-3. (SCF, however, was observed to induce reactivity of a Mr 140,000 protein with the anti-PY694-STAT-5 antibody used in these studies).

Possible SCF/Kit modulation of EpoR-PY343-STAT-5 response genes also was assessed. This involved assaying combined effects of SCF plus Epo exposure on *oncostatin-M*, *Pim-1* and *SOCS-3* expression in expanded and purified wt-EpoR $Kit^{pos}CD71^{high}$ erythroblasts (Figure 4.8). SCF per se did not significantly modulate these transcripts. In the presence of Epo, however, SCF selectively up-modulated *oncostatin-M* expression but down-modulated *SOCS-3*. At the transcript level, Epo-dependent *Pim-1* expression was not significantly affected by SCF/Kit. In repeated analyses, these effects were reproduced and the wt-EpoR supported up to 3-fold increases in SCF-induced Epo-dependent *oncostatin-M* expression, and 2.5-fold decreases in *SOCS-3* expression ($p<0.01$ at 90 minutes of Epo exposure). In EpoR-H, but not EpoR-HM erythroblasts similar SCF effects were observed.

Pim-1 recently has been reported to be regulated via post-translational stabilization [370]. Whether SCF might affect Epo-dependent levels of Pim-1 protein expression therefore was also assessed using isolated $Kit^{pos}CD71^{high}$ erythroblasts (Figure 4.9). Interestingly, Epo-dependent Pim-1 expression proved to be selectively reinforced by SCF (Figure 4.9A). Furthermore, SCF/Kit enhancement of Pim-1 expression was supported by EpoR-H, but not EpoR-HM erythroblasts (Figure 4.9B). EpoR-PY343-STAT-5 signals therefore appear to mediate Kit's effects on Pim-1 expression. In contrast, no such cooperative effects were observed for ERK1,2, a target which previously has been suggested to be synergistically regulated by SCF plus Epo [371].

Figure 4.7. SCF effects on pro-erythroblast expansion are supported by EpoR PY343 signals. A] To test possible roles for EpoR PY343 signals in a context of EpoR and Kit function, the capacities of EpoR-HM, EpoR-H and wt-EpoR erythroid progenitor cells to expand in the presence of sub-maximal SCF doses were assessed via assays of CD117 (Kit) and CD71 marker expression at 72 hours of culture. When SCF was limited to 5ng/mL, EpoR-HM erythroblasts selectively accumulated at a Kit^{pos} $CD71^{high}$ stage, and exhibited clear defects in development to $Kit^{neg}CD71^{high}$ cells (left panels). Right panels show increased progression at 50 ng SCF/mL to a $Kit^{pos}CD71^{high}$ stage, but a persistent defect in the formation of $Kit^{neg}CD71^{high}$ cells. These Kit co-signaling defects were corrected upon PY343 site restoration. **B]** SCF-dependent EpoR-HM, EpoR-H and wt-EpoR erythroblast expansion capacities also were assessed based on direct cell counts (cumulative means +/- SD). Data in A and B are representative of n=2 mice per EpoR allele, and three independent experiments. **C]** In expanded wt-EpoR Kit^{pos} erythroblasts, Stat factor activation in response to either SCF or Epo was assessed. Cells were deprived of hematopoietic growth factors for six hours and then exposed to SCF (100 ng/mL) or Epo (2.5 U/mL) for 10 minutes. Levels of phospho- (and total) STAT-5, -1 and -3 then were assayed by western blotting (P-S, phospho-Stat; S, Stat). The asterisk indexes a SCF-induced anti-PY-STAT-5 reactive phosphoprotein (Mr~140,000).

FIGURE 4.7

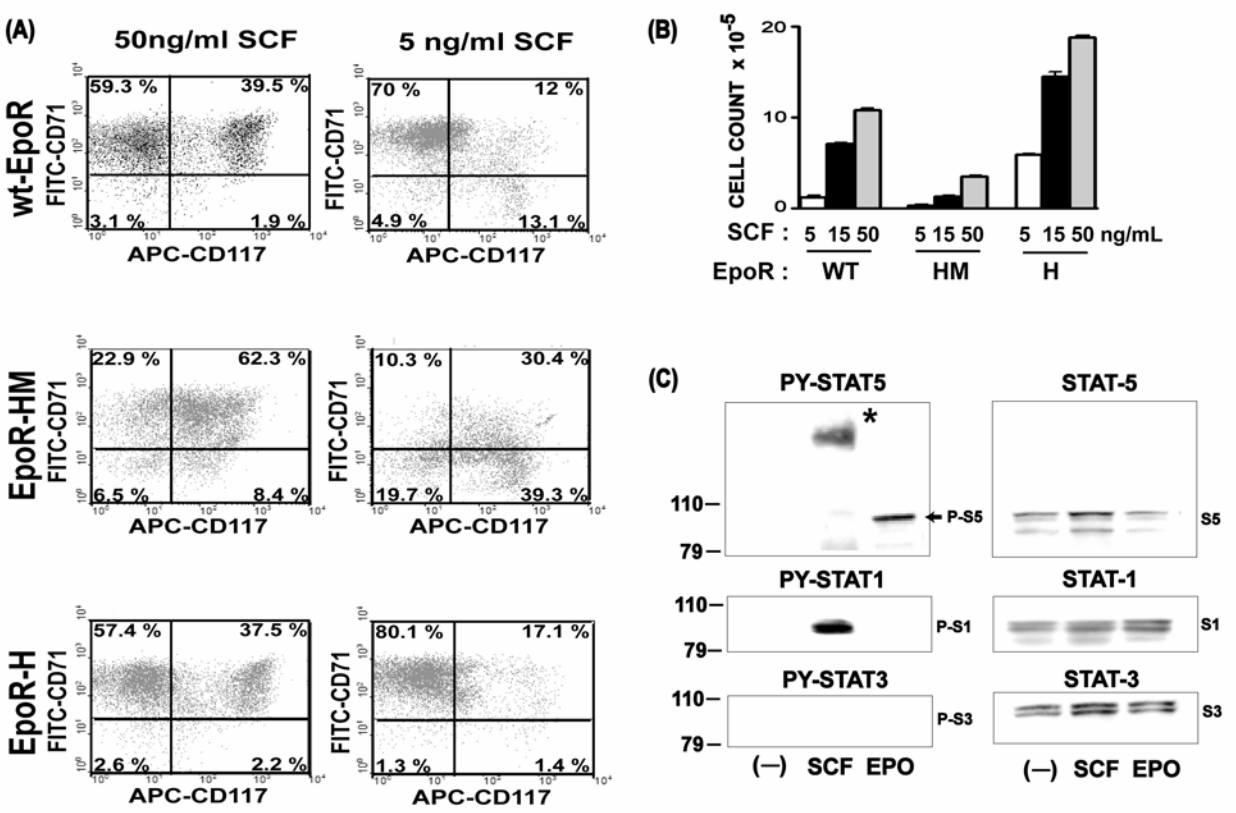

(A)
50ng/ml SCF
5 ng/ml SCF
wt-EpoR
EpoR-HM
EpoR-H
FITC-CD71
APC-CD117
59.3 %
39.5 %
3.1 %
1.9 %
70 %
12 %
4.9 %
13.1 %
22.9 %
62.3 %
6.5 %
8.4 %
10.3 %
30.4 %
19.7 %
39.3 %
57.4 %
37.5 %
2.6 %
2.2 %
80.1 %
17.1 %
1.3 %
1.4 %
(B)
CELL COUNT x 10^-5
20
10
0
SCF : 5 15 50 5 15 50 5 15 50 ng/mL
EpoR : WT HM H
(C)
PY-STAT5
STAT-5
P-S5
S5
PY-STAT1
STAT-1
P-S1
S1
PY-STAT3
STAT-3
P-S3
S3
110
79
(–) SCF EPO
(–) SCF EPO

FIGURE 4.8

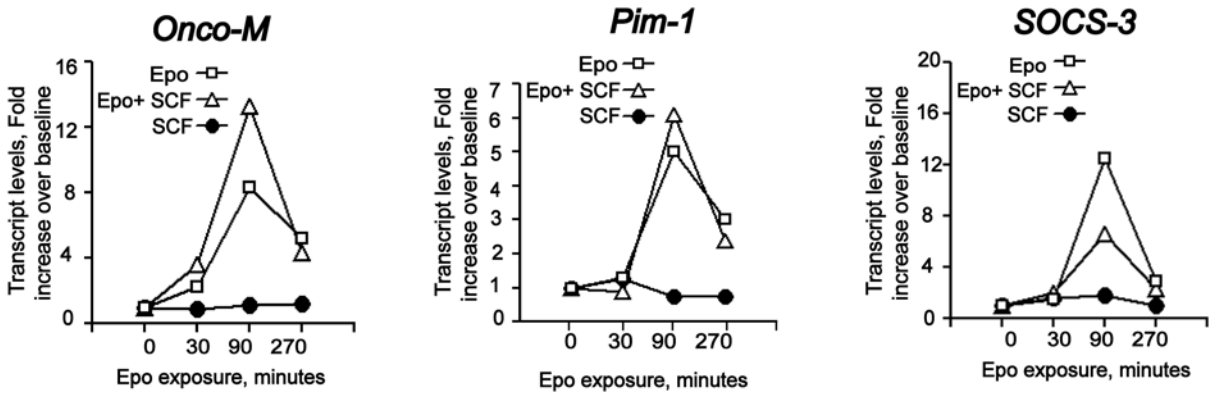
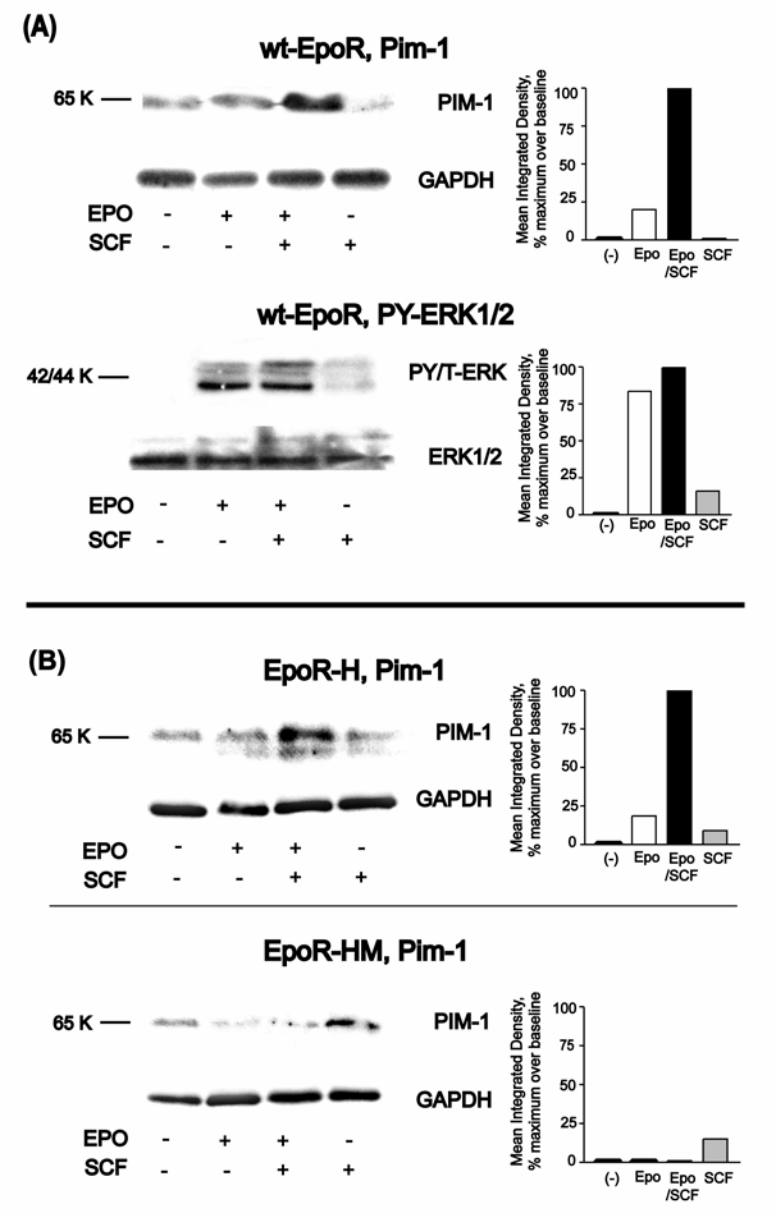


Figure 4.8. Integration of Epo and SCF signals to modulate STAT-5 response genes. *Kit signals selectively modulate EpoR-PY343- dependent oncostatin-M and SOCS-3 expression* .Erythroid progenitor cells were expanded from wt-EpoR, marrow preparations, and isolated as $Kit^{pos}CD71^{high}$ erythroblasts. Cytokines were withdrawn for six hours, and erythroblasts then were exposed to Epo (0.75 U/mL) and/or SCF (75 ng/mL) for 0, 30, 90, or 270 minutes. Induced levels of *oncostatin-M*, *Pim-1* and *SOCS-3* transcripts were determined by quantitative PCR. Note the selective SCF up-modulation of *oncostatin-M*, and down-modulation of *SOCS-3*.

Figure 4.9. Kit signals enhance EpoR-PY343-dependent Pim-1 protein expression. A] Erythroid progenitor cells were expanded from wt-EpoR bone marrow preparations and $Kit^{pos}CD71^{high}$ erythroblasts were isolated. Cytokines were withdrawn for 6 hours, and cells then were exposed to Epo (0.75 U/mL), SCF (75ng/mL) or both factors for 90 minutes. Pim-1 protein levels then were assayed by western blotting (upper sub-panel). Note the multi-fold effect of SCF on Epo-dependent Pim-1 expression. In analyses of SCF- plus Epo- regulation of ERKs (lower sub-panel), no such synergistic effects were observed. **B]** *SCF- enhancement of Epo-induced Pim-1 expression in EpoR-H, but not EpoR-HM erythroblasts*- $Kit^{pos}CD71^{high}$ erythroblasts were prepared from EpoR-H and EpoR-HM mice, and were challenged with SCF, Epo or SCF plus Epo. SCF/Kit enhancement of Pim-1 expression was efficiently supported in EpoR-H, but not EpoR-HM erythroblasts.

FIGURE 4.9


(A)
wt-EpoR, Pim-1
65 K
PIM-1
GAPDH
EPO - + + -
SCF - - + +
Mean Integrated Density, % maximum over baseline
100
75
50
25
0
(-) Epo Epo /SCF SCF
wt-EpoR, PY-ERK1/2
42/44 K
PY/T-ERK
ERK1/2
EPO - + + -
SCF - - + +
Mean Integrated Density, % maximum over baseline
100
75
50
25
0
(-) Epo Epo /SCF SCF
(B)
EpoR-H, Pim-1
65 K
PIM-1
GAPDH
EPO - + + -
SCF - - + +
Mean Integrated Density, % maximum over baseline
100
75
50
25
0
(-) Epo Epo /SCF SCF
EpoR-HM, Pim-1
65 K
PIM-1
GAPDH
EPO - + + -
SCF - - + +
Mean Integrated Density, % maximum over baseline
100
75
50
25
0
(-) Epo Epo /SCF SCF

EpoR-PY343 modulates oncostatin-M's effects on late stage erythroblast survival

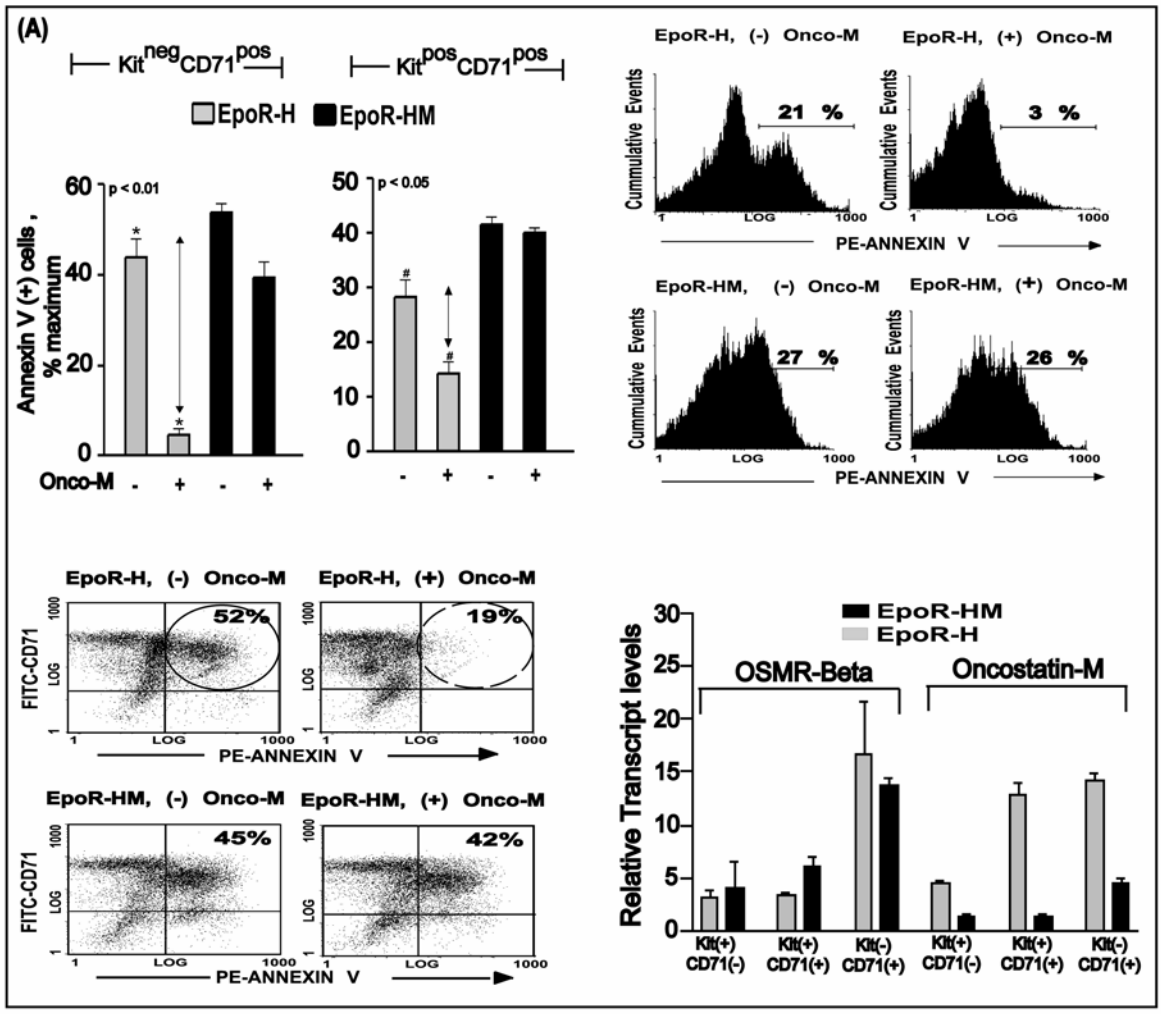
Based on Epo's marked induction of *oncostatin-M,* possible effects of oncostatin-M on erythroblast survival were investigated. EpoR-HM and EpoR-H erythroblasts initially were analyzed, and were expanded in the presence of oncostatin-M (+/-10 ng/mL), Epo (2.5U/mL, non-limiting concentration), and SCF at non-limiting (100 ng/mL) or limiting concentrations (5 ng/mL). Apoptosis among developing erythroid subpopulations then was assayed by annexin-V staining. Interestingly, when SCF concentrations were limited, marked effects of oncostatin-M on EpoR-H erythroblast survival were discovered (i.e., $\geq$ ten-fold decreases in annexin-V positivity due to oncostatin-M) primarily on late-stage $Kit^{neg}CD71^{high}$ erythroblasts. In EpoR-HM erythroblasts, however, this oncostatin-M effect was not exerted in the specific absence of EpoR PY343 signals (Figure 4.10A). Findings indicate a previously undefined role for oncostatin-M in promoting erythroblast survival, and suggest that EpoR-PY343 signals modulate this response. Oncostatin-M effects on wt-EpoR erythroblast survival were also tested (Table 4.3). Oncostatin-M proved to specifically enhance survival of wt-EpoR $Kit^{neg}CD71^{high}$ cells (on average) to ~42.5 % above levels supported by SCF and Epo in the absence of oncostatin-M. This effect, while significant, was not so marked as the survival advantage provided in EpoR-H erythroblasts. Signals relayed via wt-EpoR distal PY motifs therefore may also reinforce survival.

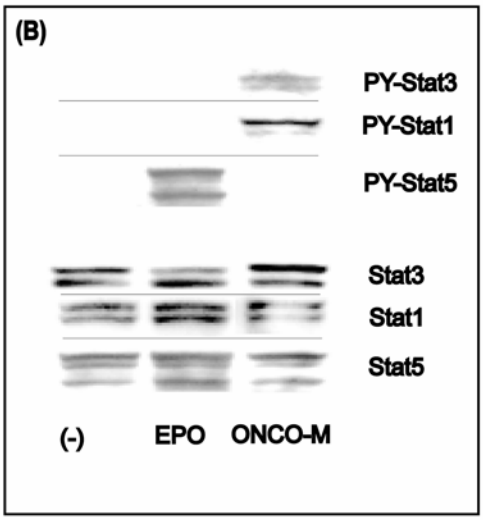
One factor that might affect differential oncostatin-M responses in EpoR-H vs EpoR-HM erythroblasts involves oncostatin-M receptor expression [372]. *Oncostatin-M beta- receptor* (*onco-MRβ)* levels therefore were analyzed in developing EpoR-H and EpoR-HM erythroblasts. O*nco-MRβ* was expressed at all stages, however, and was only modestly up-regulated in EpoR-H

erythroblasts at a late $Kit^{neg}CD71^{high}$ stage (Figure 4.10A). Finally, Stat activation by oncostatin-M also was analyzed in $CD71^{high}$ wild-type erythroblasts. Oncostatin-M proved to efficiently stimulate STAT-1 and -3, but not STAT-5 (Figure 4.10B). EpoR plus oncostatin-M effects therefore might involve the combined actions of STAT-5, plus STAT-1 and/or STAT-3.

Figure 4.10. Oncostatin-M promotes the survival of Kit^{neg}CD71^{high} erythroblasts, in part via EpoR PY343- dependent routes. A] Oncostatin-M reinforcement of EpoR-H, but not EpoR-HM erythroblast survival: EpoR-H and EpoR-HM erythroblasts were expanded in SP34-EX medium in Epo (2.5U/mL) and SCF (5ng/mL) in the presence, or absence, of oncostatin-M (onco-M, +/-10 ng/mL). At 72 hours of culture, frequencies of annexin-V- positive cells among Kit^{neg}CD71^{high} and Kit^{pos}CD71^{high} erythroblasts were determined. Note the substantial survival effect of oncostatin-M selectively on EpoR-H Kit^{neg}CD71^{high} erythroblasts (upper left panels) (*, $p<0.01$; #, $p<0.05$). Also illustrated are annexin-V staining profiles for expanded EpoR-H and EpoR-HM cells, and for CD71^{high} erythroblasts. For expanded erythroblasts from EpoR-H and EpoR-HM bone marrow preparations, Kit^{pos}CD71^{low}, Kit^{pos}CD71^{high} and Kit^{neg}CD71^{high} subpopulations also were isolated by FACS, and lysed in Trizol. Levels of *oncostatin-M receptorβ* (*onco-MRβ*) (and for comparison, *oncostatin-M* transcripts) from each population then were determined by quantitative RT-PCR. *Actin* was used as a normalizing control. **B]** Oncostatin-M selectively activates Stats-3 and -1 in CD71^{high} erythroblasts: wt-EpoR CD71^{high} erythroblasts were isolated from expansion cultures. Erythroblasts then were deprived of cytokines for six hours and exposed to Epo (2.5U/mL) or oncostatin-M (10ng/mL). At 10 minutes of exposure, lysates were prepared and levels of activated (PY) and total Stats-3, -1 and -5 were analyzed by western blotting. **C]** A model is outlined in which EpoR PY343 (and STAT-5) signals act during stress erythropoiesis to centrally support erythroblast production, reinforce Kit signals for proerythroblast expansion, and mediate EpoR plus oncostatin-M signals for late erythroblast survival.

FIGURE 4.10


(A)
Kit^neg CD71^pos
Kit^pos CD71^pos
EpoR-H
EpoR-HM
Annexin V (+) cells , % maximum
p < 0.01
p < 0.05
Onco-M
EpoR-H, (-) Onco-M
EpoR-H, (+) Onco-M
21 %
3 %
Cummulative Events
PE-ANNEXIN V
EpoR-HM, (-) Onco-M
EpoR-HM, (+) Onco-M
27 %
26 %
52%
19%
45%
42%
FITC-CD71
Relative Transcript levels
OSMR-Beta
Oncostatin-M
Kit(+) CD71(-)
Kit(+) CD71(+)
Kit(-) CD71(+)
Kit(+) CD71(-)
Kit(+) CD71(+)
Kit(-) CD71(+)


(B)
PY-Stat3
PY-Stat1
PY-Stat5
Stat3
Stat1
Stat5
(-)
EPO
ONCO-M

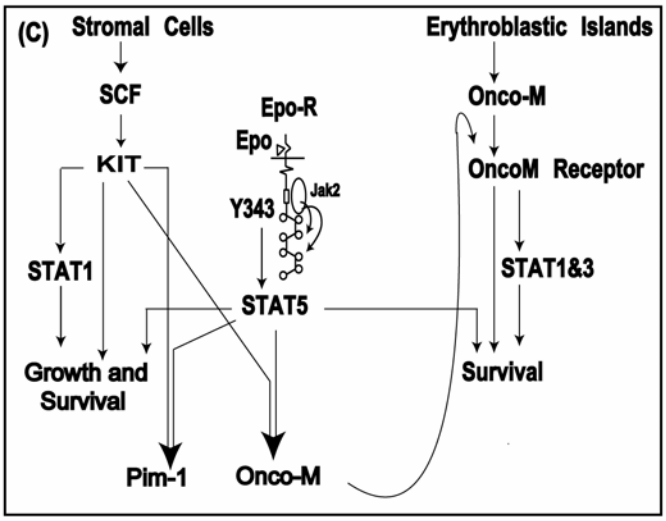

(C)
Stromal Cells
SCF
KIT
Epo-R
Epo
Jak2
Y343
STAT1
STAT5
Growth and Survival
Pim-1
Onco-M
Erythroblastic Islands
Onco-M
OncoM Receptor
STAT1&3
Survival

TABLE 4.3. wt-EpoR ERYTHROBLASTS EXHIBIT INCREASED SURVIVAL IN RESPONSE TO ONCOSTATIN-M

Oncostatin-M dependent increases in wt-EpoR erythroblast survival

	$Kit^{neg}CD71^{high}$	$Kit^{pos}CD71^{high}$
exp #1	56 +/- 9.3%	29 +/- 6.7%
exp #2	31 +/- 5.1%	19 +/- 4.3%

(Values are means of duplicate analyses, and represent percent increases in survival due to oncostatin-M exposure (10ng/mL) over no oncostatin-M assayed based on annexin-V – positive erythroblasts).

DISCUSSION

The presently defined requirement for Epo receptor PY343 signals during stress erythropoiesis distinguishes between steady-state and stress erythropoiesis at the level of Epo signaling. Mechanistically, this novel finding raises basic questions concerning the nature of key signals that are provided to erythroblasts via this EpoR-PY343-STAT-5 axis during anemia. Below, these questions are considered in the contexts of Stat activation; the nature of Epo-regulated STAT-5 response genes in primary erythroblasts; and apparent roles for EpoR-PY343-STAT-5 signals in reinforcing Kit and oncostatin-M receptor action. In Figure 9C, proposed overall roles for EpoR-PY343-STAT-5 signaling during Epo, SCF and oncostatin-M receptor activation are outlined.

In the wt-Epo receptor, PY343 is a major STAT-5 binding site [283,284]. In EpoR-H, PY343 is the singular retained PY site, while in EpoR-HM this site is mutated to F343. One prediction, therefore, is that the major difference between EpoR-H and -HM signaling capacities is an ability *vs*. inability to activate STAT-5 (and downstream events). Previously, STAT-1 and/or -3 also have been implicated in EpoR signaling [302] and in *STAT-1*$^{-/-}$ BFUe, Epo- and SCF-responsiveness is attenuated [303]. In the present primary erythroblast system, however, little to no Epo- activation of STAT-1 or -3 was detected. This discounts (but does not rule-out) roles for these Stats in EpoR signaling.

If STAT-5 is a major mediator of EpoR-H PY343 dependent stress erythropoiesis, then faltered erythroid phenotypes might also be predicted to be exhibited by *STAT-5a,b*$^{-/-}$ mice. In STAT-5-deficient mice, phenylhydrazine-induced splenic erythropoiesis, in fact, is compromised [299].

Certain *STAT-5a,b*$^{-/-}$ phenotypes, however, are controversial. Embryonic anemia has been reported, for example, in one [296] but not another study [300]. Spontaneous erythrosplenomegaly also commonly occurs in adult *STAT-5a,b*$^{-/-}$ mice, and can be accompanied by up to 100-fold increases in Epo production [299]. This phenotype, however, is suppressed within a recombination-activating gene 2 deficient background [300] suggesting that anemia in STAT-5$^{-/-}$ mice is of an auto-immune type and potentially involves T cells. In EpoR-HM mice, no such embryonic anemia, spontaneous splenomegaly or elevated base-line Epo levels were observed. Pertaining to the study of erythropoiesis, one potential advantage of EpoR-HM mice is that unlike STAT-5$^{-/-}$ mice, EpoR-HM mice do not exhibit aberrant functioning of non-erythroid hematopoietic cells like T-cells. If low-level Epo activation of STAT-5 were to occur, this might contribute to the ability of EpoR-HM mice to support steady-state erythropoiesis [300]. In repeated sensitive assays of STAT-5 and STAT-5 target gene activation, however, EpoR-HM failed to detectably stimulate STAT-5. This includes analyses of Epo induction of a well-characterized STAT-5 target gene, Cis-1 [249] which was induced ~ 25 fold via EpoR-H and the wt-EpoR, but not by EpoR-HM.

The case that EpoR-PY343-STAT-5 signals are critical for stress erythropoiesis raises questions concerning the specific nature of key STAT-5- regulated genes within developing erythroblasts. Presently, four additional genes that have been indicated in cell line models and/or non-erythroid cells to comprise Epo- and STAT-5-response genes were analyzed, i.e., *Pim-1* [293], *oncostatin-M* [294], *SOCS-3* [295] and *Bcl-x* [296]. For *Bcl-x*, failed reticulocyte formation due to conditional gene disruption illustrates Bcl-xl's role as an important erythroblast survival factor [262]. In addition, STAT-5 occupancy of a consensus element within intron-1 of the *Bcl-x* gene also has been

described in HCD-57 cells [296]. In the present primary erythroblast system, *Bcl-x* transcription unexpectedly (but reproducibly) was not modulated significantly via wt-EpoR, EpoR-H or -HM alleles. Given Epo's role as an erythroblast survival factor, this outcome emphasizes the potential importance of alternate candidate EpoR-PY343-STAT-5 target genes. In particular, *Pim-1*, *oncostatin-M* and *SOCS-3* each were observed to be strongly induced in $Kit^{pos}CD71^{high}$ erythroblasts via an EpoR PY343-STAT-5 pathway. Recently, Pim kinases have been shown to affect erythropoiesis based on decreased CFUe in *Pim-1*$^{-/-}$ mice and microcytic anemia in compound *Pim-1*$^{-/-}$/*Pim-2*$^{-/-}$ mice [365]. In addition, Pim kinases can confer resistance to rapamycin (mTOR inhibitor) and can act in parallel with mTOR to modulate eIF4E [365-367]. In EpoR-HM erythroblasts, decreased levels of Pim-1 therefore may account for increased rapamycin sensitivity. For oncostatin-M, present analyses reveal substantial expression by erythroblasts via an EpoR PY343 route, as well as novel survival effects on late-stage erythroblasts. Oncostatin-M also is expressed at high levels by macrophage [294]. Therefore, blood islands represent an interesting candidate site for suggested paracrine and/or autocrine effects of oncostatin-M on erythropoiesis. In oncostatin-M deficient mice, decreases in circulating erythrocytes interestingly have been observed [357]. Finally, SOCS factors (including SOCS-3) commonly are associated with receptor kinase inhibition, and degradation [373]. SOCS-3, however, may also affect cell survival by sequestering GTPase activating protein via a unique SOCS-box domain PY site [343].

The present experiments also raise questions regarding roles of distal EpoR PY motifs on the one hand, and EpoR-HM allele action mechanisms on the other. For distal PY sites, three observations are consistent with an exertion of net negative effects: i) lower levels of Epo production in phenylhydrazine-treated EpoR-H *vs.* wt-EpoR mice; ii) elevated ^{3}HdT response

profiles for EpoR-H erythroblasts; and iii) heightened Epo-induction of *Pim-1*, *oncostatin-M* and *SOCS-3* in EpoR-H erythroblasts (see Figures 2, 5, and 6). These results are consistent with polycythemia described by Prchal and co-workers [352] in patients, and in mice expressing EpoR carboxyl-terminal truncation mutants. With regards to mechanisms that the PY-null allele EpoR-HM may utilize to support steady-state erythropoiesis, selective retention by EpoR-HM of MEK1,2 and ERK1,2 signaling (see Chapter 3) is noted. The extent to which ERK signaling might be necessary and sufficient for EpoR-HM bio-function, however, is presently uncertain.

As illustrated by macrocytic anemia in *Kit* $^{w/wv}$ mice [309], Kit receptor tyrosine kinase signals also are important for stress erythropoiesis, and Kit is known to act in synergy with the Epo receptor. One cell line model for synergy, in fact, involves Epo receptor trans-phosphorylation by Kit [170,368]. Presently, SCF/Kit signals are shown in primary erythroblasts to selectively enhance EpoR PY343-dependent *oncostatin-M* gene expression, and to repress *SOCS-3*. For SOCS-3, repression by Kit is suggested to down-modulate SOCS-3's suppressive effects on EpoR signaling [295]. SCF enhancement of oncostatin-M expression, in contrast, may act in a positive feed-forward mode based on oncostatin-M's survival effects on Kit^{neg}CD71^{high} late-stage erythroblasts. An additional physiologically interesting feature involved enhanced oncostatin-M survival effects when SCF was limited, especially in EpoR-H erythroblasts. Specific mechanisms that integrate these SCF, Epo and oncostatin-M receptor effects, however, are not yet defined. SCF/Kit signals also selectively enhanced EpoR PY343-dependent Pim-1 protein expression. Recently, Pim-1 has been shown to be stabilized by HSP-90 [370]. Whether this mechanism is exerted in the present system remains to be tested.

Overall, the present work first illustrates defects in stress erythropoiesis via a minimal EpoR-HM allele which otherwise supports red cell formation at steady-state [300]. This includes clear in vivo erythropoietic defects in three anemia models. The rescue of efficient stress erythropoiesis via restoration of a single PY343 STAT-5 binding site furthermore distinguishes between steady-state and stress- induced red cell formation at the level of Epo receptor action. Mechanistically, Pim-1 and oncostatin-M (but not Bcl-xL) are proposed to constitute important EpoR-PY343-STAT-5 induced genes and erythroblast survival factors. An EpoR-PY343-STAT-5 signaling axis also is suggested to assist in the integration of additional survival signals relayed via both SCF, and oncostatin-M receptors. Findings advance insight into core EpoR action mechanisms [90], add to our knowledge of STAT-5 action [281,296,299,330], and also may shed light on Epo-regulated cytoprotection in non-hematopoietic tissues [121].

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

To summarize the findings presented in this thesis, EpoR-PY343 coupled STAT-5 activation is critical for accelerated erythropoiesis that is employed during conditions of stress. This hypothesis was tested in two distinct anemia models via PHZ and 5-FU administration. In addition, bone marrow cells derived from mice deficient in EpoR phosphotyrosine sites (EpoR-HM) is deficient in providing radioprotection to irradiated recipient mice as assayed via short-term transplantation experiments. Thus, EpoR-PY343/STAT-5 pathway which seems to be dispensable during steady state erythropoiesis via possible in vivo compensatory mechanisms is found to be critical during moments of stress. However, steady-state bone-marrow EPCs derived from PY-null EpoR-HM mice exhibit stark ex vivo defects in survival, proliferation and differentiation as demonstrated in a defined and conditioned medium. Thus, the in vivo compensatory mechanisms during steady-state might involve one or more critical factors which might or might not be hematopoietic. In addition, this might also involve a clonal expansion of erythroid progenitor cells with an altered EpoR PY independent signaling mechanism. These possibilities need to be tested further and at present, are merely hypothetical. In addition, at least one pathway that is intact in EpoR-HM derived bone marrow progenitor cells is the ERK pathway. EpoR PY independent activation of ERKs however was deregulated, and contributed to deficient differentiation in EpoR-HM bone marrow cells. It is currently difficult to postulate the in vivo effects of ERK hyperactivation in EpoR-HM mice and indeed, it may very well constitute one of the compensatory mechanisms for PY independent proliferation of early erythroid progenitor cells in the marrow. Also, members of STAT family seems to compensate for the lack

of one or more specific members. This is clearly observed in STAT-1 deficient mice in which erythroid progenitors exhibit sustained activation of STAT-5. In the erythroid system, STAT-1 and STAT-3 are known to be activated via EpoR, in addition to STAT-5. However, in the absence of STAT-5 activation in EpoR-HM derived EPCs, no compensatory hyperactivation of STAT-1 or STAT-3 was observed.

Future studies will involve deciphering the mechanisms activated in response to Epo in EpoR-HM erythroid cells. This will involve stage-specific affymetrix profiling of bone marrow derived erythroid progenitor cells expanded in SP34-EX medium. In combination with analysis of gene profiling results from EpoR-H (PY-343 only) and wt-EpoR EPCs, novel Epo and STAT-5 target genes could be identified and pursued. In addition, studies in STAT-$5^{-/-}$ mice are currently controversial with conflicting results obtained via different studies. The generation of conditional STAT-5 null mice in an erythroid specific context by crossing floxed STAT-5 and Gata-1-cre mice would avoid potential complications due to STAT-5's roles in other lineages. Also, Bcl-xL , a previously reported STAT-5 target gene was not found to be modulated at the transcript level via Epo in our studies. However, Bcl-xl protein was significantly lower in EpoR-HM derived EPCs as compared to EpoR-H or wt-EpoR erythroid cells. Potential post-translational mechanisms leading to Epo mediated Bcl-xL protein stabilization is presently unknown and could be investigated. In addition to Bcl-xl, EpoR PY479 mediated PI3-kinase/AKT activation constitutes an important survival pathway in erythroid cells. Low-level activation of Akt was observed in PY479 lacking EpoR-H and EpoR-HM derived EPCs. Potential PY479 independent activation of PI3-kinase pathway in EpoR-HM via adaptor proteins like GABs and IRS-2 can be further investigated. Also, mechanisms leading to EpoR PY independent activation of ERK is

presently unknown and is another area that needs further studies. In addition, it would be interesting to investigate whether retroviral overexpresion of one or more of the STAT-5 target genes in EpoR-HM derived bone marrow EPCs would rescue the multiple ex vivo defects in survival, proliferation and differentiation as well as its deficient bone marrow transplantation capabilities. Along the same lines, the critical nature of these STAT-5 target genes could be demonstrated via retroviral transduction of $EpoR^{-/-}$ or $JAK2^{-/-}$ fetal liver cells to investigate a potential rescue mechanism.

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PUBLICATIONS

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ABSTRACTS

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