

Erythropoietin critically regulates the terminal maturation of murine and human primitive erythroblasts

Jeffrey Malik,^{1,2} Ah Ram Kim,^{1,3} Kaitlin A. Tyre,¹ Anjuli R. Cherukuri,¹ and James Palis¹

¹Center for Pediatric Biomedical Research, Department of Pediatrics; ²Department of Pathology and Laboratory Medicine; and ³Department of Biomedical Genetics, University of Rochester Medical Center, Rochester, NY, USA

ABSTRACT

Primitive erythroid cells, the first red blood cells produced in the mammalian embryo, are necessary for embryonic survival. Erythropoietin and its receptor EpoR, are absolutely required for survival of late-stage definitive erythroid progenitors in the fetal liver and adult bone marrow. *Epo*- and *Epor*-null mice die at E13.5 with a lack of definitive erythrocytes. However, the persistence of circulating primitive erythroblasts raises questions about the role of erythropoietin/EpoR in primitive erythropoiesis. Using *Epor*-null mice and a novel primitive erythroid 2-step culture we found that erythropoietin is not necessary for specification of primitive erythroid progenitors. However, *Epor*-null embryos develop a progressive, profound anemia by E12.5 as primitive erythroblasts mature as a synchronous cohort. This anemia results from reduced primitive erythroblast proliferation associated with increased p27 expression, from advanced cellular maturation, and from markedly elevated rates of apoptosis associated with an imbalance in pro- and anti-apoptotic gene expression. Both mouse and human primitive erythroblasts cultured without erythropoietin also undergo accelerated maturation and apoptosis at later stages of maturation. We conclude that erythropoietin plays an evolutionarily conserved role in promoting the proliferation, survival, and appropriate timing of terminal maturation of primitive erythroid precursors.

Introduction

Primitive erythroid cells constitute a distinct lineage responsible for the first red blood cells circulating in the vertebrate embryo. Studies in the mouse indicate that this lineage emerges in the E7.5 – E9.0 yolk-sac as a transient wave of unipotential progenitors (EryP-CFC)¹ that generates a cohort of primitive erythroblasts that mature semi-synchronously in the fetal circulation, ultimately enucleating between E12.5 – E16.5 to form primitive erythrocytes.^{2,3} While primitive erythropoiesis is short-lived, it is absolutely necessary for survival of the murine embryo, since *Gata1*-null mouse embryos die by E10.5, with specific failure to mature primitive erythroblasts beyond the proerythroblast stage.⁴ Primitive erythropoiesis is superseded by definitive erythroid lineages that mature in the fetal liver⁵ and begin to enter the bloodstream of the mouse embryo as erythrocytes between E11.5 – E12.5.⁶

Signaling by erythropoietin (EPO), through its receptor EpoR, is necessary for the production of definitive erythrocytes in the murine fetal liver. Mice with targeted disruption of *Epo* or *Epor* die at E13.5 with a block in definitive erythropoiesis at the late progenitor (CFU-E) stage.⁷⁻⁹ Consistent with these observations, EPO has been shown to critically regulate CFU-E survival in the adult.¹⁰ Transgenic expression of EpoR in hematopoietic cells rescues the anemia and results in viable offspring, indicating that the erythroid effects of *Epor* deletion are responsible for fetal demise.¹¹ The function of EPO in primitive erythropoiesis has remained controversial. Early studies indicated that addition of EPO to gastrulating mouse

embryos failed to increase heme synthesis.¹² In contrast, EpoR transcripts were later detected during the initial emergence of primitive erythroid cells and exogenous EPO was demonstrated to increase embryonic globin gene expression in yolk sac explants,¹³ suggesting that primitive erythroid cells are capable of responding to EPO. Additionally, while reduced numbers of primitive erythroblasts have been observed in *Epor*-null embryos,^{7,9} their persistence has been widely interpreted to indicate that primitive erythropoiesis is independent of EpoR signaling.¹⁴

Here, we investigate the role of EPO in the emergence and maturation of the primitive erythroid lineage, making use of *Epor*-null mouse embryos and a novel primary primitive erythroid 2-step culture system. We find normal numbers of primitive erythroid progenitors are specified in the absence of *Epor*, while *Epor*-null embryos subsequently develop a progressive and profound anemia with a 90% loss of primitive erythroblasts by E12.5. Primitive erythroblasts deprived of EPO/EpoR have reduced rates of cell cycling and undergo apoptosis at late maturational stages. Surprisingly, *Epor*-null primitive erythroblasts that survive this genetic insult undergo accelerated cellular maturation.

Furthermore, human primitive erythroblasts also exhibit increased cell death and an accelerated maturational state when cultured in the absence of EPO. Unlike the critical role for EPO/EpoR in definitive erythroid progenitor survival, we conclude that EPO signaling is not required for survival of the immature primitive erythroid progenitors but instead plays an evolutionarily conserved role to promote the proliferation,

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Correspondence: james_palis@urmc.rochester.edu

survival, and appropriate timing of terminal primitive erythroid precursor maturation.

Methods

Mice

Swiss Webster (Taconic, Germantown, NY, USA) and *Epor*^{fl} mice⁸ were mated overnight and vaginal plugs checked the following morning. Embryos from *Epor*^{fl} mouse matings were genotyped as described previously.⁸ Erythroid cells per embryo were quantified using benzidine staining.¹⁵

EryP-CFC colony assays

EryP-CFC colonies were enumerated at five days of culture as described previously.¹ Alternatively, 0.2 mL benzidine dihydrochloride (Sigma, St Louis, MO, USA) solution (2 mg/mL in 0.5% acetic acid, 0.15% H₂O₂) was added to methylcellulose at two days and benzidine-positive cell clusters were enumerated.

Primitive erythroid 2-step culture

Cultures were initiated with trypsin- (Worthington Biochemical, Lakewood, NJ, USA) dissociated E8.5 embryos on gelatin-coated wells (Corning Inc., Corning, NY, USA) for 24 h in erythroid maturation media containing IMDM, 10% serum replacement (Invitrogen), 10% PFHM-II (Invitrogen), 2 mM glutamax, 150 μ M MTG (Sigma), 1% plasma-derived serum (Animal Technologies, Tyler, TX, USA), and 1 U/mL recombinant human EPO (Amgen, Thousand Oaks, CA, USA), unless otherwise stated. After 24 h, non-adherent cells were transferred to uncoated wells and cultured for another 2–3 days (*Online Supplementary Figure S1*).

Imaging flow cytometry analysis of erythroid cell maturation

To analyze primitive erythroblast maturation, blood cells were stained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA), MitoTracker Deep Red (Invitrogen), thiazole orange (Sigma-Aldrich), phycoerythrin (PE)-CD71, biotin-Ter119 (eBioscience, San Diego, CA, USA), and PE-Texas Red streptavidin (BD Biosciences, San Jose, CA, USA). Data were collected on the ImageStreamX and analyzed using IDEAS 4.0 (Amnis, Seattle, WA, USA).

Cell cycle analysis

E10.5 primitive erythroblasts were cultured for 90 min in maturation media containing bromodeoxyuridine (BrdU). BrdU incorporation was detected using a FITC BrdU flow kit (BD Biosciences), PE-CD71, APC-Ter119 and DAPI on a LSR-II flow cytometer (BD Biosciences, San Jose, CA, USA). To track cell divisions, primitive erythroblasts were stained with carboxyfluorescein diacetate, succinimidyl ester (CFSE; Invitrogen) and cultured with or without EPO for the remainder of the culture period. Baseline CFSE intensity was measured with an LSR-II flow cytometer 16 h after staining and every day thereafter.

Apoptosis analysis

Primitive erythroblasts were stained and analyzed for apoptotic nuclear morphology using the ImageStreamX as previously described.^{16,17}

Gene expression analysis

Quantitative real-time PCR (qPCR) was performed as described previously.¹⁸

Human primitive erythroblast culture

H1 human embryonic stem (hES) cells (WA01, WiCell Research

Institute) were maintained in mTeSR1 (Stem Cell Technologies), on matrigel- (BD Biosciences) coated wells. hES cells were differentiated to embryoid bodies as previously described¹⁹ in STEM^{diff} APEL Medium (Stem Cell Technologies) with cytokine supplementation. Primitive erythroblasts released from Day (d) 8–10 embryoid bodies were transferred to erythroid differentiation media (as described above) and cultured for six days.

Results

EPO expression is preferentially localized to the embryo proper between E7.5–E12.5 of mouse development

We previously detected EpoR expression within the yolk-sac blood islands beginning at E7.5 and increasing at E8.5,¹³ i.e. during the emergence of the first primitive erythroid cells in the murine embryo. We recently utilized flow cytometry to sort comparatively staged primitive, fetal definitive, and adult definitive erythroid precursors to compare global gene expression by Affymetrix microarray²⁰ and found that primitive erythroblasts express increasing levels of EpoR transcripts as they mature between E9.5 – E12.5 (Figure 1A), suggesting that the cells respond to EPO. Embryonic survival is dependent on EPO/EpoR and the identical phenotypes of Epo- and Epor-null embryos indicate that maternal EPO, even when exogenously augmented, does not impact the fetus,^{7–9} indicating that the murine embryo supplies its own EPO to ensure survival beyond E12.5. To determine the potential sources of EPO, we examined tissues from mouse embryos between E7.5 and E12.5 and found EPO transcripts localized almost exclusively in the embryo proper, increasing exponentially in the fetal liver by E10.5 (Figure 1B). The differential expression of EPO, consistent with previous findings,²¹ indicates that EPO is produced in a spatially and temporally separate compartment from emerging primitive erythroid progenitors.

Epor is not necessary for specification of the primitive erythroid lineage

To determine if EPO/EpoR regulates the initial emergence of the primitive erythroid lineage, we analyzed the number of EryP-CFC in *Epor*-null mouse embryos at E8.5, a developmental time when EryP-CFC peak in number.¹ At Day 5 of EryP-CFC culture, we found more than 80% loss of colony-forming ability in *Epor*-null embryos (Figure 2A). However, erythroid colony formation relies on the ability of progenitors to form erythroblast precursors that can proliferate and accumulate hemoglobin during their maturation. Therefore, a reduction in EryP-CFC colonies at Day 5 could also be due to defective erythroid precursor maturation. To test this hypothesis, we quantified nascent EryP-CFC-derived colonies as benzidine-positive cell clusters consisting of 8 cells or over at Day 2 of culture (Figure 2A and B). This analysis revealed near normal numbers of Day 2 EryP-CFC compared to wild-type littermates (Figure 2A). Similarly, removal of EPO from the methylcellulose media resulted in an 80% decrease in the number of primitive erythroid colonies at Day 5, but only a marginal decrease in benzidine-positive cell clusters at Day 2 of culture (Figure 2C). Therefore, like the EPO-independent specification of CFU-E in the fetal liver,⁷ these data indicate that EpoR signaling is not essential for specification of

primitive erythroid progenitors in the mammalian embryo and suggest that EPO plays an important role in the subsequent maturation of primitive erythroid precursors.

Primitive erythroblast survival is dependent upon EpoR-mediated suppression of apoptosis at late stages of maturation

Primitive erythroid precursors normally undergo several maturational cell divisions between E9.5 and E12.5 as they transition from proerythroblasts to late-stage orthochromatic erythroblasts.^{1,2} As previously described,⁷⁻⁹ the number of primitive erythroblasts in *Epor*-null embryos was approximately half the normal number of primitive erythroblasts at E10.5 and only 10% of littermate controls at E12.5 (Figure 3A). The etiology of this profound loss of maturing primitive erythroblasts has not been clarified. Since EPO is known to provide antiapoptotic signals in definitive erythroid progenitors, we utilized imaging flow cytometry to identify erythroblasts displaying nuclear morphology associated with programmed cell death (Online Supplementary Figure S2).^{16,17} At E10.5, the percentage of apoptotic primitive erythroblasts is 2-fold higher in *Epor*-null embryos compared to their wild-type littermates. However, by E11.5, more than 60% of the primitive erythroblasts *in vivo* are apoptotic in the absence of *Epor* (Figure 3B). Consistent with these data, Bcl-x1 expression is significantly reduced in primitive erythroblasts isolated from E11.5 *Epor*-null embryos (Figure 3C), suggesting that Bcl-x1 lies downstream of EpoR signaling and supports survival of late stage primitive erythroblasts.

To further explore the role of EPO during primitive erythroblast maturation, we developed a 2-step primitive erythroid culture assay (Online Supplementary Figure S1). Consistent with *in vivo* expansion of the primitive ery-

thron, EPO stimulation results in robust expansion of benzidine-positive primitive erythroblasts during the four days of *in vitro* culture (Figure 3D). EPO deprivation immediately following tissue dissociation and throughout the 4-day culture period reduced total erythroid output by 85% (Figure 3D). Since EPO withdrawal *in vitro* recapitulates the kinetics of primitive erythroblast loss in *Epor*-null embryos, we investigated whether this loss was associated with increased apoptosis. Consistent with our observations of *Epor*-null embryos, EPO deprivation in wild-type primitive erythroblasts results in a greater than 2-fold increase in apoptosis, with a majority of cells succumbing at Day 3 of culture (Figure 3E). To further characterize the anti-apoptotic response of primitive erythroblasts, we measured the expression of several genes known to influence erythroid cell survival.^{17,22,23} Strikingly, EPO withdrawal results in a 200-fold decrease of Bcl-x1 and a nearly 3-fold decrease of Mcl expression by 96 h of culture compared to EPO-stimulated primitive erythroblasts (Figure 3F). Concurrently, there is an increase in the expression of the pro-apoptotic genes Bax, Bid, and Bim upon EPO deprivation (Figure 3F). These data, taken together, provide evidence that EPO promotes primitive erythroblast survival during the terminal stages of erythroblast maturation by regulating the expression of pro- and anti-apoptotic genes.

Primitive erythroblast proliferation is dependent upon EPO/EpoR

To determine whether EPO also regulates the proliferation of maturing erythroblasts, we assessed the cell cycle status of viable primitive erythroblasts isolated from E10.5 embryos. *Epor*-null erythroblasts revealed a significant reduction in S-phase cells compared to wild-type litter-

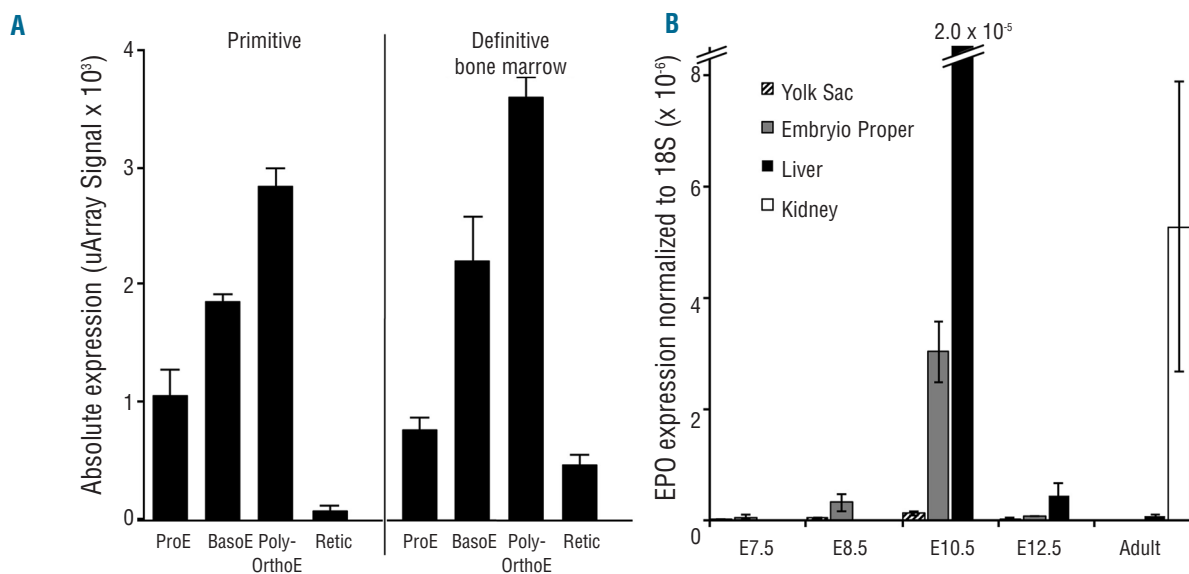


Figure 1. Epo is expressed in the embryo proper subsequent to the formation of *Epor*-expressing primitive erythroblasts. (A) Expression of EpoR transcripts during primitive and definitive erythroblast maturation as measured by Affymetrix microarray.¹⁹ Mean \pm SEM, n=5. (B) Expression of EPO transcripts by qPCR in E7.5 – E12.5 mouse embryos, and liver and kidney from adult mice. Yolk sac and embryo proper were isolated from E7.5, E8.5, E10.5, and E12.5. E10.5 and E12.5 fetal livers were isolated and processed separately from embryo proper fractions. Adult mouse liver and kidney were also isolated. Mean \pm SEM, n \geq 3 for all samples.

mates (Figure 4A and B). *Epor*-null erythroblasts also exhibited a 2-fold increase in the percentage of cells in the G0/G1 and in the G2-M phases of the cell cycle (Figure 4A). To further investigate how EPO regulates the proliferation of primitive erythroblasts, we utilized CFSE staining to track the number cell divisions in 2-step culture. Primitive erythroblasts deprived of EPO retain more CFSE than their EPO-stimulated counterparts at Day 2 (Figure 4C, left panel). By Day 3 of culture, the difference in CFSE staining indicates that EPO stimulated primitive erythroblasts have undergone one more cell division than EPO-deprived erythroblasts (Figure 4C, right panel). These data, taken together with cell cycle changes seen *in vivo*, indicate that EPO increases the proliferative capacity of maturing primitive erythroblasts, and provides another mechanism contributing to the progressive anemia that occurs in *Epor*-null embryos.

To characterize EPO-dependent transcriptional changes that may contribute to a reduction in primitive erythroblast cell cycling, we measured the expression of genes known to regulate the G1 to S phase transition. Interestingly, p21 expression, which has been shown to trigger cell cycle exit and terminal erythroid maturation in erythroleukemic and normal definitive erythroid cells,²⁴ is not altered by EPO deprivation in primitive erythroid 2-step cultures (Figure 4D). In contrast, EPO deprivation results in a 6-fold increase of p27 expression at Day 2 of culture compared to primitive erythroblasts stimulated by EPO (Figure 4D). This induction of p27 is coincident with the reduction in primitive erythroblast proliferation in the absence of EPO and is consistent with the role of p27 in promoting cell cycle exit and terminal differentiation in definitive erythroid cells.^{25,26}

Primitive erythroblast maturation is accelerated in the absence of *Epor*

Primitive erythroblasts normally undergo semi-synchronous maturation characterized by reduced cell size, nuclear condensation, decreased RNA content, and organelle loss.²³ We utilized imaging flow cytometry to quantify these cellular features as primitive erythroblasts mature over developmental time (Figure 5A and B). The persistence of some *Epor*-null primitive erythroblasts allowed us to analyze their maturational status, compared to wild-type littermate controls. After excluding cells with apoptotic morphology, we observed that *Epor*-null primitive erythroblasts have smaller nuclear and cell areas, reduced RNA content, (Figure 5C and D) and dramatically reduced CD71 cell surface expression at E10.5 compared to wild-type littermate controls (Figure 5C). While *Epor*-null primitive erythroblasts exhibit an advanced maturation phenotype at E10.5, they continue to mature as evidenced by the progressive reduction in cell size, nuclear size, RNA content, CD71 cell surface expression (Figure 5C), and mitochondrial content at E11.5 (Figure 5C and D, open histograms). Thus, as a population, *Epor*-null primitive erythroblasts appear more mature when compared to wild-type littermates by all of these measures except for mitochondrial content. These findings indicate that EPO regulates the overall rate of primitive erythroblast maturation, but does not appear to regulate mitophagy (Figure 5C and D).

Primitive erythroblasts also undergo maturational changes at the molecular level. We previously described a robust β H1- to ϵ y-globin switch during primitive ery-

throblast maturation.¹⁸ With EPO stimulation, wild-type primitive erythroblasts recapitulate the maturational globin switch *in vitro*, undergoing the transition of β H1-globin to ϵ y-globin around Day 3 of culture (Figure 5E, left panel). However, EPO-deprived primitive erythroblasts undergo the β H1- to ϵ y-globin switch almost 1.5 days earlier (Figure 5E, right panel). Consistent with these results, we also find that the less robust ζ - to α -globin switch¹⁸ has sig-

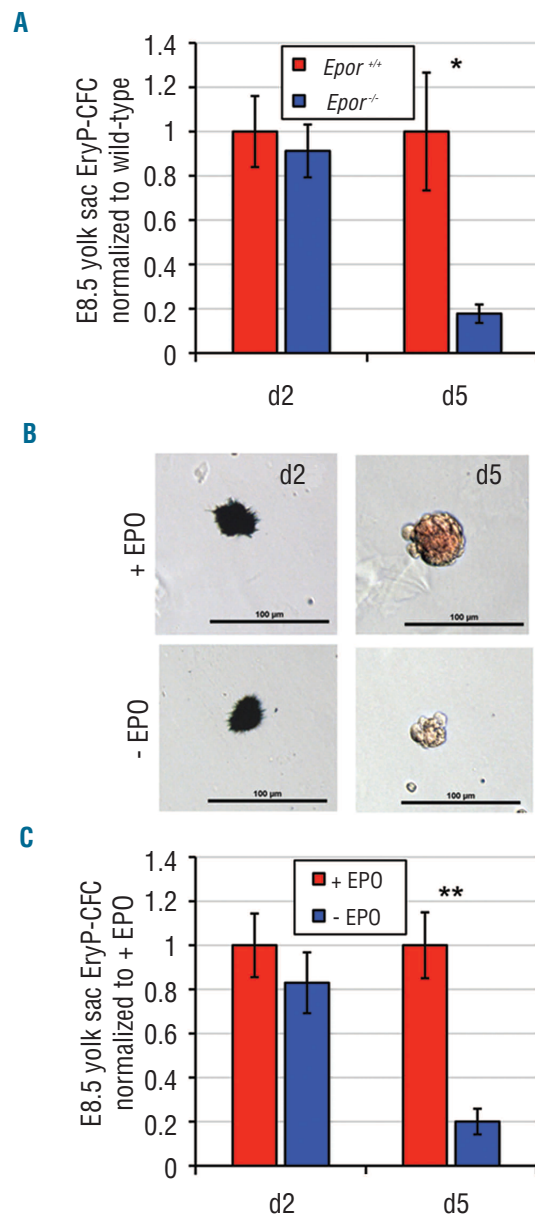


Figure 2. EPO/*Epor* is dispensable for specification of the primitive erythroid lineage. (A) EryP-CFC colonies were quantified from individual E8.5 embryonic yolk sacs following 2 days (d2) or 5 days (d5) in methylcellulose culture. EryP-CFC values for *Epor*-null (blue) and wild-type littermates (red) were normalized to wild-type (d2 = 680; d5 = 200). Mean \pm SEM, *Epor*-null n=5; wild-type n=3. (B) Representative images of benzidine-positive EryP-CFC colonies from wild-type E8.5 embryos with (top) or without (bottom) EPO stimulation during the indicated culture period. (C) EryP-CFC colony formation from wild-type E8.5 embryonic yolk sacs was tested in the presence (red) or absence (blue) of EPO in the methylcellulose media. Colonies were quantified and normalized to EPO-supplemented values (d2 = 1850; d5 = 536). Mean \pm SEM, n=3. (**P* = 0.006; ***P* = 0.00005).

nificantly progressed only in the cultures lacking EPO (Online Supplementary Figure S3). Furthermore, E11.5 Epor-null erythroblasts stain more darkly with benzidine than those from wild-type littermates (Figure 5F), suggesting a higher concentration of hemoglobin. These data, taken together, further support the concept that the lack of EPO leads to accelerated erythroid maturation.

EPO promotes survival of human primitive erythroblast survival and delays their maturation

Primitive erythroid cells constitute the first erythroid lineage in human embryos^{27,28} and the differentiation of hES cells serves as an *in vitro* model of early human development. The induced differentiation of H1 hES cells resulted in the emergence of immature primitive erythroblasts (Figure 6A) that express predominantly ϵ -globin (*data not shown*) like their *in vivo* counterparts.^{29,30} Subsequent culture of these cells in maturation media supplemented with EPO resulted in the accumulation of hemoglobin-containing cells (Figure 6B, red line) with morphological changes typical of erythroblast maturation (Figure 6A). EPO deprivation prevented the expansion of hemoglobin-containing primitive erythroblasts (Figure 6B, blue line) and increased the proportion of apoptotic cells (Figure 6C). In addition,

EPO-deprived human primitive erythroblasts display evidence of advanced maturation compared to EPO-stimulated controls. Even by Day 2 of culture, EPO-deprived human primitive erythroblasts appear smaller with greater nuclear condensation than the EPO-stimulated cells and these differences remain evident up until Day 6, when primitive reticulocytes are formed (Figure 6A). We used imaging flow cytometry to categorize primitive erythroblasts by their relative maturity based on cell and nuclear sizes as defined by the areas of CD235a and DAPI signal, respectively (Figure 6D). By Day 3 of culture, the majority of human primitive erythroblasts stimulated with EPO were classified as “hEryP B” while the remaining cells were either less mature (hEryP A) or more mature (hEryP C). In the absence of EPO, nearly 80% of primitive erythroblasts were the most mature (hEryP C) cells with a complete absence of the least mature cohort of “hEryP A” cells (Figure 6E).

Like the mouse, human primitive erythroblasts undergo ζ - to α -globin switching during maturation.³¹ We asked whether this switch was accelerated in cultures of human erythroblasts deprived of EPO. As shown in Figure 6F, the relative proportion of ζ -globin expression was lower in EPO-deprived human primitive erythroblasts

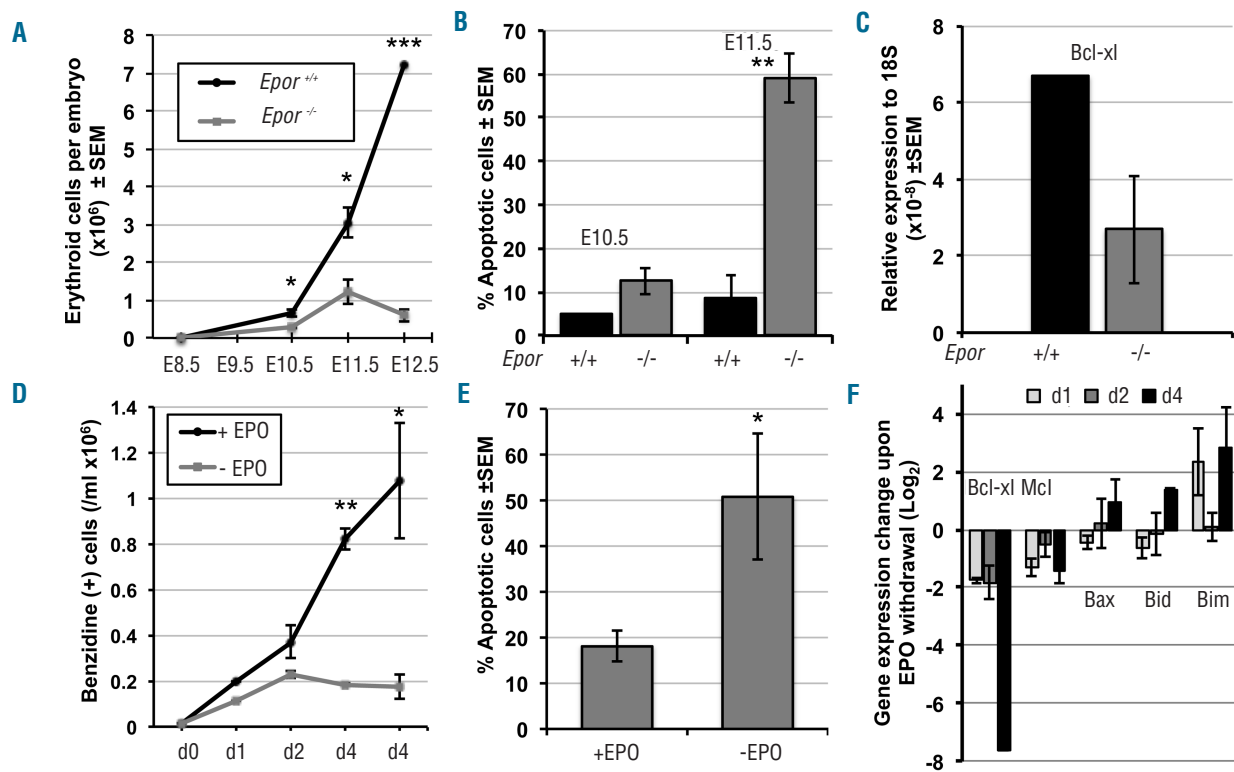


Figure 3. EPO/*Epor* is required for primitive erythroblast survival and to promote an anti-apoptotic transcriptional state at late maturational stages. Primitive erythroblasts were isolated from *Epor*-null and wild-type littermate embryos. (A) Hemoglobin-expressing, benzidine-positive cells were quantified from individual embryos at E10.5, E11.5, and E12.5. E8.5 values represent the number of EryP-CFC per embryo (Figure 1A.). (B) Primitive erythroblasts were analyzed for apoptotic nuclear morphology by imaging flow cytometry and the percentage of apoptotic cells per embryo was calculated. (C) Expression of the anti-apoptotic gene *Bcl-x1* in E11.5 primitive erythroblasts was measured by qPCR, and normalized to 18S levels. (D-F) Wild-type primitive erythroblasts from primitive erythroid 2-step cultures were analyzed following EPO stimulation or deprivation. (D) Primitive erythroblasts, as defined by benzidine positivity, were enumerated daily throughout the culture period. (E) Primitive erythroblasts were analyzed for apoptotic nuclear morphology by imaging flow cytometry following 3 days of culture. (F) Primitive erythroblast expression of anti-apoptotic genes (*Bcl-x1*, *Mcl*) and pro-apoptotic genes (*Bax*, *Bid*, and *Bim*) were measured by qPCR and normalized to 18S levels. The difference in gene expression between EPO supplementation and EPO withdrawal was calculated and reported on a Log₂ basis. (A-C) mean ± SEM, n≥2 with the exception of (A) E12.5 wild-type, (B) E10.5 wild-type, and (C) wild-type; n=1. (D-F) mean ± SEM, n=3. (*P<0.05; **P<0.01; ***P<0.0001).

compared to EPO-stimulated cells. In addition, EPO-deprivation resulted in higher total α -globin transcript accumulation at Day 2 of culture, comparable to levels present in erythroblasts stimulated with EPO for three days (Figure 6F). Taken together, these data support the concept that EPO regulates the rate of maturation of human primitive erythroblasts and suggests that this regulation is evolutionarily conserved in mammalian species.

Discussion

Both definitive and primitive erythropoiesis are characterized by colony-forming progenitor compartments that generate a wave of maturing erythroblast precursors. Similar to BFU-E,⁷⁻¹⁰ we determined that EryP-CFC are specified independently of *Epor*. However, unlike CFU-E, EryP-CFC are not dependent on EPO for their survival. Rather, our data indicate that EPO critically regulates the maturation of downstream primitive erythroblast precursors. These findings are consistent with the temporal and spatial expression of EPO which has been shown previously to first take place in the vitelline vessel.²¹ Here, we confirmed the lack of significant EPO transcript expression in the yolk sac as EryP-CFC emerge. Instead, EPO is subsequently expressed at increasing levels within the

embryo proper, which occurs concomitant with the circulation of primitive erythroblasts that are dependent on EPO for their maturation and survival.

Compared to wild-type littermates, *Epor*-null embryos exhibit progressively higher rates of primitive erythroblast apoptosis from E10.5 – E11.5, with reduced expression of Bcl-xl. These findings indicate that EPO-mediated survival mechanisms are required in late-stage primitive erythroblasts, consistent with findings from ES cell-derived primitive erythroid cells.³² Furthermore, primitive erythroblasts require EPO in order to progress to an anti-apoptotic transcriptional state whereby Bcl-xl and Mcl expression increase and Bid, Bax, and Bim decrease, similar to changes observed in bone marrow-derived definitive erythroblasts.^{17,33} The dependence of CFU-E on EPO/EpoR for survival severely limits the study of EPO function during late stages of definitive precursor maturation. CFU-E have different thresholds of EPO activity required to promote their survival.³⁴ A similar mechanism may exist in primitive erythroblasts allowing some primitive erythroblasts to survive while most ultimately die in the absence of EpoR signaling. Interestingly, a small population of primitive erythroblasts also persists in zebrafish following *epor* morpholino injection,³⁵ suggesting that a significant, but not complete, dependence of primitive erythroblasts on EPO is evolutionarily conserved.

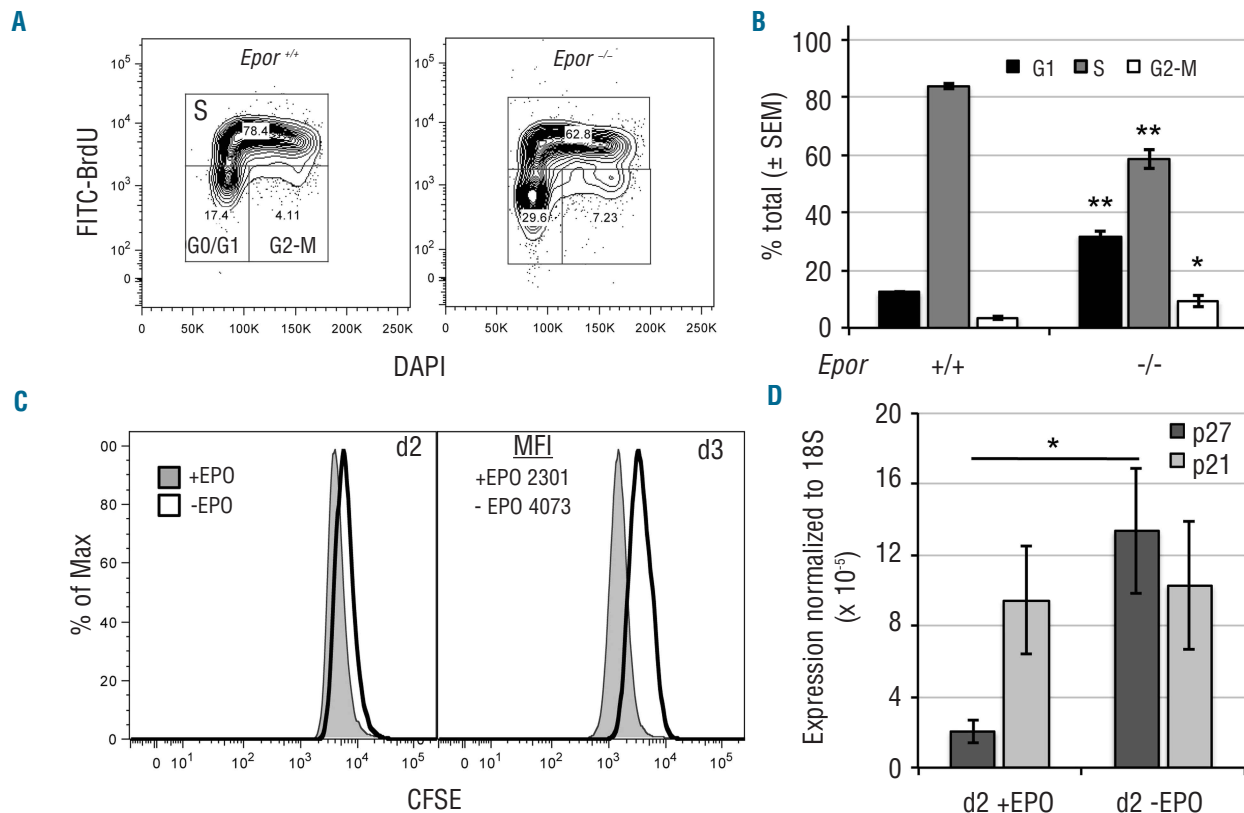


Figure 4. EPO/*Epor* promotes primitive erythroblast proliferation and delays cell-cycle exit. (A) Cell cycle status of primitive erythroblasts from individual E10.5 *Epor*-null and wild-type littermate embryos analyzed by flow cytometry to measure BrdU incorporation and DNA content. (B) Cell cycle analysis, mean \pm SEM of G0/G1, S, and G2/M phases. Asterisks denote statistical significance between genotypes. (C) Primitive erythroid cells in 2-step culture were loaded with CFSE and matured in the presence or absence of EPO. CFSE intensity, which decreases 50% with each cell division, was measured by flow cytometry on successive days of culture. Representative CFSE histograms and median fluorescence intensity (MFI) is reported. (D) Primitive erythroblast expression of cell cycle regulators p21 and p27 in response to EPO *in vitro* was measured by qPCR. Mean \pm SEM, n=3. (* P <0.05; ** P <0.005).

Gene deletions, e.g. *c-myb*, that solely ablate definitive, but not primitive, erythropoiesis, lead to fetal anemia only after 12.5 and fetal death beginning at E15.5.^{36,37} In contrast, *Epor*-null embryos all succumb by E13.5, and the fetal death can be rescued by the expression of EPO-R in hematopoietic tissues.¹¹ Taken together, these data indicate that the profound loss of primitive erythroid cells in

EpoR-null embryos contributes significantly to the etiology of fetal demise.

The terminal maturation of erythroid precursors is traditionally defined by the progressive decrease in cell size concurrent with the condensation of the nucleus and the shift from a basophilic to an acidophilic cytoplasm as RNA content decreases and hemoglobin accumulates. We and

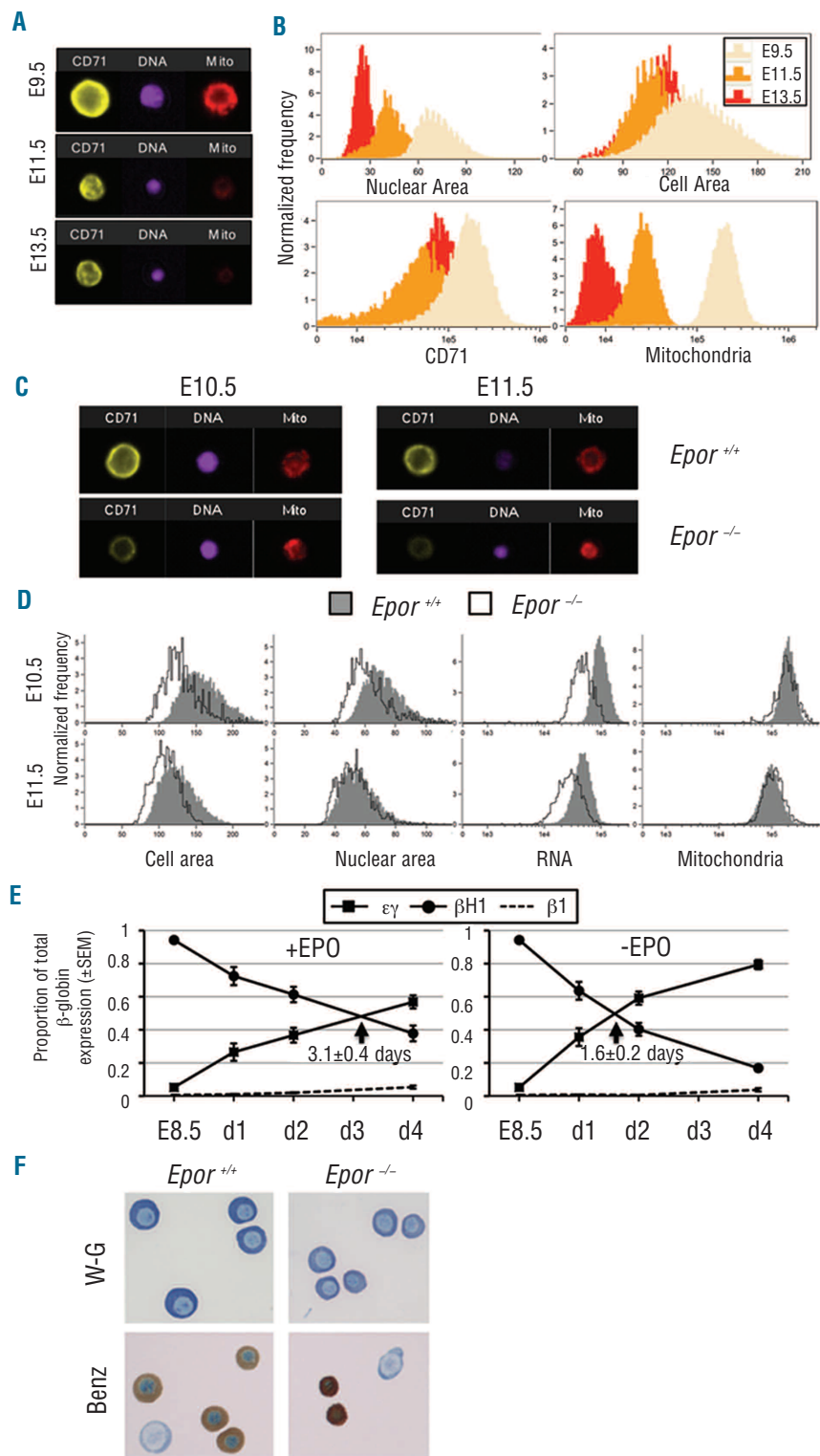


Figure 5. Lack of EPO/*Epor* accelerates the phenotypic maturation of primitive erythroblasts. (A) Representative images from imaging flow cytometry analysis of wild-type, outbred E9.5, E11.5, and E13.5 primitive erythroblasts. Images from channels detecting PE-CD71, Hoechst (DNA), and MitoTracker Deep Red (Mito) signals are shown. (B) Histogram plots reporting the nuclear area, cell area, CD71, and mitochondrial signal intensity distributions for each population of E9.5, E11.5, and E13.5 primitive erythroblasts. (C) Representative images from imaging flow cytometry analysis of non-apoptotic, wild-type littermate and *Epor*-null E10.5 and E11.5 primitive erythroblasts. (D) Representative histogram plots comparing the population distributions for cell area, nuclear area, RNA, and mitochondrial signal intensity of wild-type littermate (gray) and *Epor*-null (open) primitive erythroblasts from representative individual E10.5 (top row) and E11.5 (bottom row) embryos. (E) Wild-type primitive erythroblast β -globin gene expression was analyzed by qPCR in 2-step primitive erythroid maturation cultures stimulated (left), or deprived (right) of EPO (1 U/mL). Proportional β -globin expression levels were calculated by measuring the absolute expression of each gene normalized to 18S and dividing by the total β -globin gene expression (i.e. $\epsilon\gamma/(\epsilon\gamma + \beta\text{H1} + \beta1)$). $\beta2$ -globin expression was negligible and omitted from the analysis. Mean \pm SEM, $n=3$. Time of globin switch was determined by calculating the x-value for the intersection of βH1 -globin and $\epsilon\gamma$ -globin expression using the equations of each line ($y = mx + b$) as defined by their data points ($P < 0.02$). Mean \pm SEM, $n=3$. (F) E11.5 primitive erythroblasts from wild-type and *Epor*-null embryos stained with Wright-Giemsa (“W-G”) for morphological inspection or benzidine/field stain to detect hemoglobin content.

others have recognized that similar morphological changes occur during the maturation of primitive erythroblasts.^{2,3,38} Flow cytometry has been used to classify maturing erythroblasts by surface expression of Ter119, CD71, and CD44.³⁸⁻⁴² Previously, we used imaging flow cytometry to quantify traditional morphological features, including cell and nuclear size, as well as immunophenotypic features to characterize progressive stages of erythroid maturation.^{17,43} Here, we have applied imaging flow cytometry to quantify multiple cellular features of primitive erythroblasts as they mature as a cohort from proerythroblasts at E9.5 to orthochromatic erythroblasts at E12.5 – E13.5. The persistence of some primitive erythroblasts in *Epor*-null embryos allowed us to analyze the functional role of EPO signaling compared to their wild-type littermates.

At E10.5, *Epor*-null primitive erythroblasts have a smaller size, more condensed nucleus, and lower RNA content compared to primitive erythroblasts in wild-type littermates. Similar differences were evident between *Epor*-null and wild-type primitive erythroblasts at E11.5. Importantly, *Epor*-null primitive erythroblasts show progressive changes in all of the maturational parameters examined between E10.5 and E11.5, consistent with their accelerated maturation *in vivo*. Cell surface expression of CD71 was extremely low in *Epor*-null primitive erythro-

blasts, consistent with CD71 being a direct target of Stat5 signaling downstream of EpoR in definitive erythropoiesis.^{22,44} However, cell surface expression of CD71 decreases between E10.5 and E11.5, suggesting that the maturational reduction of CD71 also occurs independent of its starting level. Changes in cell size, nuclear size and RNA content have been associated with apoptosis. To ensure that the changes we observed in primitive erythroblasts lacking EpoR were due to *Epor* ablation and not a secondary defect of apoptosis, we excluded morphologically apoptotic erythroblasts^{16,17} from this analysis.

Previously, we determined that a hallmark of primitive erythroblast maturation is a tightly controlled β H1- to ϵ -globin switch.¹⁸ Removal of EPO induced an earlier transition of the β -globin maturational switch in our primitive erythroid 2-step culture compared to the EPO-stimulated cells. Furthermore, E11.5 primitive erythroblasts from *Epor*-null embryos exhibited a higher ratio of ϵ - to β H1-globin expression compared to wild-type littermates (*data not shown*), consistent with an accelerated maturational state. These changes in maturational state are associated with the reduced cell cycle progression and elevated expression of p27. Definitive erythroblast accumulation of p27 is associated with cell cycle exit during terminal maturation.^{25,45} The increase in p27 in EPO-deprived primitive erythroblasts may be responsible for the decrease in their

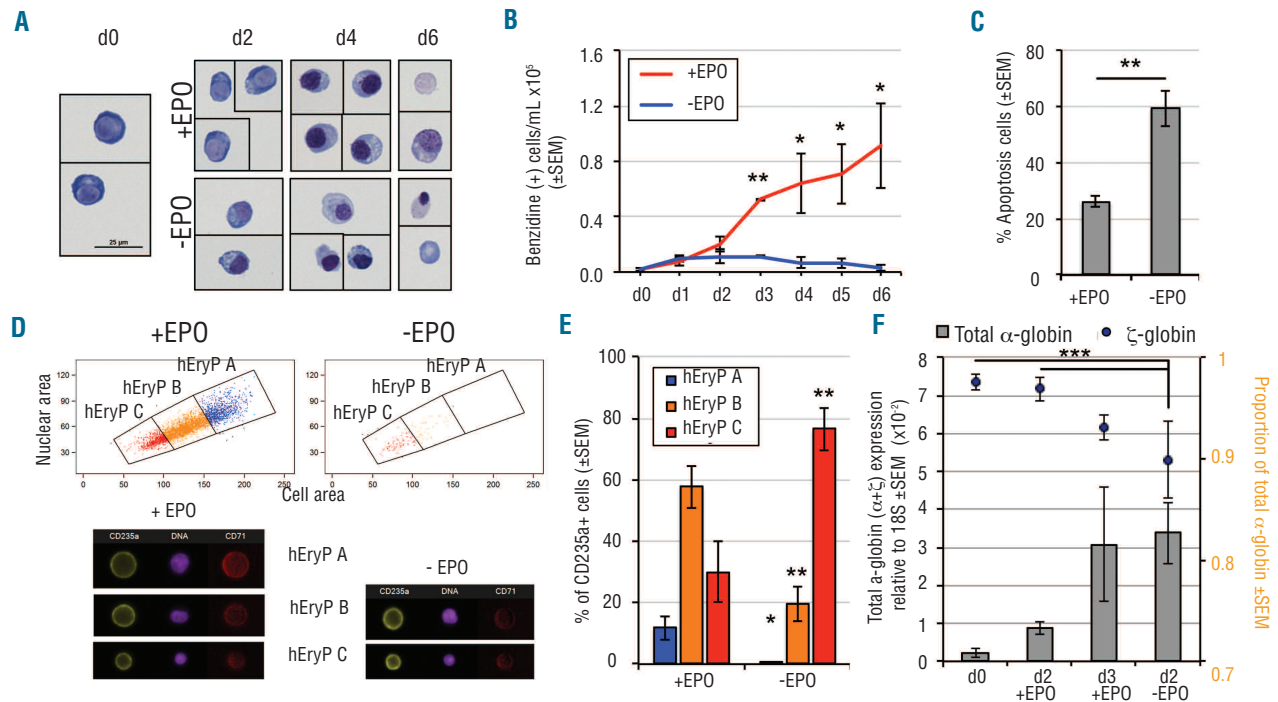


Figure 6. EPO promotes human primitive erythroblast survival and delays terminal maturation. Human primitive erythroblasts were harvested from hEBs cultured for 8–10 days and differentiated *in vitro* in the presence or absence of EPO. (A) Representative images of human primitive erythroblasts isolated from Day 8 hEBs (d0). (B) Human primitive erythroblasts were enumerated during the maturation culture period by benzidine staining. (C) Apoptotic nuclear morphology was measured in CD235-positive cells by imaging flow cytometry following 3 days of maturation culture in the presence (+EPO) or absence of EPO (-EPO). (D) The effect of EPO on the maturation of human primitive erythroblasts was assessed by imaging flow cytometry at 3 days. Populations hEryP A, B, and C represent least (hEryP A) to most (hEryP C) mature primitive erythroblasts present among the CD235a-positive cells. (E) Quantification of hEryP A, B, and C among the CD235a-positive cells, in +EPO and -EPO maturation culture for 3 days. Nuclear morphology was measured by imaging flow cytometry to identify apoptotic primitive erythroblasts in d3 maturation culture with or without EPO for 3 days. (F) α -globin gene family, α -globin and ζ -globin, expression was measured by qPCR in human primitive erythroblasts. Total α -globin (α - + ζ -globin) expression is reported on the left axis and the level of ζ -globin as a proportion of total α -globin is reported on the right axis. (Mean \pm SEM, n=3; * P <0.05, ** P <0.01, *** P <0.005).

proliferative capacity. Therefore, in addition to providing a survival signal to primitive erythroblasts, EPO regulates the process of terminal erythroblast maturation, potentially by coordinating the timing of cell cycle exit.

Interestingly, mitochondrial loss during erythroblast maturation appears to be independent of EPO/EpoR, since primitive erythroblasts that are more mature based on smaller cell size, nuclear size, and lower RNA content retain mitochondria at levels comparable to a more immature state. This dysregulation raises an interesting question about the interplay of erythroblast maturation and the establishment of an anti-apoptotic intracellular milieu through EpoR signaling. Mitochondrial depolarization and subsequent cytochrome c release are critical steps in the initiation of apoptosis.⁴⁶ The anti-apoptotic role of EPO/EpoR functions through the induction of Bcl-xl, which directly inhibits mitochondrial depolarization. Therefore, EpoR signaling may coordinate Bcl-xl induction at the appropriate cytoplasmic maturational state to ensure erythroblast survival. In the case of *Epor*-null primitive erythroblasts, Bcl-xl transcript levels are reduced and mitochondrial content is elevated relative to the apparent advanced maturational state of the cell, potentially further predisposing the mutant erythroblasts to apoptosis.

In order to examine whether human primitive erythroblasts respond to EPO like their murine counterparts, we generated immature primitive proerythroblasts from hES cells for further maturation in culture. We identified human primitive erythroblasts by cell morphology, ζ -globin gene expression and CD235a cell surface expression, and found that EPO promotes their survival and terminal maturation. Importantly, maturation of human primitive erythroblasts is accelerated in the absence of EPO stimulation, highlighting the analogous role of EPO in the mouse and human systems. To our knowledge, this is the first analysis of EPO function in human primitive erythroid cells.

Taken together, our data indicate that EpoR signaling is a critical regulator of multiple aspects of mammalian primitive erythropoiesis. While primitive erythroblasts are produced in the absence of EPO/EpoR, EpoR signaling is required for their optimal expansion by delaying cell cycle exit and terminal maturation, and by providing a critical survival signal to prevent apoptosis. These studies highlight the value of primitive erythropoiesis as a model system to investigate and better understand the regulation of terminal erythroid cell maturation. Given the many common attributes of primitive and definitive erythropoiesis, it will be of interest to determine if EPO functions analogously at late stages of definitive erythroblast maturation.

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