Signals for stress erythropoiesis are integrated via an erythropoietin receptor–phosphotyrosine-343–Stat5 axis

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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 on an Epo receptor–phosphotyrosine-343–Stat5 signaling axis. In mice expressing a phosphotyrosine-null (PY-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 WT levels upon the selective restoration of an EpoR PY343 Stat5-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 Stat5 target genes *proviral integration site-1* (*Pim-1*) and *oncostatin-M*. *Bcl2-like 1* (*Bcl-x*), in contrast, was not significantly induced via WT-EpoR, EpoR-HM, or EpoR-H alleles. In Kit$^+$CD71$^+$ erythroblasts, EpoR-PY343 signals furthermore enhanced SCF growth effects, and SCF modulation of Pim-1 kinase and *oncostatin-M* expression. In maturing Kit$^-$CD71$^+$ erythroblasts, oncostatin-M exerted antiapoptotic effects that likewise depended on EpoR PY343–mediated events. Stress erythropoiesis, therefore, requires stage-specific EpoR-PY343-Stat5 signals, some of which selectively bolster SCF and oncostatin-M action.

Introduction

As a central hormonal regulator of red cell production, erythropoietin (Epo) is required for development beyond the colony-forming unit–erythroid (CFUe) stage and functions primarily as an erythroblast survival factor (1). Via mechanisms regulated by hypoxia-inducible factors-1α/β through -3α/β (2), Epo is expressed in the adult kidney and is secreted as a complex sialoglycoprotein (3). Its actions on erythroid progenitor cells then depend on Epo binding to preformed Epo receptor (EpoR) dimers (4). Extensive studies of Epo and EpoR interactions have assisted the development of highly active recombinant erythropoietins as important antianemia agents (5). Nonetheless, resistance to Epo during chronic anemia and myelodysplasia (6, 7), Epo hyperresponsiveness in congenital and familial polycythemias (8), and efforts to develop novel Epo orthologues (5, 9) 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 (10) and by roles for Epo during angiogenesis (10) and possibly tumorigenesis (11).

EpoR signals for erythroblast formation involve first the activation of Jak2, an essential upstream Janus kinase that preassembles at a conserved EpoR box-1 domain (3). Jak2 then mediates the phosphorylation of 8 conserved EpoR cytoplasmic phosphotyrosine (PY) motifs (3). These EpoR PY sites function as a scaffold for the binding of a complex, yet fairly well defined set of Src-homology 2 (SH2) and phosphotyrosine-binding protein domain signal transduction factors. One subset of EpoR PY site–recruited factors coordinates negative feedback. PY429 binds protein tyrosine phosphatase, nonreceptor type 6 (SHP-1), which can dephosphorylate Jak2 (12). PY401 together with PY429 and PY431 binds SOCS-3 and cytokine-inducible SH2-containing protein 1 (Cis-1) (13), which (as suppressors of cytokine signaling) can interfere with Jak2 and/or Stat5 activation and can also target interacting factors for ubiquitination (14). In addition, SH2-containing inositol phosphatase-1 (Ship-1; an inhibitory phosphatase for phosphatidylinositol 3,4,5-triphosphate) associates with activated EpoR complexes and downmodulates PI3K-stimulated events (15).

Epo's predominating positive signals are linked to a distinct subset of EpoR PY sites, and coupled effectors (3). PI3K binding at EpoR PY479 leads to Akt, mammalian target of rapamycin (mTOR), and p70S6K activation (16, 17). Growth factor receptor–bound protein 2 (Grb2)/Shc binding at PY464 (together with Syp phosphatase binding at PY425) (18) has been linked to murine homologue of Drosophila son of sevenless (mSos)/Ras/Raf/MEK regulation, while phospholipase Cγ1 activation and calcium flux mediated by transient receptor potential cation channel, subfamily C, member 2 (TRPC2) appear to couple to PY460 (19). EpoR PY site–dependent signals, in addition, have been implicated in Gab docking protein (20) and NF-κB modulation (21). Finally,

Nonstandard abbreviations used: Bcl-x, Bcl2-like 1; BFUe, burst-forming unit(s)–erythroid; CFUe, colony-forming unit(s)–erythroid; Cis, cytokine-inducible Src-homology 2–containing protein; Epo, erythropoietin; EpoR, Epo receptor; EpoR-H, knocked-in EpoR allele truncated at amino acid 361; EpoR-HM, Y343F-mutation within EpoR-H to yield a PY-null allele; Gas6, growth arrest–specific 6; IMDM, Iscove's modified Dulbecco's medium; mTOR, mammalian target of rapamycin; Pim, proviral integration site; PY, phosphotyrosine; SH2, Src-homology 2; SOCS, suppressor of cytokine signaling; SP34-EX, SP34-containing serum-free erythroid expansion medium.

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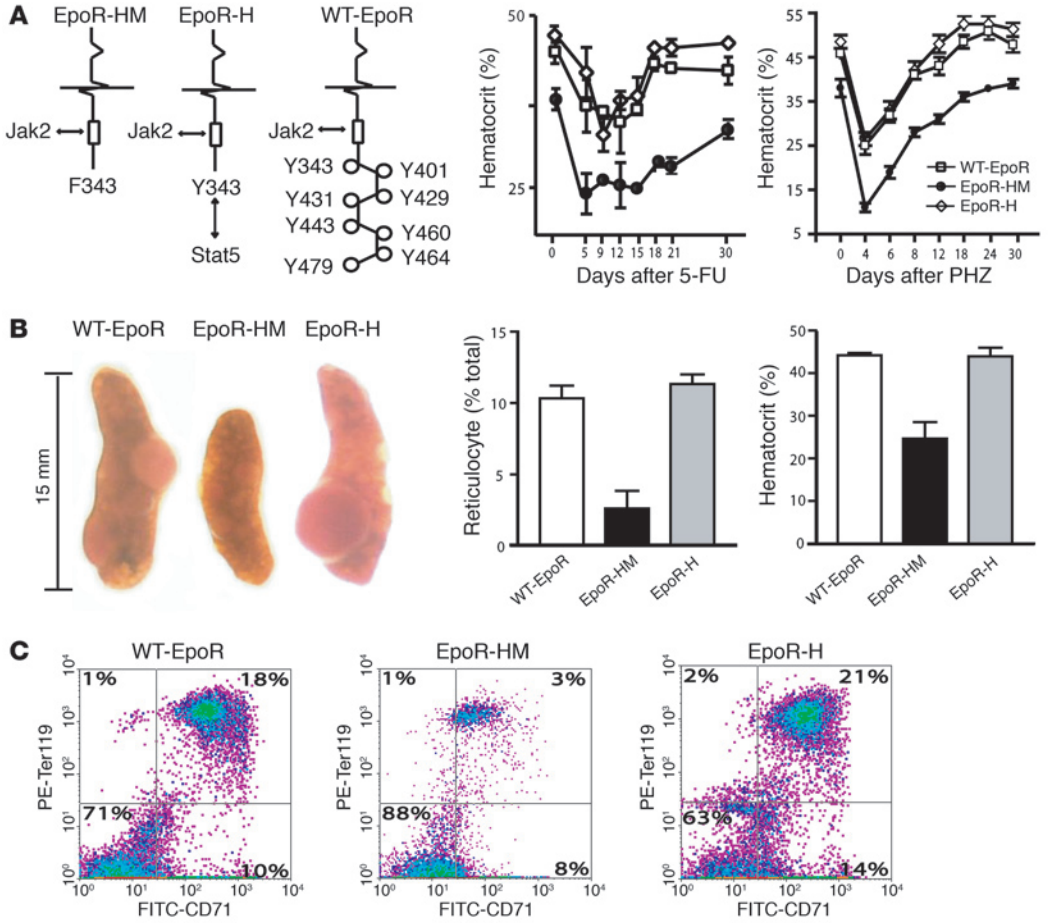

Figure 1

Efficient stress erythropoiesis in response to 5-fluorouracil, hemolytic anemia, or bone marrow transplantation is not supported by a minimal PY-null EpoR-HM allele but is rescued by EpoR-H–PY343–Stat5 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 ± SEM for 4 mice per time point. (**B**) In transplantation experiments, marrow preparations from WT-EpoR, EpoR-HM, and EpoR-H mice were transplanted into lethally irradiated Ly5.1-marked recipients. For mice with at least 95% Ly5.2 donor cell contributions, day 15 reticulocyte levels and hematocrits were determined and representative spleens were photographed. (**C**) Frequencies of $CD71^{high}Ter119^{+}$ erythroblasts in recipient spleens also were analyzed.

Stat5 activation occurs predominantly via PY343 (22) and may promote Bcl-x_L expression (23).

Despite this conserved evolution of EpoR PY signaling scaffolds (24), steady-state erythropoiesis unexpectedly has been shown to be supported by a minimal PY-null EpoR allele (EpoR-HM; a Y343F-mutation within EpoR-H to yield a PY-null allele) as expressed from the endogenous murine *EpoR* locus (25). Specifically, EpoR-HM mice maintain hematocrits within 8 points of normal, rbc counts at approximately 80% of normal, and essentially WT levels of burst-forming units–erythroid (BFUe) and colony-forming units–erythroid (CFUe). Core EpoR-Jak2–dependent, EpoR PY site–independent signals therefore appear to be sufficient for steady-state erythropoiesis.

The present studies focus on the concept that 1 or more positively acting EpoR PY sites might be required for erythropoiesis during anemia. Recently, selective stress-erythropoietic roles have been ascribed for several distinct ligand-receptor systems. These include bone morphogenic protein 4 (BMP4)/mothers against decapentaplegic homologue 5 (Madh5) regulation of splenic erythroid progenitor cell expansion (26); enhancement of erythropoiesis during chronic anemia by growth arrest–specific 6 (Gas6; a Tyro3/Axl/Mer RTK ligand) (27); and differential effects of oncostatin-M on extramedullary versus bone marrow erythropoiesis (28). Herein, mice expressing knocked-in PY-mutated EpoR alleles were used to discover and characterize EpoR-associated events that selectively affect stress erythropoiesis. 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 (knocked-in EpoR allele truncated at amino acid 361), in which a PY343 Stat5-binding site is selectively restored. In a unique ex vivo system, EpoR-PY343-Stat5 signals furthermore are shown to be important for Epo-dependent $Kit^{+}CD71^{+}$ erythroblast growth and survival, and for the selective induction of proviral integration site-1 (*Pim-1*) *kinase* and *oncostatin-M* expression. Finally, EpoR-PY343-Stat5 signals are also revealed to enhance the effects of both SCF and oncostatin-M on primary erythroblast development.

Results

Signals transduced via an EpoR-PY343-Stat5 axis are essential for efficient stress erythropoiesis. The minimal EpoR allele, EpoR-HM, retains a Jak2-binding domain but otherwise lacks all additional known cytoplasmic signaling motifs (25, 29). Unexpectedly, mice expressing this allele effectively support steady-state erythropoiesis (25). In response to phenylhydrazine-induced anemia, however, splenomegaly in EpoR-HM mice appears to be limited (29). To extend this basic observation, EpoR-HM mice together with mice expressing an EpoR-H allele with a singularly restored PY343 Stat5-binding site (25) were treated with either phenylhydrazine or 5-fluorouracil, and resulting anemias were characterized over 30-day time courses (Figure 1A). In EpoR-HM mice, 5-fluorouracil depletion of proliferating progenitor cells resulted in an abnormally sharp and persistent anemia. In contrast, no such defect was observed in EpoR-H mice, and PY343-mediated signals proved to restore stress erythropoiesis at WT levels. In response to phenylhydrazine, similar defects in rebound erythropoiesis were observed in EpoR-HM mice but, notably, were again restored to a WT response in EpoR-H mice.

The EpoR also is expressed in vascular, cardiac, and renal cells (10), and EpoR-HM actions in these tissues might impact on stress erythropoiesis. To address this potential issue, and to test EpoR allele function in a distinct and clinically relevant stress context, short-term repopulating capacities of hematopoietic progenitor cells from EpoR-HM and EpoR-H mouse bone marrow were assessed. In the spleens of WT recipients, transplanted EpoR-HM donor cells essentially failed to give rise to the large colonies of engrafted cells observed for WT-EpoR donor controls (Figure 1B). Furthermore, reticulocyte levels in recipients transplanted with EpoR-HM cells were decreased at least 4-fold; hematocrits were approximately 2-fold below control levels; and a 6-fold deficit in splenic CD71$^+$Ter119$^+$ erythroblast frequencies was observed (Figure 1C). Monocytic (Mac1$^+$) and lymphoid (B220$^+$) lineages, in contrast, were represented at essentially WT frequencies (data not shown).

For transplanted EpoR-H cells, the above EpoR-HM–associated defects again were corrected to essentially WT erythropoietic capacities (Figure 1, B and C).

One factor that might contribute to defective stress erythropoiesis in EpoR-HM mice relates to Epo production. Specifically, possible high-level Epo expression in untreated EpoR-HM mice might effectively blunt responses to phenylhydrazine and/or 5-fluorouracil. In *Stat5a,b*$^{-/-}$ mice, for example, up to 100-fold spontaneous increases in Epo production (and erythrosplenomegaly) have been described (30). *Epo* levels in untreated EpoR-HM mice, however, were not markedly elevated (Figure 2) (nor was splenomegaly observed at steady state among more than 100 untreated mice examined). Phenylhydrazine treatment, however, did induce renal *Epo* transcript expression in EpoR-HM mice at levels severalfold 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 3 independent experiments. For EpoR-HM, a case for blunted erythropoietic responses due to elevated steady-state Epo levels therefore is discounted. For EpoR-H, somewhat lower-level *Epo* production is consistent with suggested overall negative roles for distal EpoR cytoplasmic domains (31).

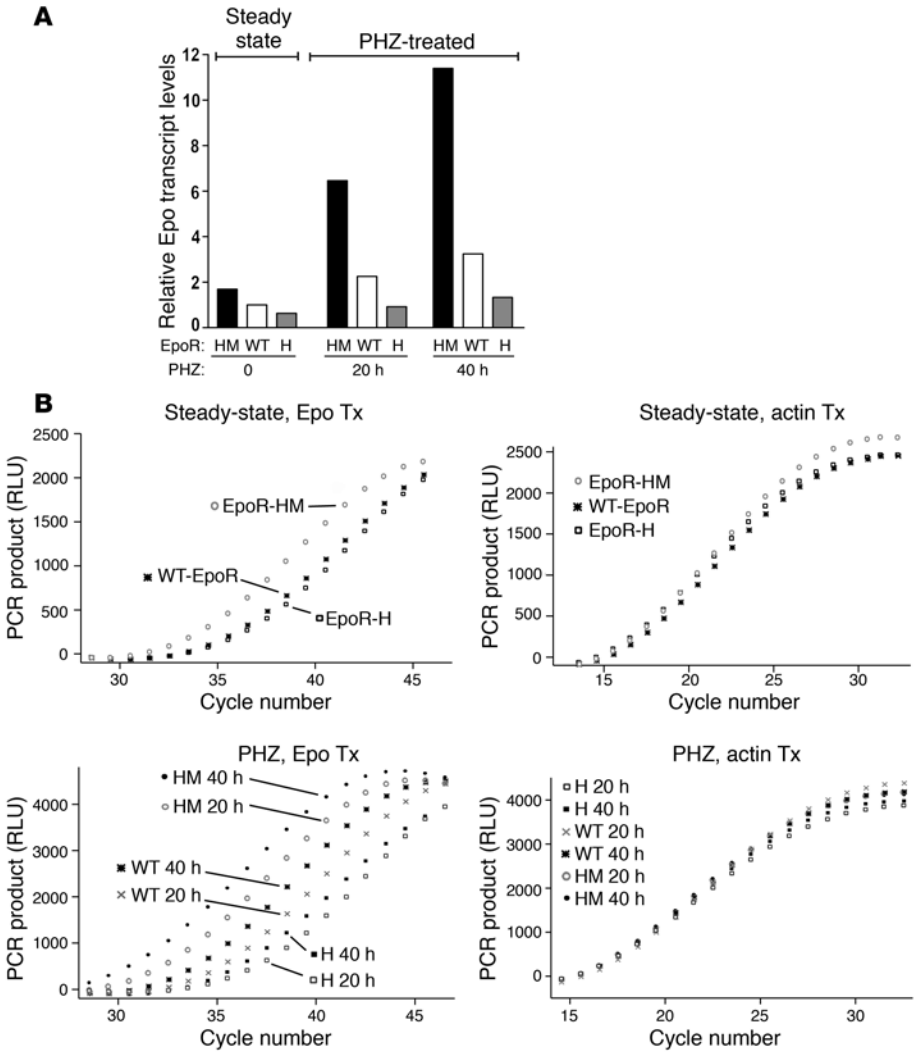

Figure 2
Epo expression in EpoR-HM mice is not substantially elevated during steady-state erythropoiesis but is hyperactivated in response to anemia. (**A**) EpoR-HM, WT-EpoR, and EpoR-H mice were treated with phenylhydrazine (PHZ, at 100 mg/kg, or PBS only). Base-line (steady-state) and phenylhydrazine-induced levels of renal *Epo* transcripts (at 0, 20, and 40 hours after phenylhydrazine dosing) then were determined by quantitative RT-PCR (and are normalized for coassayed levels of *actin* transcripts). (**B**) Representative primary PCR data also are shown. Tx, transcript.

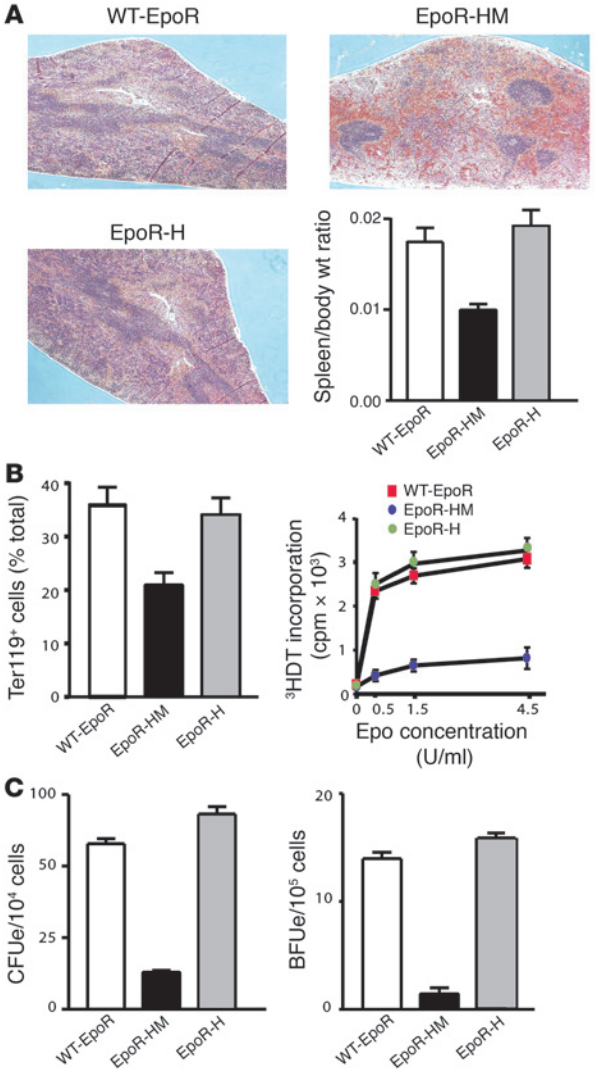

Figure 3
Aberrant splenic architecture and CFUe and BFUe formation in phenylhydrazine-treated EpoR-HM mice, and rescue of extramedullary erythropoiesis by EpoR-H. (**A**) At day 4 after phenylhydrazine treatment, spleens from EpoR-HM, EpoR-H, and WT-EpoR mice were fixed, sectioned, stained (H&E), and examined for white and red pulp architecture. Histomorphologies are representative of 4 mice per group (and 20 sections per mouse) and are presented at ×40 magnification. Also illustrated are EpoR-HM–specific deficiencies in splenomegaly. (**B**) EpoR-HM–specific defects in splenic Ter119$^+$ erythroblast formation and Epo-dependent proliferation, and rescue by EpoR-H. Frequencies of Ter119$^+$ erythroblasts in splenocyte preparations from phenylhydrazine-treated WT-EpoR, EpoR-HM, and EpoR-H mice are shown. Also graphed are rates of Epo-induced ^{3}HdT incorporation for splenic erythroid progenitor cells (prepared at day 3). (**C**) At day 2 after phenylhydrazine treatment, frequencies of splenic CFUe and BFUe were determined. For each analysis, mean values ± SEM are illustrated (n = 3 mice per group). Results are representative of 3 independent experiments.

Factors underlying defective erythropoiesis in EpoR-HM mice during hemolytic anemia were considered further based on spleen histomorphologies, and erythroid progenitor cell formation (Figure 3). Spleen architectures in phenylhydrazine-treated EpoR-HM mice were aberrant as compared with those in congenic controls. This included broad regions of apparently rapidly maturing eosinpositive erythroblasts, and abnormal retention of white pulp (Figure 3A). Signals relayed via PY343 within EpoR-H restored both WT spleen architecture and efficient splenomegaly. Flow cytometric analyses of EpoR-HM splenocytes further revealed decreased levels of Ter119$^+$ cells, and assays of Epo-dependent ^{3}HdT incorporation indicated limited proliferative responsiveness (Figure 3B). In contrast, each response in EpoR-H erythroblasts was essentially normal. Furthermore, CFUe and BFUe pools were markedly decreased in EpoR-HM spleens, while frequencies in EpoR-H spleens approximated those in WT-EpoR controls (Figure 3C). Core signals relayed by EpoR-HM and Jak2 therefore apparently fail at a relatively early progenitor cell level to effectively support extramedullary stress erythropoiesis (despite high-level Epo production in EpoR-HM mice). This overall process, however, is efficiently rescued via EpoR-H PY343–propagated signals.

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 versus bone marrow erythropoiesis have been described recently (26–28). In addition, spleen contains a discrete progenitor pool that rapidly expands during stress erythropoiesis (26). Efforts therefore were directed toward studies of EpoR-HM and EpoR-H action within adult bone marrow–derived erythroblasts. Specifically, a unique in vitro expansion system was established that supports the stepwise development of primary marrow-derived proerythroblasts (Figure 4). This involved Kit$^+$ progenitor cell isolation and expansion in SP34-EX, an SP34 serum-free medium supplemented with optimized levels of SCF, Epo, dexamethasone, β-estradiol, transferrin, and BSA. In this medium, erythroid progenitor cells developed over a 3-day course from a Kit$^+$CD71^{low} cohort, to a Kit$^+$CD71^{high} population, and further to Kit$^-$CD71^{high} late-stage erythroblasts (Figure 4A). (CD71 and CD117 correspond to the transferrin receptor and Kit, respectively). To further assess developmental capacities, expanded erythroblasts were shifted to an insulin- and Epo-containing medium. Within 40 hours, at least 80% of cells differentiated to Ter119$^+$ erythroblasts with low forward-angle light scatter (Figure 4B). Subsequently, this novel system was used to investigate stage-specific EpoR-HM, EpoR-H, and WT-EpoR bioactivities (and action mechanisms) in adult bone marrow–derived erythroblasts at both early and late developmental stages.

For ex vivo analyses, EpoR-HM, EpoR-H, and WT-EpoR erythroblasts were expanded and were isolated as Kit$^+$CD71^{high} populations either by fluorescence-activated cell sorting (FACS) or by lineage depletion and Kit$^+$ magnetic-activated cell sorting (MACS). These Kit$^+$CD71^{high} cells were observed to form colonies with CFUe-like morphologies (Figure 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 (32). In EpoR allele biosignaling analyses, Epo-dependent ^{3}HdT incorporation responses first were assessed (Figure 5B). WT-EpoR and EpoR-H Kit$^+$CD71^{high} erythroblasts proved to be highly Epo-responsive. EpoR-HM cells, in contrast, exhibited an at least 30-fold defect in Epo-induced ^{3}HdT incorporation rates (and this defect was not reversed by exposure to Epo at concentrations up to 5 U Epo/ml; data not shown). Response profiles are represen-

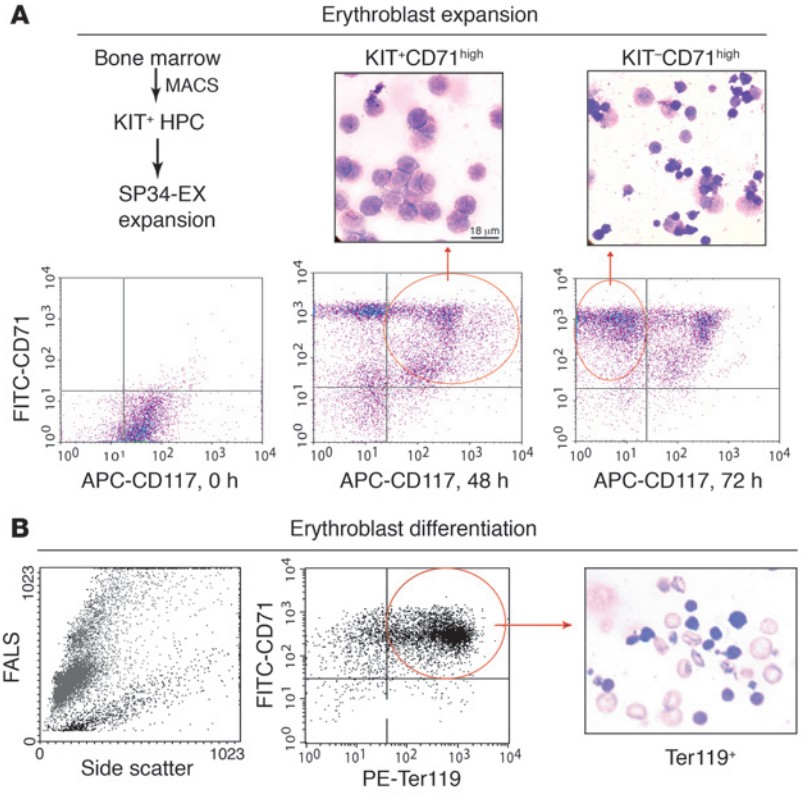

Figure 4
In vitro expansion system for analyses of primary adult bone marrow–derived erythroblast development. (**A**) In the system outlined, hematopoietic progenitor cells (HPCs) were isolated (here, from WT control bone marrow preparations) as Kit+ cells, and expanded in cytokine- and dexamethasone-supplemented SP34-EX. During expansions, CD117 (Kit) and CD71 (transferrin receptor) expression was monitored (lower panels). Morphologies of expanded and FACS-isolated Kit+CD71high and Kit–CD71high erythroblasts also were analyzed and are presented at ×400 magnification. MACS, magnetic-activated cell sorting; APC, allophycocyanin. (**B**) Differentiation capacities of expanded erythroblasts were assessed based on forward-angle light scatter (FALS), Ter119 and CD71 marker expression, and morphologies after transfer to a medium containing insulin, Epo, and transferrin. Magnification, ×1000.

tative of 3 independent experiments, and significance testing of mean response values at 0.1 U/ml confirmed significant differences between EpoR-HM and WT-EpoR or EpoR-H erythroblasts ($P < 0.001$) (as well as between WT-EpoR and EpoR-H erythroblasts, $P < 0.05$). In these erythroblast populations, and in Kit–CD71high erythroblasts, apoptosis also was analyzed. Annexin V staining revealed at least 3-fold increases in apoptosis for EpoR-HM erythroblasts in each population as compared directly with WT-EpoR and EpoR-H erythroblasts (Figure 5C).

The ability of EpoR-H and EpoR-HM Kit+CD71high erythroblasts to support Epo induction of candidate Epo and Stat5 response genes was next studied. This included *Pim-1*, *oncostatin-M, SOCS-3*, and Bcl2-like 1 (*Bcl-x*). For comparison, *Pim-2, Gas6* (27), and *EDR* (erythroid differentiation regulator) (33) 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 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 3 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 (28, 34, 35). Here (as for Epo-induced rates of ^{3}HdT incorporation — see above), enhanced responses for EpoR-H may reflect an absence of inhibitory motifs within EpoR distal cytoplasmic domains (31). Unexpectedly, *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 1). During anemia, erythroid progenitor cells therefore appear to be a significant source of *oncostatin-M*.

With further regard to Pim-1, Pim kinases recently have been shown to modulate eIF4E, and to regulate hematopoietic cell survival (36–38). Possible contributions of Pim-1 to Kit+CD71high erythroblast survival therefore were tested indirectly, but functionally, based on possible differential sensitivity of EpoR-H versus EpoR-HM erythroblasts to rapamycin (an mTOR inhibitor). Based on annexin V staining, EpoR-HM cells reproducibly exhibited a more than 8-fold increase in rapamycin sensitivity (Table 2). This difference may reflect increased dependence of EpoR-HM on mTOR activation in the absence of Pim-1's effects on eIF4E (36–38).

Integration of Kit and EpoR-PY343 signals during early erythroblast formation. Kit supports stress erythropoiesis and can act in synergy with the EpoR (39, 40). Recent cell line studies of PY-mutated human EGFR/mouse EpoR chimeras have further suggested that Kit's signals might selectively integrate with EpoR-PY343 signals (41). This concept was tested in primary erythroblasts

Table 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.

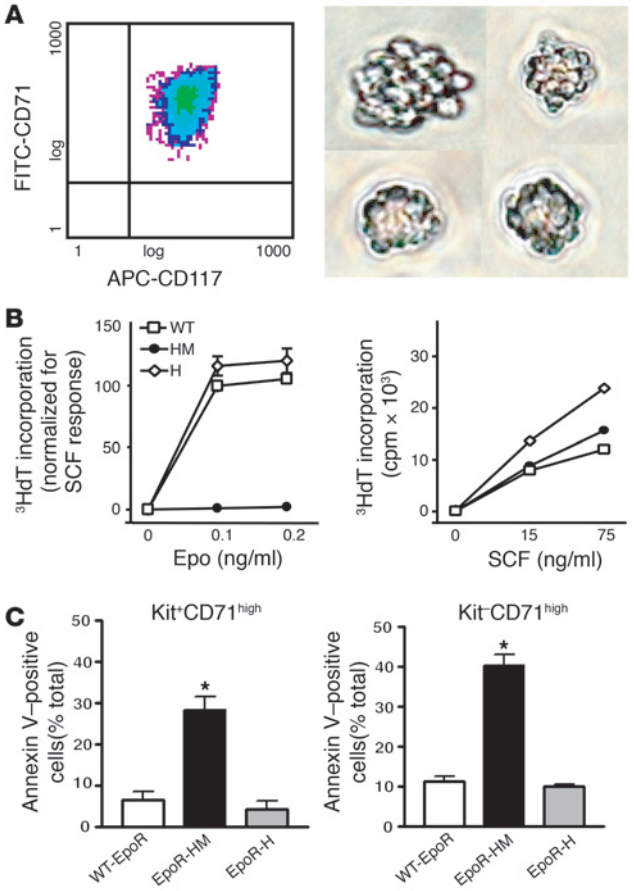

Figure 5
At a Kit$^+$CD71^{high} stage, EpoR-HM erythroblasts exhibit proliferation and survival defects that are corrected by EpoR-H PY343 signals. (**A**) Erythroid progenitor cells from WT-EpoR mice were expanded in SP34-EX. At 72 hours of culture, Kit$^+$CD71^{high} erythroblasts were isolated by FACS and plated in methylcellulose. Colonies with CFUe morphologies uniformly formed as shown in the right panels (and as confirmed by benzidine staining, not shown). Magnification, ×250. (**B**) Isolated Kit$^+$CD71^{high} WT-EpoR, EpoR-HM, and EpoR-H erythroblasts were cultured in SP34-EX 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 2 independent experiments (n = 3 WT-EpoR, EpoR-HM, and EpoR-H mice per experiment). (**C**) For Kit$^+$CD71^{high} and Kit$^-$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 (*P < 0.01 vs. WT-EpoR and EpoR-H).

expanded from EpoR-HM, EpoR-H, and WT-EpoR mice initially by the decreasing of SCF levels to physiologic concentrations, and assessment of associated effects on Kit$^+$CD71^{high} and Kit$^-$CD71^{high} erythroblast formation. Interestingly, and specifically for EpoR-HM progenitor cells, this resulted in deficient progression of Kit$^+$ cells to a Kit$^-$CD71^{high} stage (Figure 7A). In particular, when SCF dosing was decreased from 50 to 5 ng/ml, WT-EpoR and EpoR-H erythroblasts continued to progress from a Kit$^+$CD71^{high} stage to a Kit$^-$CD71^{high} stage (59% to 70% and 57% to 80% progression, respectively). For EpoR-HM erythroblasts, this event faltered, and frequencies of Kit$^-$CD71^{high} erythroblasts decreased when SCF was limited (i.e., from 22% to 10%). Results (representative of 3 independent experiments) therefore indicate important roles for EpoR-PY343 (and Stat5) signals in Kit-mediated proerythroblast expansion, especially beyond a Kit$^+$CD71^{high} stage. Direct cell counts further revealed defects in the capacities of EpoR-HM progenitor cells to expand in an SCF dose–dependent context (Figure 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 (42, 43). In bone marrow–derived primary erythroblasts, Epo efficiently activated Stat5 but did not detectably activate Stat1 or Stat3 (Figure 7C). In contrast, SCF efficiently activated Stat1 but did not detectably activate Stat5 or Stat3. (SCF, however, was observed to induce reactivity of a 140,000-M_r protein with the anti–PY694-Stat5 antibody used in these studies.)

Possible SCF/Kit modulation of EpoR-PY343-Stat5 response genes also was assessed. This involved assaying of the combined effects of SCF plus Epo exposure on *oncostatin-M*, *Pim-1,* and *SOCS-3* expression in expanded and purified WT-EpoR Kit$^+$CD71^{high} erythroblasts (Figure 7D). SCF per se did not significantly modulate these transcripts. In the presence of Epo, however, SCF selectively upmodulated *oncostatin-M* expression but downmodulated *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 (data not shown).

Pim-1 recently has been reported to be regulated via posttranslational stabilization (44). Whether SCF might affect Epo-dependent levels of Pim-1 protein expression therefore was also assessed using isolated Kit$^+$CD71^{high} erythroblasts (Figure 8). Interestingly, Epo-dependent Pim-1 expression proved to be selectively reinforced by SCF (Figure 8A). Furthermore, SCF/Kit enhancement of Pim-1 expression was supported by EpoR-H but not by EpoR-HM erythroblasts (Figure 8B). EpoR-PY343-Stat5 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 that previously has been suggested to be synergistically regulated by SCF plus Epo (45).

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 or absence of oncostatin-M (10 ng/ml) and in the presence of Epo (2.5 U/ml, nonlimiting concentration), and SCF at nonlimiting (100 ng/ml) or limiting concentrations

Table 2
EpoR-HM erythroblasts exhibit increased sensitivity to rapamycin

	Rapamycin-induced annexin V positivity (% over background)	
	Kit$^-$CD71^{high}	Kit$^+$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.

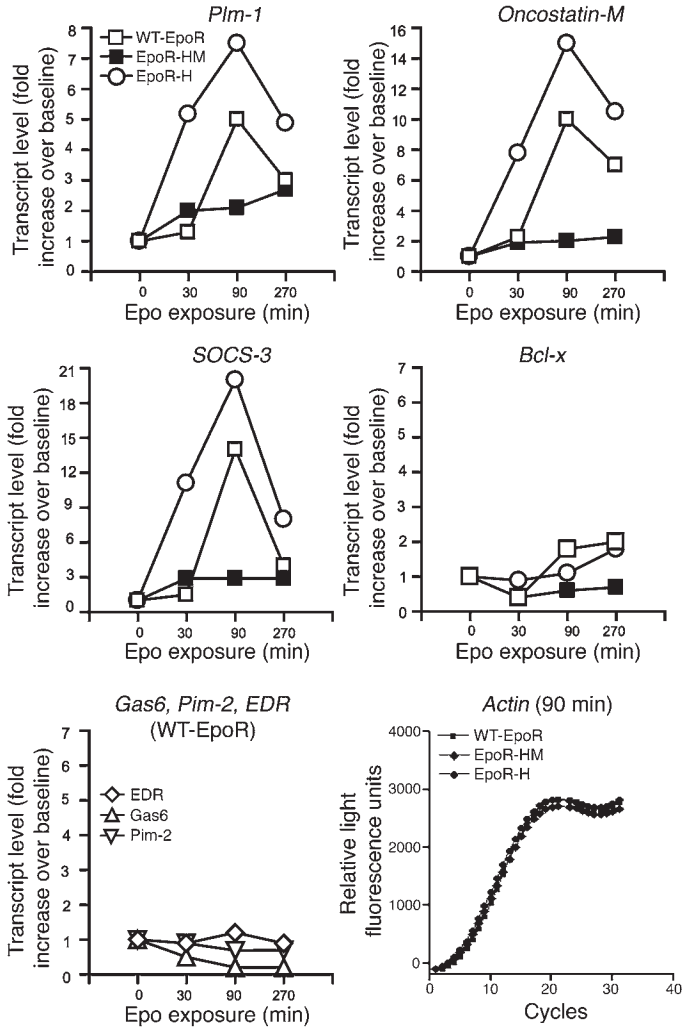

Figure 6
EpoR-PY343 signals support Epo induction of *Pim-1*, *oncostatin-M,* and *SOCS-3*, but not *Bcl-x*. Kit^{+}CD71^{high} erythroblasts were isolated from expanded WT-EpoR, EpoR-HM, and EpoR-H marrow preparations, and cultured for 6 hours in 0.5% BSA, 10 ng/ml insulin, and 0.1 mM 2-mercaptoethanol in IMDM. At 0, 30, 90, and 270 minutes of subsequent Epo exposure (1 U/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-0 base lines). In all RT-PCR reactions, *actin* was used as an internal control.

(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., at least 10-fold decreases in annexin V positivity due to oncostatin-M) primarily among late-stage Kit^{-}CD71^{high} erythroblasts. In EpoR-HM erythroblasts, however, this oncostatin-M effect was not exerted in the specific absence of EpoR-PY343 signals (Figure 9A). 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 3). Oncostatin-M proved to specifically enhance survival of WT-EpoR Kit^{-}CD71^{high} cells (on average) to approximately 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 versus EpoR-HM erythroblasts involves oncostatin-M receptor expression (46). *Oncostatin-M receptor* β levels therefore were analyzed in developing EpoR-H and EpoR-HM erythroblasts. *Oncostatin-M receptor* β was expressed at all stages, however, and was only modestly upregulated in EpoR-H erythroblasts at a late Kit^{-}CD71^{high} stage (Figure 9A). Finally, Stat activation by oncostatin-M also was analyzed in CD71^{high} WT erythroblasts. Oncostatin-M proved to efficiently stimulate Stat1 and Stat3, but not Stat5 (Figure 9B). EpoR plus oncostatin-M effects therefore might involve the combined actions of Stat5 plus Stat1 and/or Stat3.

Discussion

The presently defined requirement for EpoR-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-Stat5 axis during anemia. Below, these questions are considered in the contexts of Stat activation; the nature of Epo-regulated Stat5 response genes in primary erythroblasts; and apparent roles for EpoR-PY343-Stat5 signals in reinforcing Kit and oncostatin-M receptor action. In Figure 9C, proposed overall roles for EpoR-PY343-Stat5 signaling during Epo, SCF, and oncostatin-M receptor activation are outlined.

In the WT-EpoR, PY343 is a major Stat5-binding site (22, 47). 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 EpoR-HM signaling capacities is an ability versus inability to activate Stat5 (and downstream events). For these EpoR alleles, this assumes comparable Jak2 activation kinetics (as well as similar Stat5 activation profiles for EpoR-H and the WT-EpoR). Importantly, in control experiments these basic assumptions were quantitatively confirmed (see Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI25227DS1). Previously, Stat1 and/or Stat3 also have been implicated in EpoR signaling (42), and in *Stat1*$^{-/-}$ BFUe, Epo and SCF responsiveness is attenuated (48). In the present primary erythroblast system, however, little or no Epo activation of Stat1 or Stat3 was detected. This discounts (but does not rule out) roles for these Stats in EpoR signaling.

If Stat5 is a major mediator of EpoR-H PY343–dependent stress erythropoiesis, then faltered erythroid phenotypes might also be predicted to be exhibited by *Stat5a,b*$^{-/-}$ mice. In Stat5-deficient mice, phenylhydrazine-induced splenic erythropoiesis, in fact, is compromised (30). Certain *Stat5a,b*$^{-/-}$ phenotypes, however, are controversial. Embryonic anemia has been reported, for example, in one study (23) but not another (25). Spontaneous erythrosplenomegaly also commonly occurs in adult *Stat5a,b*$^{-/-}$ mice and can be accompanied by up to 100-fold increases in Epo production

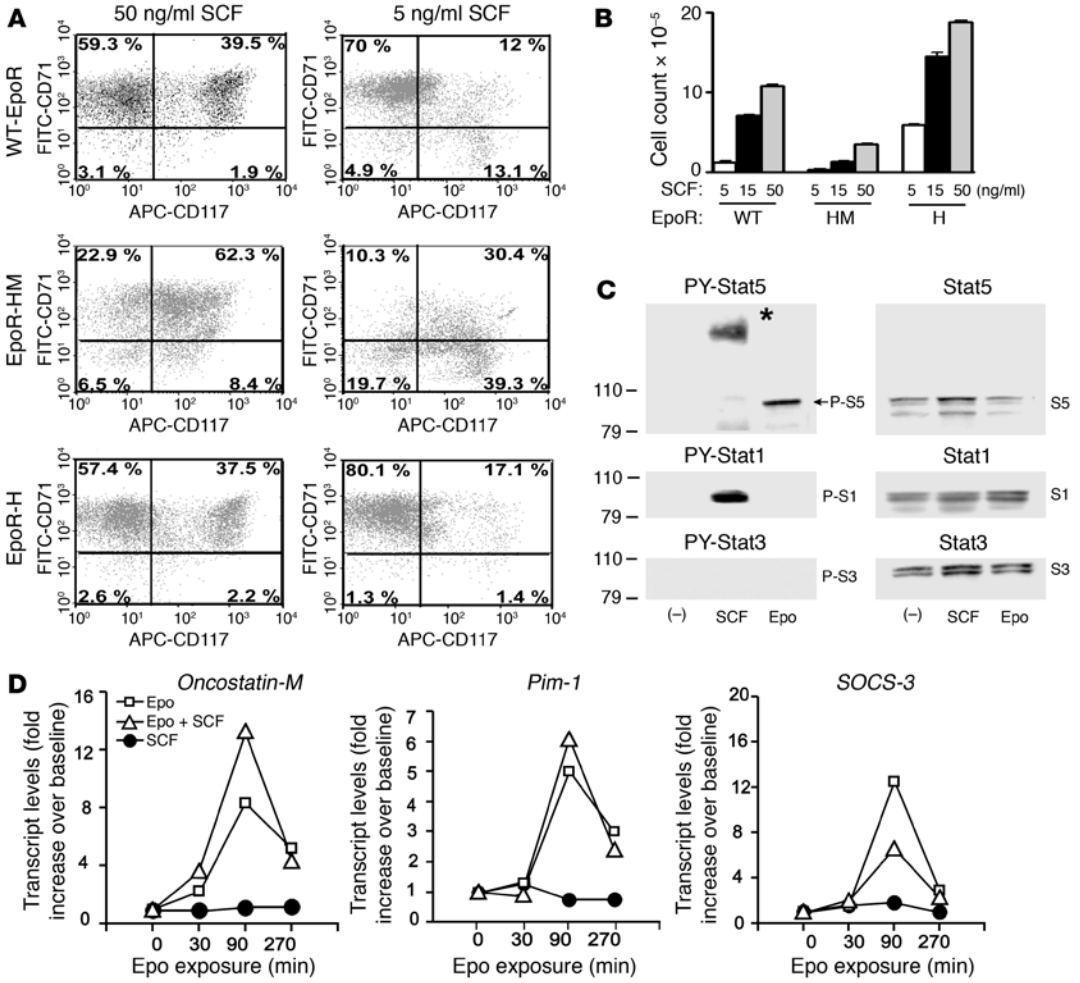

Figure 7

SCF-dependent erythroblast expansion involves EpoR-PY343 signals and is associated with SCF modulation of Epo-dependent *oncostatin-M* and *SOCS-3* expression. (**A**) To test roles for EpoR-PY343 signals in a context of Kit action, capacities of EpoR-HM, EpoR-H, and WT-EpoR (pro)erythroblasts to expand in limited SCF doses were assessed via CD117 (Kit) and CD71 marker expression assays. In 5 ng/ml SCF, EpoR-HM erythroblasts accumulated at a $Kit^{+}CD71^{high}$ stage and exhibited defective development to $Kit^{-}CD71^{high}$ cells (left panels). At 50 ng/ml SCF, $Kit^{+}CD71^{high}$ cell development proceeded, but deficits in $Kit^{-}CD71^{high}$ cell formation persisted. Upon EpoR-PY343 site restoration, these defects were corrected. APC, allophycocyanin. (**B**) SCF-dependent EpoR-HM, EpoR-H, and WT-EpoR erythroblast expansion also was assayed via direct cell counts (means ± SEM). Data are representative of 2 mice per EpoR allele and 3 independent experiments. (**C**) In WT-EpoR Kit^{+} erythroblasts, Stat activation by SCF or Epo was assessed. Cells were deprived of cytokines and exposed to SCF (100 ng/ml) or Epo (2.5 U/ml). After 10 minutes, phospho- (and total) Stat5, Stat1, and Stat3 levels were assayed by Western blotting (P-S, phospho-Stat; S, Stat). The asterisk indicates an anti–PY-Stat5–reactive, 140-kD phosphoprotein. (**D**) Kit signals selectively modulate EpoR-PY343–dependent *oncostatin-M* and *SOCS-3* expression. Cells were expanded from WT-EpoR marrow and isolated as $Kit^{+}CD71^{high}$ erythroblasts. Cytokines were withdrawn, and erythroblasts were then exposed to Epo (0.75 U/ml) and/or SCF (75 ng/ml). Induced levels of *oncostatin-M*, *Pim-1,* and *SOCS-3* transcripts were determined by quantitative RT-PCR. Note selective SCF upmodulation of *oncostatin-M* and downmodulation of *SOCS-3*.

(30). This phenotype, however, is suppressed within a recombination-activating gene 2–deficient background (25). In EpoR-HM mice, no such embryonic anemia, spontaneous splenomegaly, or elevated base-line Epo levels were observed. In addition to these differences, we recently have discovered that the exon deletion strategy used to knock out Stat5 alleles appears to result in substantial expression of N-terminal truncated Stat5 with potential signaling activities (unpublished observations). The present minimal EpoR-H and EpoR-HM allele model avoids these potential complications. In EpoR-HM mice, if low-level Epo activation of Stat5 were to occur, this might contribute to the ability of EpoR-HM mice to support steady-state erythropoiesis (25). In repeated sensitive assays of Stat5 and Stat5 target gene activation, however, EpoR-HM failed to detectably stimulate Stat5. This includes analyses of Epo induction of a well-characterized Stat5 target gene, Cis (49), which was induced approximately 25-fold via EpoR-H and the WT-EpoR, but not by EpoR-HM (see Supplemental Figure 1C).

The case that EpoR-PY343-Stat5 signals are critical for stress erythropoiesis raises questions concerning the specific nature of key Stat5-regulated genes within developing erythroblasts.

Presently, 4 additional genes that have been indicated in cell line models and/or nonerythroid cells as Epo and Stat5 response

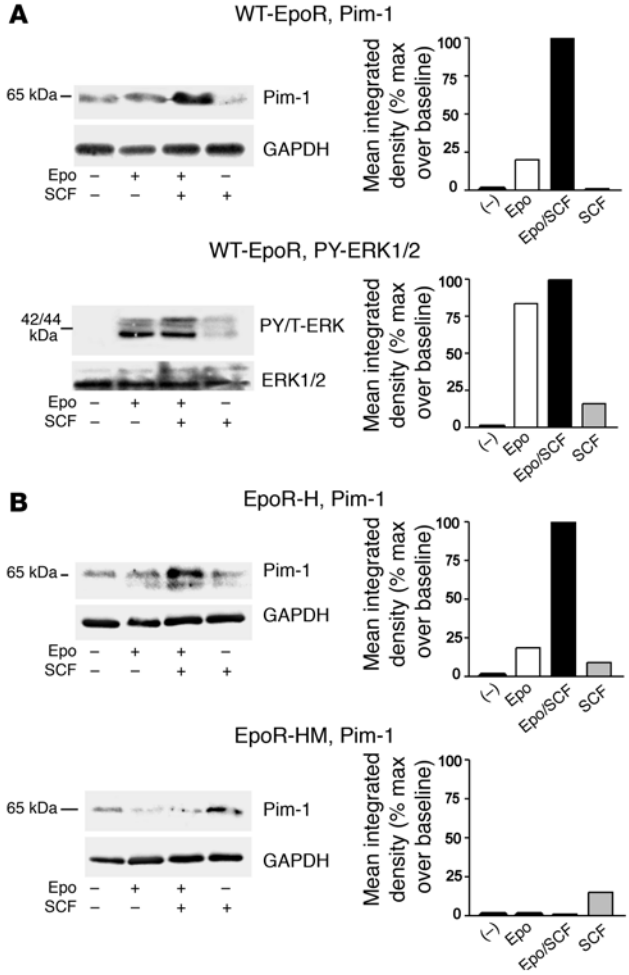

Figure 8
Kit signals enhance EpoR PY343–dependent Pim-1 protein expression. (**A**) Erythroid progenitor cells were expanded from WT-EpoR bone marrow preparations, and Kit$^+$CD71^{high} erythroblasts were isolated. Cytokines were withdrawn for 6 hours, and cells then were exposed to Epo (0.75 U/ml), SCF (75 ng/ml), or both factors for 90 minutes. Pim-1 protein levels then were assayed by Western blotting (upper panel). Note the multifold effect of SCF on Epo-dependent Pim-1 expression. In analyses of SCF plus Epo regulation of ERKs (lower panel), no such synergistic effects were observed. (**B**) SCF enhancement of Epo-induced Pim-1 expression in EpoR-H but not in EpoR-HM erythroblasts. Kit$^+$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 in EpoR-HM erythroblasts. max, maximum.

genes were analyzed, i.e., *Pim-1* (34), *oncostatin-M* (50), *SOCS-3* (51), and *Bcl-x* (23). For *Bcl-x*, failed reticulocyte formation due to conditional gene disruption illustrates Bcl-x_L's role as an important erythroblast survival factor (52). In addition, Stat5 occupancy of a consensus element within intron 1 of the *Bcl-x* gene also has been described in HCD-57 cells (23). In the present primary erythroblast system, *Bcl-x* transcription unexpectedly (but reproducibly) was not modulated significantly via WT-EpoR, EpoR-H, or EpoR-HM alleles. Given Epo's role as an erythroblast survival factor, this outcome emphasizes the potential importance of alternate candidate EpoR-PY343-Stat5 target genes. In particular, *Pim-1*, *oncostatin-M,* and *SOCS-3* each were observed to be strongly induced in Kit$^+$CD71^{high} erythroblasts via an EpoR-PY343-Stat5 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 (36). In addition, Pim kinases can confer resistance to rapamycin (an mTOR inhibitor) and can act in parallel with mTOR to modulate eIF4E (36–38). 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 macrophages (50). 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 (28). Finally, SOCS factors (including SOCS-3) commonly are associated with receptor kinase inhibition, and degradation (14). SOCS-3, however, may also affect cell survival by sequestering GTPase-activating protein via a unique SOCS-box domain PY site (35).

Our 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, 3 observations are consistent with an exertion of net negative effects: (a) lower levels of Epo production in phenylhydrazine-treated EpoR-H versus WT-EpoR mice; (b) elevated ^{3}HdT response profiles for EpoR-H erythroblasts; and (c) 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 coworkers (8) in patients, and in mice expressing EpoR carboxyl-terminal truncation mutants. With regard to mechanisms that the PY-null allele EpoR-HM may use to support steady-state erythropoiesis, we recently have observed selective retention by EpoR-HM of MEK1/2 and ERK1/2 signaling (unpublished observations). The extent to which ERK signaling might be necessary and sufficient for EpoR-HM biofunction, however, is presently uncertain.

As illustrated by macrocytic anemia in *Kit*$^{w/wv}$ mice (53), Kit receptor tyrosine kinase signals also are important for stress erythropoiesis, and Kit is known to act in synergy with the EpoR. One cell line model for synergy, in fact, involves EpoR *trans*-phosphorylation by Kit (39, 40). 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 downmodulate SOCS-3's suppressive effects

Table 3
WT-EpoR erythroblasts exhibit increased survival in response to oncostatin-M

	Oncostatin-M–dependent increases in WT-EpoR erythroblast survival	
	Kit$^-$CD71^{high}	Kit$^+$CD71^{high}
Experiment 1	56% ± 9.3%	29% ± 6.7%
Experiment 2	31% ± 5.1%	19% ± 4.3%

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

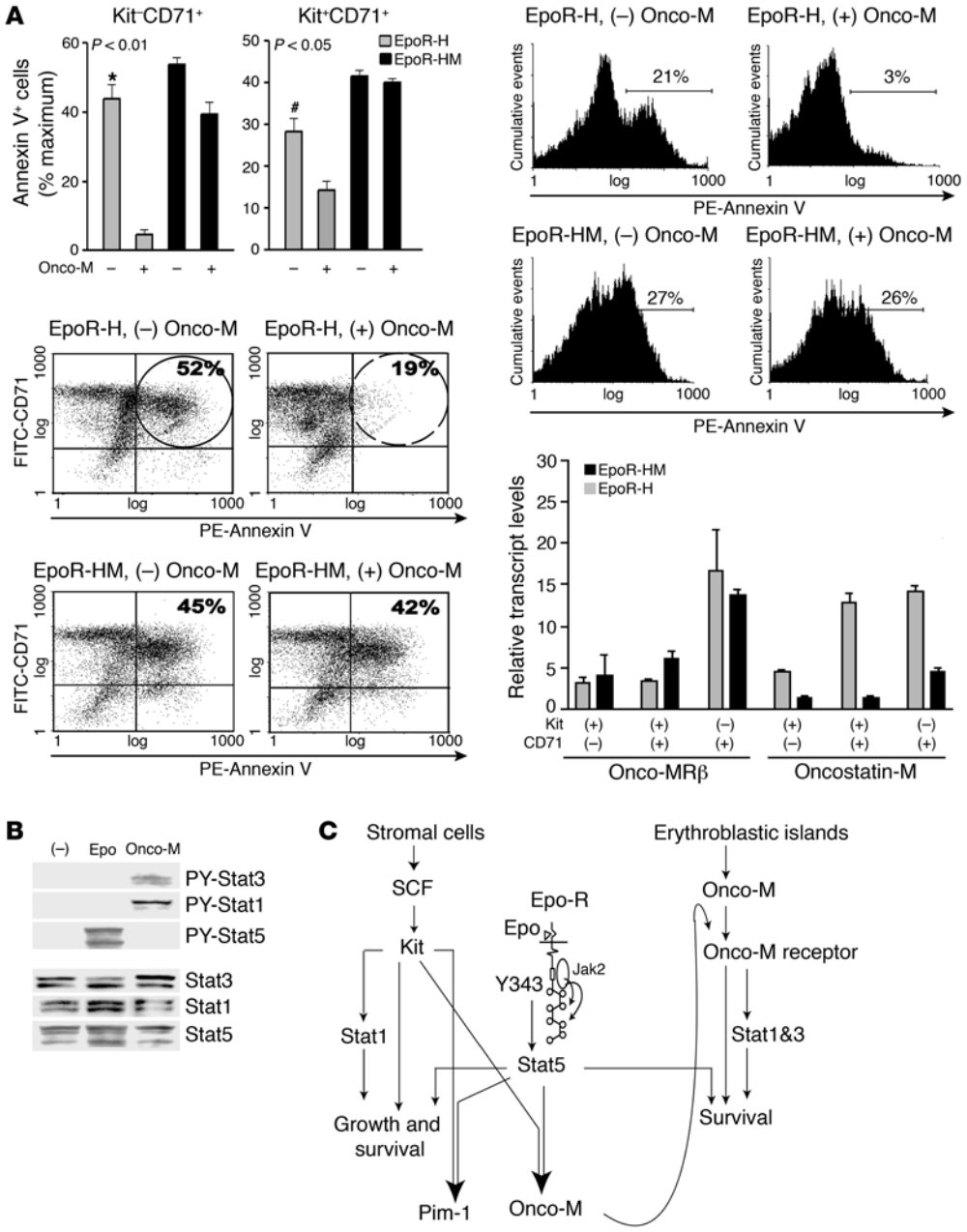

Figure 9

Oncostatin-M promotes the survival of Kit^{-}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 in Epo (2.5 U/ml) and SCF (5 ng/ml) in the presence or absence of oncostatin-M (onco-M) at 10 ng/ml. At 72 hours of culture, frequencies of annexin V–positive cells among Kit^{-}CD71^{high} and Kit^{+}CD71^{high} erythroblasts were determined. Note the substantial survival effect of oncostatin-M selectively on EpoR-H Kit^{-}CD71^{high} erythroblasts (upper left panels) ($^{*}P < 0.01$ vs. EpoR-H with oncostatin-M; $^{\#}P < 0.05$ vs. EpoR-HM with oncostatin-M). 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^{+}CD71^{low}, Kit^{+}CD71^{high}, and Kit^{-}CD71^{high} subpopulations also were isolated by FACS, and lysed in TRIzol. Levels of *oncostatin-M receptor* β (*onco-MR*β) (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 Stat3 and Stat1 in CD71^{high} erythroblasts. WT-EpoR CD71^{high} erythroblasts were isolated from expansion cultures. Erythroblasts then were deprived of cytokines for 6 hours and exposed to Epo (2.5 U/ml) or oncostatin-M (10 ng/ml). At 10 minutes of exposure, lysates were prepared, and levels of activated (PY) and total Stat3, Stat1, and Stat5 were analyzed by Western blotting. (**C**) A model is outlined in which EpoR-PY343 (and Stat5) 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.

on EpoR signaling (51). 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$^-$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 (44). 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 that otherwise supports red cell formation at steady state (25). This includes clear in vivo erythropoietic defects in 3 anemia models. The rescue of efficient stress erythropoiesis via restoration of a single PY343 Stat5-binding site furthermore distinguishes between steady-state and stress-induced red cell formation at the level of EpoR action. Mechanistically, Pim-1 and oncostatin-M (but not Bcl-x_L) are proposed to constitute important EpoR-PY343-Stat5–induced genes and erythroblast survival factors. An EpoR-PY343-Stat5 signaling axis also is suggested to assist in the integration of additional survival signals relayed via both SCF and oncostatin-M receptors. These findings advance insight into core EpoR action mechanisms (3), add to our knowledge of Stat5 action (23, 30, 41, 54), and also may shed light on Epo-regulated cytoprotection in nonhematopoietic tissues (10).

Methods

Mice and anemia models. Mice with knocked-in EpoR-HM and EpoR-H alleles (and congenic controls; Jackson Laboratory, stock no. 1009003) were as described previously (25, 29). Each group was maintained as a C57BL/6-129 line, and mice were used at 8–12 weeks. Phenylhydrazine (60 mg/kg) was administered s.c. at 1 and 24 hours. 5-Fluorouracil (150 mg/kg) was administered i.p. In transplantations, bone marrow cells were depleted of T cells (CD90.2 negative selection) and injected (1×10^6 cells) into lethally irradiated Ly5.1 B6.SJL recipients (Jackson Laboratory, stock no. 002014). Hematocrits and reticulocyte levels were assayed by capillary microcentrifugation and flow cytometry. Spleens were paraformaldehyde-fixed, paraffin-embedded, sectioned (4 μm), and stained with H&E. All procedures were ethical and approved by the Institutional Animal Care and Use Committee of the Maine Medical Center Research Institute.

Erythroid progenitor cell preparation and culture. Splenocytes from phenylhydrazine-treated mice were disrupted, passed through 40-μm mesh, exposed to NH_4Cl (StemCell Technologies), and collected through 50% FBS in PBS (Invitrogen Corp., no. 14190-144). In BFUe and CFUe assays, splenocytes were plated in triplicate (1.2×10^5 and 3.5×10^5 cells/ml). Hemoglobinized colonies were benzidine-stained and scored. Marrow cells were prepared as described previously (29). Kit$^+$ cells were isolated using CD117 microbeads (Miltenyi Biotec) and cultured (7.5×10^5 cells/ml) in StemPro-34 medium (Invitrogen Corp.) supplemented with 2.5 U/ml Epo, 100 ng/ml mouse SCF (PeproTech), 1 μM dexamethasone, 1 μM β-estradiol, 75 μg/ml human transferrin, 1% BSA (StemCell Technologies), and 0.1 mM 2-mercaptoethanol (SP34-EX). At 24 hours, 0.6 volumes of SP34-EX was added. At 48 hours, cells were replated (7.5×10^5 cells/ml) in 0.8 volumes of new and 0.2 volumes of residual conditioned media. In differentiation experiments, expanded erythroblasts were transferred to Iscove's modified Dulbecco's medium (IMDM; Invitrogen Corp., no. 12440-053) containing Epo (2.5 U/ml), transferrin (150 μg/ml), insulin (10 μg/ml), 0.1 mM 2-mercaptoethanol, and 10% FBS. Slide centrifugation (1×10^5 cells) was for 15 minutes (100 *g*; Hettich AG). Staining was with Dip Stain reagent (Volu-Sol Inc.).

FACS and MACS. FACS of Kit$^+$CD71^{high} and Kit$^-$CD71^{high} erythroid progenitor cells involved allophycocyanin-CD117 plus FITC-CD71 sorting (FACSVantage; BD). Alternatively, magnetic-activated cell sorting (MACS) was performed via lin$^+$ cell depletion (StemCell Technologies, no. 19756) plus Kit$^+$ selection (Miltenyi Biotec, no. 130-091-224). Subpopulation purities were at least 95%.

Flow cytometry and ^{3}HdT incorporation assays. In flow cytometry (FACSCalibur; BD), washed cells (1×10^6 per 0.2 ml PBS, 0.1% BSA) were incubated with rat IgG (1 μg), and stained with 1 μg of allophycocyanin-CD117 plus PE-Ter119 and/or FITC-CD71, FITC-Mac1, or FITC-B220 (BD Biosciences). Allophycocyanin-Ly5.2 and -Ly5.1 antibodies were from eBioscience. Annexin V binding (BD Biosciences) was in 140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES (pH 7.4). In ^{3}HdT incorporation assays, expanded erythroblasts were plated in SP34-EX (1×10^5 cells/ml) with the indicated cytokines. Splenocytes were plated at 5×10^6 cells/ml. At 20 hours, 1 μCi of ^{3}HdT was added (29), and incorporation rates (per 5 hours) were determined.

RT-PCR and Western blotting. RNA was prepared using TRIzol reagent (Invitrogen Corp.). cDNA was prepared with SuperScript III (Invitrogen Corp.). Quantitative PCR (iCycler) used SYBR Green reagents (Bio-Rad Laboratories) (for primer pairs, see supplemental material; available online with this article; doi:10.1172/JCI25227DS1). For Western blotting, erythroblasts were incubated for 6 hours in 0.5% BSA, 10 μg/ml transferrin, 10 ng/ml insulin, and 0.1 mM 2-mercaptoethanol in IMDM (8×10^5 cells/ml) and exposed to Epo, SCF, or oncostatin-M as indicated. Cells were then combined with 4 volumes of 2°C PBS, collected, and lysed in 1% Igepal, 150 mM NaCl, 50 mM NaF, 2 mM Na_2EDTA, 10 mM sodium pyruvate, 25 mM β-glycerol phosphate, 10% glycerol, 1 mM DTT, 50 mM HEPES (pH 7.5) supplemented with 0.25 mg/ml phenylmethylsulfonylfluoride, 1× protease inhibitor, and 1× phosphatase inhibitor cocktails (Sigma-Aldrich, nos. P8340 and P5726) (0.15 ml lysis solution per 1×10^7 cells). An equal volume of 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 112.5 mM NaCl, 37.5 mM Tris-HCl (pH 7.4) then was added, and cleared extracts were denatured, electrophoresed (25 μg), and blotted. In chemiluminescence, HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) and SuperSignal West Dura reagent (Pierce) were used. For the sources of the primary antibodies, see the supplemental material (available online with this article; doi:10.1172/JCI25227DS1).

Statistics. Statistical tests used for comparing means include 2-tailed Student's *t* test and ANOVA (for F-distribution testing of differences in means among 3 or more treatment groups). A *P* value of less than 0.05 was used to define significance.

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