CORE ERYTHROPOIETIN RECEPTOR SIGNALS FOR LATE ERYTHROBLAST DEVELOPMENT

Running Title: Epo minimal receptor allele action mechanisms

Madhu P. Menon1,2, Jing Fang1, and Don M. Wojchowski1-3

1Maine Medical Center Research Institute and Program in Stem Cell Biology & Regenerative Medicine, Scarborough, ME; 2Molecular Medicine Program, The Pennsylvania State University, University Park, PA

3Corresponding Author:

Don M. Wojchowski
Program in Stem Cell Biology & Regenerative Medicine
Maine Medical Center Research Institute
81 Research Drive
Scarborough, ME 04074
Tel: 207-885-8258
Fax: 207-885-8179
Email: wojchd@mmc.org

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ABSTRACT

Critical signals for erythroblast formation are transduced by activated, tyrosine-phosphorylated erythropoietin receptor (EpoR) complexes. Nonetheless, steady-state erythropoiesis is supported effectively by EpoR alleles that are deficient in cytoplasmic phosphotyrosine sites. To better define core EpoR action mechanisms, signaling capacities of minimal PY-null (EpoR-HM) and PY343-retaining (EpoR-H) alleles were analyzed for the first time in bone marrow-derived erythroblasts. Jak2 activation via each allele was comparable. Stat5 (and several Stat5- response genes) were induced via EpoR-H but not via EpoR-HM. Stat1 and -3 activation was nominal for all EpoR forms. For both EpoR-HM and EpoR-H, Akt and p70S6-kinase activation was decreased multi-fold, and JNK activation was minimal. ERKs, however, were hyperactivated uniquely via EpoR-HM. In vivo, Epo expression in EpoR-HM mice was elevated, while Epo-induced reticulocyte production was diminished. In vitro, EpoR-HM erythroblast maturation also was attenuated (based on DNA content, forward-angle light-scatter and hemoglobinization). These EpoR-HM-specific defects were corrected not only upon PY343 site restoration in EpoR-H, but also upon MEK1,2 inhibition. Core EpoR PY site-independent signals for erythroblast formation therefore appear to be Stat-5,-1,-3, p70S6-kinase- and JNK-independent, but ERK-dependent. Wild-type signaling capacities, however, depend further upon signals provided via an EpoR/PY343/Stat5 axis.
INTRODUCTION

Signals provided by Epo and its single transmembrane receptor (EpoR) are essential for erythroblast formation. Physicochemical studies have revealed unique mechanisms for Epo binding, and conformation-dependent activation of EpoR-Jak2 kinase complexes. Epo-activated signaling pathways also are well-studied, yet gaps in knowledge persist concerning the nature of key signals for EpoR biofunction. Interest in this basic problem also is provoked by apparent EpoR cytoprotection of injured myocardial, neuronal, endothelial and renal cells, and by the association of EpoR action with angiogenesis, VHL carcinomas, melanoma, and myoma formation. To better elucidate core signals for erythroid progenitor cells, we presently have performed first-time analyses of the signaling capacities of minimal knocked-in murine EpoR alleles in primary bone marrow-derived erythroblasts.

Epo binding occurs via Epo high-affinity A, B, D helix site-1, and low-affinity A, C helix site-2 interactions with bipartite seven beta-strand ligand binding sites in appositioned EpoR dimers. Via a cytoplasmic juxtamembrane box-1 domain, the EpoR also preassembles with Jak2 kinase. Epo-EpoR interactions stimulate Jak2 phosphorylation at Y1007/Y1008 sites, 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 particular, within the EpoR of mouse, man and zebra fish, eight distal phosphotyrosine motifs are conserved that possess established binding specificities for SH2-domain encoding effectors. These include: PY343 binding of Stat5; 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; PY429 and PY431 binding of SOCS-3 and/or SHP-1; PY460 binding of CrkL and regulation of intracellular calcium flux; PY464 and/or PY479 binding of Lyn; and PY479 binding of alpha-p85/PI3 kinase. In the human EpoR, an additional juxtamembrane
cytoplasmic PY285 site also exists and has been demonstrated in 32D cells to modulate Stat5 and Stat1 activation \(^{29}\).

Based on the evolutionary conservation of these EpoR PY sites and their demonstrated role as an assembling scaffold for the above effectors \(^{4}\), EpoR phosphotyrosine motifs are predicted to be important for Epo’s actions. The extent to which these EpoR regions (and linked pathways) act in central or perhaps only modulatory capacities, however, is controversial. This point is highlighted by the ability of PY-deficient EpoR alleles to support steady-state erythropoiesis \textit{in vivo} \(^{30}\). 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% \(^{30}\). Unexpectedly, this suggests that core signals provided by Jak2 (in the absence of EpoR PY- signals) efficiently support Epo-dependent erythroblast formation. These findings raise basic questions concerning the nature of core signaling pathways that are utilized by these minimal EpoR alleles (and the wt-EpoR). Due to the challenges of working with low abundance progenitor cells, however, analyses of molecular signals relayed by minimal murine EpoR forms in bone marrow-derived erythroblasts are limited to date to single-point EMSA analyses of Stat5 activity \(^{30}\).

To address the above basic problems in Epo signaling, primary culture systems presently have been 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 Stat5 binding site is selectively restored \(^{30}\). These analyses provide several new lines of insight into signals that are relayed via EpoR-HM/Jak2 and EpoR-H/Jak2/Stat5 axes. Overall, core EpoR-plus-Jak2-activated erythropoietic signals appear to be relayed primarily via Stat-5,-1,-3, JNK- and p70S6K-independent, but ERK 1,2-dependent routes. EpoR-PY343 signals, by comparison, restore wild-type erythropoietic capacities via suggested actions of key Stat5-target genes. This dissection of
core EpoR signal transduction mechanisms in primary marrow-derived erythroblasts also may shed useful light on cytoprotective mechanisms that are stimulated by Epo in injured cardiac, neuronal, endothelial and renal cells.\(^5\)
MATERIALS AND METHODS

Mice and primary erythroid progenitor cell culture - Mice expressing knocked-in EpoR-HM and EpoR-H alleles were those described by Zang et al. Bone marrow progenitor cells were prepared as follows. Femurs and tibiae were isolated, 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, Carlsbad, CA, #12440-053) plus 2% fetal bovine serum (FBS). 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 Na₂EDTA (ethylenediaminetetraacetic acid) solution, pH 7.5. 10 x PBS (1.1 mL) was then added. Cells were collected through 16 mL of 50% FBS in PBS, and washed in IMDM, 2% FBS. In expansions, cells were cultured (at 7.5x10⁵ cells/mL) in StemPro-34 medium (Invitrogen) supplemented with 2.5U/mL Epo (Epoetin-alpha, Amgen), 100ng/mL mSCF (Peprotech Inc., Rocky Hill, NJ), 1uM dexamethasone, 1uM beta-estradiol, 40ng/mL IGF-1, 75ug/mL h-transferrin, 0.5% BSA (Stem Cell Technologies, Vancouver, BC), 0.1 mM 2-mercaptoethanol 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 7.5x10⁵ cells/mL in 80% new media plus 20% residual conditioned media. For certain experiments, CD71<sup>high</sup>-Ter119<sup>neg</sup> erythroblast populations were prepared (at day 3.5 of culture) via two rounds of Ter119<sup>pos</sup> cell depletion (Miltenyi Biotech, Auburn, CA #130-049-901). In differentiation experiments, expanded erythroblasts (at day-3 of expansion) were cultured (at 7.5x10⁵ cells/mL) in 2.5U/mL Epo, 150ug/mL transferrin, 10ug/mL insulin, 0.5% BSA, 0.1mM 2-mercaptoethanol, 10% FBS (Hyclone Logan, UT #SH30070.03) in IMDM. Differentiation was assayed based on side and forward-angle light-scatter, DRAQ5 (ALEXIS, SanDiego, CA) (10µM) staining of DNA content.
hemoglobinization \(^{32}\), and Ter119 expression. PP2, PP3, SB202190, SP600125 and U0126 (Calbiochem, San Diego, CA) were prepared upon use in DMSO (at 1000x concentrations) and were added directly to SP34-EX cultures (with supplementation at 24 and 48 hours). U0126, SB202190 and SP600125 also were included in differentiation medium.

**Flow cytometry, FACS, and cytospins** - Cells (1×10⁶) 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 CaCl₂, 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. FACS was performed with a Vantage-SE system. Cytospin analyses (1×10⁵ cells) involved slide-centrifugation (15 minutes, 300 rpm, Hettich Universal-16A cyto-centrifuge) and Dip-Stain reagent staining (Volu-Sol, Salt Lake City, UT).

**RT-PCR** - 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-AGT-TCT-GTGG-AGA-GG-3' and 5'-GCT-CCT-CGT-TG-GTG-ATA-AA-3'; *Oncostatin-M* - 5'-AAC-TGA-GCA-AGC-CTC-ACT-TCC-3' and 5'-ATG-CCG-AGG-ATA-TTG-TGC-CG-3'; *SOCS-3* - 5'-CCG-CTT-CGA-CTG-TGT-ACT-CAG-3' and 5'-TGT-TCC-TCT-CGC-CCC-CAG-AAC-ATA-3'; *Bcl-x* - 5'-ACC-TGG-AGT-TCT-GCC-CTT-GCA-AGG-3' and 5'-TGC-TGC-ATT-GTT-CCC-ATA-AGG-3'; *Epo* - 5'-ACA-ATA-GAT-GGC-GAG-GGA-AAT-3' and 5'-CTG-GTG-GCT-GGG-AG-GAG-AGG-AAT-TG-3'; *Actin* - 5'-CTG-GCG-CAC-GAT-ATA-TAA-AGG-G-3' and 5'-TGG-ATG-CAC-CAG-GAT-TCC-ATA-3'.
Signal transduction factor analyses - In signal transduction experiments, 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. Upon washing in three volumes of 2°C PBS, cells were lysed in 0.2 mL of 1% Igepal, 150mM NaCl, 50mM NaF, 2mM Na2EDTA, 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: Stat5 (#9352), PY-Stat5 (#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). Certain western blots represent composites of more than one experiment with matched ECL exposures.
Epo dosing and reticulocyte assays - At 0 and 24 hours, EpoR-HM, EpoR-H and wt-EpoR mice were injected intraperitoneally with Epo (2.5U per g-mouse). On day 5, reticulocyte levels were determined via thiazole orange staining, and flow cytometry.
RESULTS

_Jak, Stat, and Stat5-target gene activation via minimal EpoR-HM and EpoR-H alleles_

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 NH₄Cl, and culture in serum-free SP34 media with optimized supplements. Supplement sources were important, as were subculture details. At day 3.5 of culture, 2x10⁷ cells were propagated on-average per mouse – and 45% to 50% of these cells reproducibly were highly Epo-responsive CD71<sup>high</sup>Ter119<sup>low</sup> erythroblasts (see below). The balance of cells included Mac-1<sup>pos</sup> (12%), B220<sup>pos</sup> (5%) and CD41<sup>pos</sup> (5%) cells (data not shown). 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 1A and 1-B1).

In expanded wt-EpoR, EpoR-HM and EpoR-H erythroblast preparations, Epo-induced Jak2 activation first was analyzed (Figure 1-B1). 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<sup>pos</sup> subpopulations, analyses were repeated using Ter119-depleted preparations (and extended time-courses) (Figure 1-B2). 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. No Epo-dependent activation of Jak1, Jak3 or Tyk2 was detected (data not shown).
Stat activation via EpoR alleles next was studied. Stat5 is most frequently linked to Epo signaling and was first analyzed. In time course experiments, EpoR-H activation of Stat5 paralleled that of the wt-EpoR. Stat5, however, was not detectably activated via EpoR-HM (Figure 1-C1). As above, analyses of Stat5 activation were repeated in independent erythroblast preparations following Ter119POS cell depletion. Essentially equivalent results were obtained (1-C2). 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 Stat5- 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 90 minutes (see supplemental Figure S-2). Pim1, oncostatin-M, SOCS-3 and Cis-1 each were induced via the wt-EpoR and EpoR-H between 5 to 35-fold (Table 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 Stat5 activation via EpoR-HM. In cell line models, Epo-activation of Stat-1 and -3 has been reported. In addition, Stat1-deficient mice exhibit decreased erythroid progenitor cell levels, and Stat1-/- BFU-E show decreased Epo-responsiveness. Activation of these Stats via EpoR alleles in primary marrow-derived erythroblasts therefore was analyzed. Stat3 activation was undetectable. Stat1 activation was detected, but only at the limits of optimized ECL sensitivity (see supplemental Figure S-1). This suggests limited contributions of these Stats to Epo bioactivities in this primary erythroblast system.

Epo receptor allele regulation of Akt, p70S6-kinase, p60-Src and MAPKs

In cell lines and in fetal liver cells, Akt and p70S6K each have been shown to be activated by Epo. These response pathways are linked in that PI3-kinase stimulates Akt, Akt activates mTOR, and mTOR comprises a major p70S6K regulator. Each response also can affect progenitor cell survival...
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 2A). This outcome is consistent with an indicated role for an EpoR PY479 site recruitment of PI3-kinase upstream of p70S6K activation \(^{28}\) (and p70S6K therefore may be non-essential for efficient EpoR function). 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 2B). 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 \(^{11}\). 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 2C). In cytokine-deprived wt-EpoR cells, background levels of activated p60-Src, however, were sustained (as contrasted with Jak2 and Stat5, for example). In EpoR-HM erythroblasts, Epo detectably (but nominally) activated p60-Src. 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 Stat5. Epo can also activate Lyn kinase. The possibility that the SFK studied might be Lyn, however, was discounted by the parallel blotting of lysates from expanded Lyn\(^{-/-}\) erythroblasts (i.e., Lyn migrated at a lower apparent Mr, and did not react with anti- p60-Src antibodies) (data not shown). Possible differential effects of PP2 (a SFK inhibitor) on the \textit{in vitro} expansion of wt-EpoR, EpoR-HM and EpoR-H erythroblasts also were assessed (Figure 2C-3). 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 (data not shown). 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.

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 (see supplemental Figure S-3). For JNKs, two isoforms were rapidly stimulated by Epo via the wt-EpoR (Figure 3A). Little to no JNK activation was detected however via EpoR-HM or EpoR-H alleles. 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 3B-1). This latter finding was also examined further, and was confirmed, in Ter119pos-depleted erythroblast preparations (Figure 3B-2).

Faltering late-stage development of EpoR-HM erythroblasts, and rescue by PY343 signals or MEK1,2 inhibition

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 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 4A). 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 4-B1, left panels) in parallel with a $\geq 2.5$-fold disadvantage in the formation of low forward-angle light scatter late-stage erythroblasts (Figures 4-B1 and 4-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 5). 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 5A). 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 also visually obvious in pelleted cells, and cytospin preparations (Figures 5B and 5C).

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 might impact on Epo
receptor-dependent erythroblast differentiation (especially as supported by EpoR-HM). In pilot experiments, U0126 at 10μM was observed to have little effect on expansion (but at 20μM detectably affected viability, data not shown). 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 6A). As assayed based on Ter119 and CD71 marker expression, U0126 also promoted the maturation of a sub-population of EpoR-HM Ter119\textsuperscript{pos}CD71\textsuperscript{low} erythroblasts (Figure 6B). In contrast, U0126 at this dose had no significant effects on the maturation of wt-EpoR erythroblasts (or on EpoR-H erythroblasts, data not shown). Dose-dependency of this U0126 effect on EpoR-HM erythroblasts also is illustrated (Figure 6C). 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 6D). 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 (data not shown).

**EpoR-H and EpoR-HM erythroblast survival potential, and Bcl-x, Bax and DAPK2 expression**

Based on Epo’s primary role as an anti-apoptotic factor\textsuperscript{43}, possible differences among EpoR-HM, EpoR-H and wt-EpoR erythroblast survival, and Bcl-xl, Bax and DAPK-2 expression were analyzed (Figure 7). In expanded EpoR-HM erythroblasts, Bcl-xl levels proved to be decreased several-fold (Figure 7-A1). Bax expression levels, by comparison, were similar for each EpoR allele. In repeated
analyses in Ter119-depleted CD71\textsuperscript{high} wt-EpoR, EpoR-HM and EpoR-H populations, findings were similar (Figure 7-A2). The extent to which altered EpoR-HM signaling capacities might correlate with compromised erythroblast survival potential also was assessed. In these experiments, Kit\textsuperscript{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\textsuperscript{high} Kit\textsuperscript{neg} as well as differentiated Ter119\textsuperscript{pos} erythroblasts as compared directly to wt-EpoR and EpoR-H cells (Figure 7B), 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, our laboratory recently reported on predominant late-stage erythroid expression of the proapoptotic death-associated protein kinase-2 (DAPK2) \textsuperscript{44}. 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 7C). This observation is consistent with the decreased survival potential of EpoR-HM cells (and with possible roles for EpoR PY343, and Stat5, in down-modulating DAPK-2).
DISCUSSION

As revealed through transgenic mouse models, the EpoR is essential for erythroblast formation \(^{45}\), and its conditional hematopoietic expression is sufficient for normal overall development \(^{45}\).

Crystallographic and structural analyses have provided detailed insight into Epo-mediated dimerization of its receptor \(^{2,46}\), and conformation-dependent mechanisms of Jak2 activation \(^{3}\). 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 \(^{47,48}\).

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 \(^{49,50}\), and to support ERK activation at wild-type levels \(^{51}\). In the present investigations we analyzed EpoR-HM’s capacity in primary erythroblasts to activate Jak2, Stat5, Stat5-target genes, \(Bcl-x\) and \(Myc\) (see below). We also examined possible EpoR-HM regulation of Akt, p70S6-kinase, p60Src, p38-MAPK, JNKs and ERKs. Jak2 activation profiles were essentially normal, and in the absence of PY343, no significant induction of Stat5, or Stat5-target genes was detected. In addition, EpoR-HM essentially failed to activate JNKs or p70S6-kinase, and was substantially compromised in its ability to simulate Akt (as well as p60-Src).

As observed for the wt-EpoR (and EpoR-H), EpoR-HM modestly stimulated p38-MAPK. The major intact (and selectively altered) response pathway for EpoR-HM, however, involved ERKs. This finding (i.e., EpoR-HM hyperactivation of ERKs) was unexpected, and prompts considerations of first, how
the EpoR might couple to ERKs and second, what signals ERK might provide to developing primary erythroblasts.

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; a recently described PY479 plus PLC-gamma dependent route; a Shc plus Grb2 pathway to mSos and Ras; a CrkL plus C3G- coupled route; as well as a SOCS-3 mediated mechanism involving PY-SOCS box sequestration of GAP. In contrast, the presently observed strong activation of ERKs via EpoR-HM in primary erythroblasts suggests that PY sites may be non-essential for ERK activation. Candidate factors that might couple EpoR-HM (and perhaps the wt-EpoR) to ERKs are presently undefined, but G-proteins as well as PKCs stimulate MAPK modules and represent two sets of potentially Epo-regulated candidate effectors. Based on the substantial in vivo activity exerted by EpoR-HM, such Epo-regulated pathways should be of interest to define. In addition, a specific loss of PY343 signaling is associated with EpoR-HM mediated ERK hyper-activation. By speculation, this effect might involve decreased MAPK phosphatase action. MKP-7 haploinsufficiency, for example, has been associated with BCR-ABL induced proliferation, while increased MKP-1 activity is associated with pre-adipocyte differentiation.

Biofunctional roles that Epo-regulated ERKs might play in developing erythroblasts also merit consideration. In context-specific settings, ERK signaling can affect differentiation, survival and proliferation (reviewed Roux et al and Wada et al). However, ERKs are most commonly activated via growth factor receptors. Moreover, ERKs have been shown in primary erythroid cells to be the prime effector of H-Ras induced transformation. It therefore is suggested that ERK hyperactivation in EpoR-HM erythroblasts may inappropriately enforce proliferation at the expense of differentiation. This case also is supported by restoration of wild-type differentiation profiles for EpoR-HM.
erythroblasts upon exposure to the MEK1,2 inhibitor U0126 (see Figure 6). It is noted, however, that chronic activation of ERKs also can induce apoptosis, and this response also might contribute to the elevated apoptosis observed among maturing EpoR-HM cells. Finally, for EpoR-HM, two additional responses described previously in cell line models are *c-Myc*, and *Bcl-x* transcript induction. In the present system, *c-Myc* (and *n-Myc*) induction by Epo was analyzed. Each was up-modulated approximately 1.5-fold uniformly in EpoR-HM, EpoR-H and wt-EpoR erythroblasts (unpublished results). This therefore was not a strong or differential response. For Bcl-x, possible regulation via EpoR alleles is discussed below.

In EpoR-H erythroblasts, the selective restoration of PY343 restored efficient Epo-activation of Stat5 (and also increased p60-Src activation). As indicated above, ERK activation also was down-modulated as compared to EpoR-HM (to approximately wt-EpoR levels). JNK and p70S6 kinases, however, remained essentially uncoupled, and Akt activation also was inefficient (as observed for EpoR-HM). Based on these results, enhanced bioactivities of EpoR-H are suggested to depend primarily upon Stat5 activation, and the actions of select Stat5-target genes within developing erythroblasts. Five genes that previously have been suggested in cell line models to comprise Epo and Stat5-regulated genes were assessed for Epo regulation in EpoR-H, EpoR-HM and wt-EpoR erythroblasts, i.e., *Pim-1*, *oncostatin-M*, *SOCS-3*, *Cis-1* and *Bcl-x*. With the exception of *Bcl-x*, each was induced at least 5-fold by Epo via EpoR-H and the wt-EpoR, but not via EpoR-HM. SOCS-3 and Cis-1 are known to provide negative feedback, in part via inhibition of Jak2 at activated EpoR complexes. *Pim-1* and *oncostatin-M*, in contrast, may well act in positive modes. Pim kinases recently have been shown to act in parallel with mTOR to promote hematopoietic progenitor cell survival. *Oncostatin-M*, by comparision, is a pleiotropic cytokine which is expressed primarily by macrophage and activated T-cells. Interestingly, however, disruption of oncostatin-M receptor-beta expression limits bone marrow erythroid progenitor cell formation. Whether erythroblast-derived oncostatin-M might act via autocrine routes, or perhaps
on neighboring macrophage in blood islands is under investigation. Finally, present findings for Epo-regulated $Bcl-x$ expression are negative ones, and therefore should be conservatively interpreted. Two points to be considered here, nonetheless, are first, that Epo (and Stat5) may not act to strongly or rapidly up-regulate $Bcl-x$ gene transcription. In recent studies by Rhodes et al 68 this possibility also has been raised. Second, decreases in Bcl-xL levels presently observed in EpoR-HM erythroblasts therefore may only correlate with decreased Epo-dependent survival potential.

With further regards to Stat5, the ability of PY343 (and Stat5) signals in EpoR-H to restore wild-type erythroblast development (vs. faltered development for EpoR-HM) predicts that erythroid defects should exist in Stat5-deficient mice. In phenylhydrazine-treated $Stat5a,b^{-/-}$ mice, faltered splenic erythropoiesis has been characterized 69, and certain studies also have outlined deficient embryonic erythropoiesis 33. Spontaneous (erythro)splenomegaly, however, has been reported to involve immune cell by-stander effects 30. In addition, the Stat5 exon targeting strategy employed may allow for expression of at least partially functional Stat5 proteins (unpublished observations). The recent development of mice with floxed $Stat5a$ and $b$ alleles 70 offers an alternate future approach to Stat5 action mechanisms (i.e., erythroid lineage specific disruption). In these mice, conditional Stat5 disruption at the oocyte stage results in embryonic anemia 70.

Finally, and as compared to the wt-EpoR, PY-deficient EpoR-HM and EpoR-H alleles faltered in their abilities to support Epo-induction of Akt, p70S6-kinase and JNKs. For Akt and p70S6-kinase (and as discussed above) this likely reflects uncoupling from PI3-kinase 37,38. By comparison, less is known concerning Epo and EpoR- regulation of JNKs, or roles played by JNKs during erythropoiesis. Epo-activation of JNK1 and -2 has been observed in SKT6 and HCD57 cell lines. In SKT6 cells, JNK activation was linked to Epo-dependent differentiation 71. In HCD57 cells, antisense and inhibitor-based knock-down of JNKs was observed to limit proliferation 72. JNK-2 deficiency, however,
appears to increase erythroid progenitor cell proliferative potential. In primary erythroblasts, Epo appears to activate at least two JNK isoforms, but this proved to depend upon EpoR carboxyl-terminal PY sites beyond PY343. Factors that link the EpoR to MEK4/7 and JNK pathways remain to be discovered, as do the contributions of this typically stress-activated module to erythropoiesis.

Overall, the present studies reveal that core Jak2-mediated, Epo receptor PY-independent signals for erythroblast formation are associated primarily with PY-null EpoR-HM activation of ERKs, and are essentially uncoupled from Stat-5,-1,-3, JNK kinases or p70S6-kinase pathways. Whether ERK-activation is necessary-and-sufficient for EpoR-HM action nonetheless is uncertain, and it is possible that other lateral pathways are engaged (e.g., IRS1,2 and/or Gab1,2)\textsuperscript{20,74}. Such possibilities are under investigation, as are possible contributions of residual low-level Akt activation. It is clear, however, that U0126- attenuation of ERK hyper-activation corrects a late stage developmental defect in maturing EpoR-HM erythroblasts, and this result fortifies the notion that ERK activation is functionally consequential. In addition EpoR PY343 signaling is shown to be essential for Stat5 activation, and Stat5-stimulated events are shown to rescue wild-type (or higher) levels of Epo bioresponses \textit{in vitro}, and \textit{in vivo}. Here, it is possible that EpoR PY343 stimulates additional pathways beyond Stat5. However, this does not appear to include Stat-1 or -3, p70S6-kinase, JNKs or Akt. EpoR-H's activity (and that of the wt-EpoR) therefore may more likely depend upon the actions of one or more key Stat5- target genes in developing erythroblasts. Two candidate targets are Pim-1 and oncostatin-M, but others are being actively sought using the presently developed primary erythroblast system.
ACKNOWLEDGMENTS

The authors thank the following collaborators for their generous indicated contributions: Dr. James Ihle (St. Jude Children’s Research Hospital, Memphis) for provision of EpoR-H and EpoR-HM mice, Jane Mitchell (MMCRI) for expert assistance with flow cytometry and FACS.
REFERENCES


### TABLE 1. INDUCTION OF CANDIDATE STAT5-RESPONSE GENES IN WT-EPOR, EPOR-HM AND EPOR-H ERYTHROBLASTS

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<tr>
<th></th>
<th>Pim-1</th>
<th>onco-M</th>
<th>SOCS-3</th>
<th>Cis-1</th>
<th>Bcl-x</th>
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<td>wt-EpoR</td>
<td>6.3 +/- 0.8</td>
<td>10.6 +/- 1.2</td>
<td>16 +/- 1.1</td>
<td>37.3 +/- 1.7</td>
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<td>EpoR-HM</td>
<td>1.9 +/- 0.5</td>
<td>1.4 +/- 0.3</td>
<td>1.8 +/- 0.5</td>
<td>1.0 +/- 0.4</td>
<td>0.9 +/- 0.4</td>
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<td>EpoR-H</td>
<td>8.1 +/- 0.4</td>
<td>15.6 +/- 1.2</td>
<td>19.7 +/- 0.8</td>
<td>31.3 +/- 5.0</td>
<td>1.1 +/- 0.2</td>
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[Values are mean levels, +/- SE, of transcript induction over baseline at 90 minutes of Epo exposure, 2.5 U/mL]
**TABLE 2. STEADY-STATE RENAL EPO TRANSCRIPT LEVELS IN EPOR-HM, EPOR-H AND WT-EPOR MICE.**

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<tr>
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<td><em>Epo</em> transcript levels, <em>% wt-EpoR</em></td>
<td>186 +/- 8 %</td>
<td>55 +/- 2 %</td>
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*Mean values +/- SE, n=3*
FIGURE LEGENDS

Figure 1. Jak2 and Stat5 activation via EpoR-HM and EpoR-H alleles in primary bone marrow-derived 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] Jak2 activation profiles via minimal EpoR alleles – B-1] 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<sup>high</sup> erythroblasts. 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. In all expansion experiments, CD71 and Ter119 marker expression was assessed, and representative distributions are shown. B-2] Jak2 activation was analyzed as above, but for erythroblast preparations from which Ter119<sup>pos</sup> cells were depleted. C] Stat5 activation via EpoR-H, but not EpoR-HM alleles - In parallel analyses, EpoR allele-mediated activation of Stat5 was assessed both for expansion cultures (C1), and for Ter119-depleted cultures (CD71<sup>pos</sup>Ter119<sup>neg</sup> populations) (C2).

Figure 2. EpoR-HM, EpoR-H and wt-EpoR modulation of p70S6-kinase, Akt and p60-Src.  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. C-1, C-2] Deficient PY-416 p60-Src expression via EpoR-HM – In the
above cells and samples (panel B), levels of phospho-p60-Src (and p60-Src) were assayed by western blotting (and digital densitometry imaging). **C-3** 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. PP3 also was tested, but was without significant effects (data not shown).

**Figure 3. Epo receptor allele activation of JNKs, and ERKs**  

A] Efficient JNK activation via the wt-EpoR, but not via EpoR-H or -HM alleles - 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. The upper panel (A-1) illustrates results for the wt-EpoR, and includes co-analyzed positive controls (Cell Signaling #9253). In the lower panel (A-2), note the nominal activation of JNKs via EpoR-HM and -H alleles. B] ERK hyperactivation via EpoR-HM – B-1] 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-2] Parallel analyses of EpoR allele activation of ERKs were performed using expanded, Ter119-depleted wt-EpoR, EpoR-HM and EpoR-H CD71high erythroblasts (and Epo exposure was extended to 60 minutes). For comparison, levels of Epo-stimulated phosphorylated p38-MAPK (PT180 and PY182) (and total p38-MAPK) also were assayed (see supplemental Figure S-3).

**Figure 4. 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<sup>pos</sup> Ter119<sup>pos</sup> 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<sup>high</sup>Ter119<sup>pos</sup> 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.

Figure 5. 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<sup>neg</sup>Ter119<sup>pos</sup> 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 6. 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<sup>pos</sup> 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). C] U0126 dose-dependent reversal of EpoR-HM erythroblast differentiation defects also was observed based on U0126-dependent increases in frequencies of Ter119<sup>pos</sup> erythroblasts. D] 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 7. Altered Bcl-xl and DAPK-2 expression in EpoR-HM erythroblasts.** A-1] 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. A-2] Bcl-xl expression in wt-EpoR, EpoR-HM and EpoR-H erythroblasts also was analyzed in Ter119-depleted, CD71<sup>high</sup> erythroblasts. B] Defective survival of EpoR-HM CD71<sup>high</sup>Kit<sup>neg</sup> erythroblasts, and rescue of survival potential by PY343 in EpoR-H – Kit<sup>pos</sup> 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<sup>pos</sup> 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. C] 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 2

(A)

84k—PY-p70S6K

64k—p70S6K

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(B)

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PY-p60Src

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(C2)

PY-SRC

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(C3)

15μM PP2

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FIGURE 3

(A1)  wt-EpoR

CD71<sup>high</sup>TER119<sup>neg</sup> population

(+)<sup>+</sup> control

PYT-p54
SAPK/JNK
PYT-p46
SAPK/JNK

mins: 0 4 15 60 120

(A2)  CD71<sup>high</sup>TER119<sup>neg</sup> population

PYT-p54 JNK
PYT-p46 JNK

p54 JNK
p46 JNK

mins
wt-EpoR EpoR-HM EpoR-H

PYT-p54/46 SAPK/JNK,
15min

PYT-p54/48 SAPK/JNK,
4-60min

(B1)  PYT-ERK1/2

0 3 8 24 0 3 8 24 0 3 8 24 mins

wt-EpoR EpoR-HM EpoR-H

PYT-ERK1/2, 3min
PYT-ERK1/2, 0-24mins

(B2)  CD71<sup>high</sup>TER119<sup>neg</sup> population

PYT-ERK1/2

0 4 15 60 0 4 15 60 0 4 15 60 mins

wt-EpoR EpoR-HM EpoR-H

PYT-ERK1/2, 4min
PYT-ERK1/2, 4-60mins

(B2)  CD71<sup>high</sup>TER119<sup>neg</sup> population

PYT-ERK1/2

0 4 15 60 0 4 15 60 0 4 15 60 mins

wt-EpoR EpoR-HM EpoR-H

PYT-ERK1/2, 4min
PYT-ERK1/2, 4-60mins

(B2)  CD71<sup>high</sup>TER119<sup>neg</sup> population

PYT-ERK1/2

0 4 15 60 0 4 15 60 0 4 15 60 mins

wt-EpoR EpoR-HM EpoR-H

PYT-ERK1/2, 4min
PYT-ERK1/2, 4-60mins
FIGURE 4

(A)

![Graph showing events vs. fluorescence intensity.

(B1)

- wt-EpoR
  - Fc-CD71: 14%
  - PE-Ter119: 80%
  - EpoR-HM: 5.61%
  - Side Scatter: EpoR-HM

- wt-EpoR
  - Forward Scatter: EpoR-HM

(B2)

- wt-EpoR
  - EpoR-HM
  - EpoR-H

High FALS cells, % Total
FIGURE 5

(A) 

wt-EpoR

EpoR-HM

EpoR-H

(B) 

wt-EpoR

EpoR-HM

EpoR-H

Benzidine-stained Colonies

(C)

Hemoglobin (DAF assay)

wt-EpoR

EpoR-HM

EpoR-H
FIGURE 6

(A) (-) UO126  (+) UO126

(B) (-) UO126  (+) UO126

(C)

(D)

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Core erythropoietin receptor signals for late erythroblast development

Madhu P Menon, Jing Fang and Don M Wojchowski