Published in final edited form as:

Apoptosis. 2008 August ; 13(8): 1022–1030. doi:10.1007/s10495-008-0236-3.

Targeting Erythroblast-specific Apoptosis in Experimental Anemia

Abhinav Diwan^{1,3,4}, Andrew G Koesters¹, Devan Capella¹, Hartmut Geiger², Theodosia A. Kalfa², and Gerald W Dorn II^{1,2,4}

¹Center for Molecular Cardiovascular Research, University of Cincinnati, Cincinnati, OH

²Department of Pediatrics, University of Cincinnati, Children's Hospital Medical Center, Cincinnati, OH

³St. Louis Veterans Administration (VA) Medical Center, U.S. Department of Veterans Affairs, St. Louis, MO

⁴Center for Pharmacogenomics, Washington University, St. Louis, MO

Abstract

Erythrocyte production is regulated by balancing precursor cell apoptosis and survival signaling. Previously, we found that BH3-only proapoptotic factor, Nix, opposed erythroblast-survival signaling by erythropoietin-induced Bcl-xl during normal erythrocyte formation. Since erythropoietin treatment of human anemia has limitations, we explored the therapeutic potential of abrogating Nix-mediated erythroblast apoptosis to enhance erythrocyte production. Nix gene ablation blunted the phenylhydrazine-induced fall in blood count, enhanced hematocrit recovery, and reduced erythroblast apoptosis, despite lower endogenous erythropoietin levels. Similar to erythropoietin, Nix ablation increased early splenic erythroblasts and circulating reticulocytes, while maintaining a pool of mature erythroblasts as erythropoietic reserve. Erythrocytes in Nix-deficient mice showed morphological abnormalities, suggesting that apoptosis during erythropoiesis not only controls red blood cell number, but also serves a “triage” function, preferentially eliminating abnormal erythrocytes. These results support the concept of targeting erythroblast apoptosis to maximize erythrocyte production in acute anemia, which may be of value in erythropoietin resistance.

Keywords

apoptosis; anemia; erythropoiesis; erythropoietin

Introduction

Dynamic regulation of erythropoiesis is largely determined by activation of erythroblast survival pathways by erythropoietin (EPO), secreted by the kidney in response to anemia or hypoxic stress¹. EPO and its longer acting analogue, darbopoietin are widely employed therapeutically to treat anemia in cancer, myelodysplastic syndromes, renal disease and rheumatoid arthritis²⁻⁵. While this approach of enhancing erythroblast survival is effective, therapy with these ‘erythropoiesis stimulating agents’ has raised concerns for venous thromboembolism⁶, stimulation of cancer growth⁷ and induction of a rare form of red-cell aplasia⁸. EPO treatment has hypertensive effects that may increase mortality in patients with renal failure^{9,10}, and between 10-50% of the patients are hyporesponsive to EPO treatment,

Correspondence to: Gerald W. Dorn II, M.D., Washington University Center for Pharmacogenomics, 660 S. Euclid Ave., Campus Box 8086, St. Louis, MO 63110. Phone: 314-362-8901. FAX: 314-362-0186. Email: gdorn@im.wustl.edu.
Abhinav Diwan and Andrew G. Koesters contributed equally to this work.

depending on the underlying condition ¹¹. A better understanding of the molecular mechanisms involved in erythroblast development may help identify novel targets to treat anemia.

EPO secretion by the kidney is the major stimulus for increased erythrocyte production in anemia ¹². Although multiple cellular mechanisms may be involved, one of the effects of EPO is to shift the balance between erythrocyte precursor (erythroblast) survival and programmed death ¹³. An important mechanism by which EPO stimulates survival signaling in erythroblasts is transcriptional upregulation of anti-apoptotic Bcl-xl via activation of the JAK2/Stat5 pathway ^{13,14}. As a consequence of increased Bcl-xl during erythroblast maturation, there is increased production of circulating reticulocytes from erythroblast precursors. Accordingly, mouse models lacking EPO, its receptor, or its critical downstream effectors exhibit increased erythroblast apoptosis and anemia ^{1,14-16}.

Given that Bcl-xl binds to and inhibits pro-apoptotic BH3-only members of the Bcl2 family of mitochondrial apoptosis factors, ^{17,18} there must be an erythroblast death pathway opposed by Bcl-xl. Recently, using gene targeting in the mouse, we identified Nix as the critical pro-apoptotic BH3 factor that causes apoptosis of maturing erythroblasts ¹⁹. The hallmark characteristic of *Nix* knockout mice was a reticulocytosis that occurred in the context of low/normal in vivo circulating EPO levels, leading us to conclude that absence of pro-apoptotic Nix dis-inhibits EPO/Bcl-xl-stimulated erythroblast survival pathways. These findings also suggest that inhibition of erythroblast apoptosis might be an effective therapeutic approach for anemia. Here, we performed proof-of-principle studies demonstrating that inhibiting erythroblast apoptosis by *Nix* ablation can promote rapid reconstitution of the circulating erythrocyte compartment in response to an anemic stress.

Materials and Methods

Mice

Nix knockout mice ¹⁹ were maintained in a mixed 129SVJ/C57BL/6 background and were housed and studied according to procedures approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Splenocyte isolation and immunophenotyping

Splenocytes were separated from parenchyma by filtration through a 70µm nylon cell strainer (BD Biosciences, CA). As described ¹⁹, freshly isolated splenocytes were suspended at a density of 10⁶ cells per 100µl of phosphate-buffered saline (PBS), stained with Ter 119-PE (1:200) and CD71-FITC (1:100) (BD Pharmingen, CA), Annexin V-APC (1:20), and 7AAD (1:20), and 5X10⁴ cells analyzed by flow cytometry using a FACSCalibur instrument from BD Pharmingen, CA. Data analysis was performed using CellQuestTMPro software from BD Biosciences, CA.

Anemia induction by phenylhydrazine

Mice were injected intraperitoneally on each of days 0, 1, and 3 with 40mg/kg of phenylhydrazine hydrochloride (Sigma, MO) solution in PBS ²⁰. Blood was obtained by sub-mandibular bleed on days 0, 3, 4, 6, and 9 for hematocrit and reticulocyte count measurements. Reticulocytes were enumerated by staining blood smears with Reticulocyte Stain (Sigma, MO) and counted using oil immersion microscopy. Flow cytometry of splenocytes was performed on the day after the final phenylhydrazine injection.

In vivo effects of erythropoietin

Mice were injected subcutaneously with 300IU/kg of Epogen (Amgen, Thousand Oaks, CA) solution in PBS for five consecutive days (days 0-4)²¹. Blood was obtained by sub-mandibular bleed on days 0, 2, 5, 7, and 9 for spun hematocrit and manual reticulocyte count measurements. Reticulocytes were enumerated by staining blood smears with Reticulocyte Stain (Sigma, MO) and counted using a 100x oil objective (Olympus SPlan 100PL) on a Nikon Diaphot 300 microscope. FACS analysis was performed the day after the final erythropoietin dose.

Erythropoietin measurement

Serum erythropoietin was assayed with a murine erythropoietin ELISA kit from R & D Systems (Minneapolis, MN), according to the manufacturer's instructions.

Enucleation studies

To study red cell enucleation, we used a modification of an ex vivo erythroid differentiation culture protocol, previously²². Briefly, low density bone marrow cells were cultured in serum-free medium StemPro-34TM (Invitrogen, CA) supplemented with StemPro-34 medium supplement, 1% BSA, 10 µg/ml insulin, 200 µg/ml transferrin (BIT-9500, StemCell Technologies), 900 ng/ml ferrous sulfate, 90 ng/ml ferric nitrate, in three steps. On days 0-7, the medium was supplemented with 10⁻⁶ M hydrocortisone, 100ng/ml SCF, 5ng/ml IL-3, and 2 IU/ml EPO. On days 8-10, 2 X 10⁵ cells/ml were resuspended in 6-well plates coated with fibronectin (50 µg/ml) in fresh medium supplemented with EPO. Finally, on days 10-12, the cells were cultured on fibronectin in fresh medium without cytokines. The percentage of enucleated cells in ex vivo erythropoiesis cultures were evaluated by flow cytometry on day 12. The cells from each well were collected and stained with anti-Ter119-PECy7 (BD Biosciences, CA) in 100µl PBS + 0.5% BSA + 5% mouse serum, for 20 minutes on ice. After gentle washing to remove unbound antibody, the cells were resuspended in 10mM HEPES buffer pH 7.4 containing 140mM NaCl and 5mM MgCl₂ with 0.25µM SYTO16 (Molecular Probes, Invitrogen, CA), a green-fluorescent cell-permeable nucleic acid-staining dye. After gating on Ter119-positive cells, the SYTO16-low and negative population represent the enucleated red blood cells.

Imaging of mitochondria in circulating red blood cells

Peripheral blood smears were stained with MTR CMXRos (Molecular Probes, CA) and imaged using a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Thornwood, NY) equipped with a 100x Plan-Apo oil-immersion objective (N.A. 1.4), with Zeiss LSM 510 software, version 3.2.

Osmotic fragility of red blood cells

Freshly obtained red blood cells were washed and suspended in PBS to obtain a hematocrit of 20%. 10µl of resuspended RBCs were incubated with a gradient of concentrations of hypotonic to isotonic NaCl solution for 15 minutes. Hemolysis was quantitated as absorbance of released hemoglobin at 550nm as described²³.

Enrichment of Ter119 splenocytes and western blotting

Splenocytes were labeled with iron particle tagged anti-Ter119 antibody and magnetically enriched using MACS^R cell separation columns (Miltenyi Biotech, CA). Enriched splenocytes were lysed in a buffer containing 10mmol/l HEPES, pH 7.2, 320mM sucrose, 3 mM MgCl₂, 25 mM Na₂P₄O₇, 1mM DTT, 5mM EGTA and 20mM NaF and centrifuged at 100,000g for 1 hour. The resulting pellet (membrane fraction: M) and the supernatant (S) were subjected to

SDS-PAGE, western transfer and immunoblotted for Nix (antiBnip3L antibody, Abcam, MA). Red blood cell ghosts were prepared as previously described ²⁴.

Statistics

Results are expressed as mean $\pm$ SEM. Statistical differences were assessed with ANOVA, unpaired t test, or paired t tests as indicated. A nonparametric test was applied when the data were not normally distributed. Significance was determined by a two-tailed $P < 0.05$ for t test.

Results

Erythroblast apoptosis is decreased during acute anemia recovery

To characterize the effects of anemia on apoptosis of maturing erythroblasts, we treated mice with phenylhydrazine (PHZ) to induce acute hemolytic anemia ²⁵ and examined externalization of phosphatidylserine by annexin V staining on the surface of Ter119 positive splenocytes at day 4. In wild-type mice, the nadir of PHZ-induced anemia (hematocrit $\sim 30\%$, see below) was associated with a striking decrease in annexin V positivity of splenic erythroblasts throughout the early and middle stages of the maturation sequence (Figure 1). This lower level of apoptosis during anemia recovery in wild-type mice is similar to the previously reported low rate of erythroblast apoptosis observed in mice lacking the gene for *Nix* (Figure 1 and reference 19). Induction of acute anemia in *Nix* knockout mice with PHZ had only a minor additional suppressive effect on erythroblast apoptosis (Figure 1), suggesting that apoptosis inhibition during erythroblast maturation is already maximal after *Nix* ablation.

Nix null mice show accelerated recovery from acute hemolytic anemia

To determine if chronically inhibiting erythroblast apoptosis would favorably affect clinical parameters in acute anemia, we injected ten pairs of young adult *Nix* null and wild-type mice with PHZ ²⁵, and examined the time course of hematocrit decline and recovery over nine days. As previously observed with this protocol ²⁶, the hematocrits of wild-type mice had declined by almost half on day four (Figures 2A, B), and the corrected reticulocyte count peaked at approximately 60% on the sixth day after the first PHZ injection, representing a 40-fold increase over baseline (Figures 2C, D). In contrast, the nadir of hematocrit for *Nix* null mice occurred on day three and declined by only 20%, compared to the starting hematocrit value (Figures 2A, B). Reticulocyte counts of *Nix* null mice, which are characteristically elevated at baseline, increased only modestly in response to PHZ (Figures 2C, D). Recovery from anemia was also more rapid in *Nix* null mice, with hematocrit values rebounding to above baseline on day six, at which time the hematocrits of wild-type mice were still depressed. The hematocrits of both groups of mice had fully recovered on day nine. Hematocrit recovery in *Nix* null mice was not the consequence of increased EPO secretion, as serum EPO levels measured four days after PHZ were less than one third those of wild-type mice (2815 ± 384 pg/ml *Nix* null (n=5) vs. $10,354 \pm 3565$ pg/ml wild type (n=4); $P < 0.05$).

Nix ablation recapitulates an anemia recovery erythroblastosis phenotype

In rodents, the spleen is more important than bone marrow in EPO-mediated stress erythropoiesis ^{27,28}. Therefore, to assess in more detail the consequences of *Nix* ablation on erythroblast survival and maturation during acute anemic stress, we immunophenotyped splenocytes four days after PHZ treatment. In wild-type spleens, the overall proportion of erythroid cells (Ter119 positive) to lymphoid cells increased from $51 \pm 3\%$ to $73 \pm 7\%$ on day four after the first PHZ injection (Table 1). The numbers of cells from earlier stages of the erythroid maturation sequence (pro-erythroblasts, basophilic erythroblasts, and chromatophilic erythroblasts) increased dramatically (Figure 3, Table 1), and there was depletion of the more mature orthochromatic erythroblasts (Table 1, Figure 3). These changes reflect mobilization

of the more mature cells into the peripheral circulation and increased splenic erythropoiesis. By contrast, *Nix* null mice started the experiment with a characteristic early stage erythroblastosis because intrinsic apoptosis suppression in the absence of *Nix* has the greatest effect in the early stages of the maturation sequence (Figure 3). While PHZ depleted mature erythroblasts in *Nix* null mice (Table 1, Figure 3), the pre-existing erythroblast reserve was available for mobilization and more rapid anemia recovery.

Enhanced erythroblastosis in response to exogenous EPO in *Nix* null mice

Reduced severity of anemia and early recovery after PHZ in *Nix* null mice in the context of lower circulating levels of erythropoietin could be because: 1. Erythropoiesis in *Nix* null mice is essentially independent of EPO, in which case there would be insensitivity to exogenous EPO administration. 2. Or, *Nix* ablation may greatly increase sensitivity to EPO, in which case we would observe a robust erythroblastosis and reticulocytosis in response to exogenous EPO, and resistance to anemia.

To assess the integrity of in vivo EPO responsiveness in *Nix* null mice, we profiled circulating red blood cells and splenic erythroblasts in ten pairs of wild-type and *Nix* null mice that received EPO, 10U daily for five days. As previously observed with this EPO treatment protocol²¹, the hematocrits of wild-type mice peaked on day seven (i.e. two days after the last EPO dose; Figures 4A, B). In contrast, hematocrit peaked two days earlier in *Nix* null mice (Figures 4A, B), and in proportion to the individual respective starting hematocrit values, tended to increase more rapidly in response to EPO (Figure 4B). The time course for reticulocyte count to increase with EPO treatment was similar in wild-type and *Nix* null mice, peaking on day five, i.e. the day following the last EPO dose (Figures 4C, D).

Spleens of EPO-treated wild-type and *Nix* null mice showed similar increases in the proportion of total Ter119 positive cells (Table 1). In wild-type mice this was largely attributable to a ~25-fold increase in the basophilic erythroblast population compared to baseline, with qualitatively similar, but quantitatively more modest changes in the EPO-treated *Nix* null mice due to the pre-existing large numbers of erythroblasts at this maturational stage (blue colored cells in Figure 3, Table 1).

Abnormal erythrocyte morphology suggests a triage function for *Nix*

Along with characteristic reticulocytosis, *Nix* null mice are reported to exhibit abnormal erythrocyte morphology in the form of polychromasia and poikilocytosis^{19,29} (Figure 5A). Given the high rate of erythroblast development in these mice, we considered that rapid red blood cell production might be associated with an escape from normal mechanisms of quality control during erythroblast maturation. Indeed, recent studies have identified an additional role for *Nix* in removal of mitochondria during terminal erythroid maturation^{29,30}. Alternately, *Nix* ablation might create an abnormality of late erythroblast development, such as terminal enucleation, or it could even have a protective effect in mature erythrocytes. Accordingly, we examined these possibilities. We compared enucleation in cultured bone marrow cells from *Nix* null and wild-type mice. As shown in Figure 5B, at day 12, the percent of enucleated erythroblasts was similar in *Nix* null and wild type mice, which does not support a role for *Nix* in removal of erythroblast nuclei. Analogous to the recent studies^{29,30}, we also found persistence of mitochondria in the circulating red blood cell population, in *Nix* null mice (Figure 5C). Additionally, *Nix* null erythrocytes demonstrated increased osmotic fragility (Figure 5D), suggesting an additional mechanism for the decreased survival of *Nix* null erythrocytes reported by Sandoval et al³⁰, in addition to increased phagocytosis of erythrocytes.

Since increased fragility could be due to a role of *Nix* in stabilizing circulating erythrocyte membranes, as postulated for Bcl-xl³¹, we first determined whether *Nix* was present in

circulating erythrocytes. Immunoreactive Nix was detected in splenic Ter119 positive erythroblasts and Ter119 negative splenocytes, but not in circulating erythrocytes (Figure 5E). As previously reported³¹, Bcl-xl was present in erythrocytes (Figure 5E). These studies suggest a dual role for Nix during erythroblast maturation, namely, regulation of erythroblast apoptosis as a mechanism to regulate ‘quantity’ or cell number and clearance of mitochondria to regulate ‘cell quality’ resulting in mature circulating erythrocytes.

Discussion

The current studies were undertaken to test the approach of promoting erythropoiesis by suppressing endogenous erythroblast apoptosis. We used *Nix* null mice as the experimental model in these studies because Nix acts in opposition to EPO/Bcl-xl in erythroblast maturation, and elimination of Nix should de-repress erythroblast survival at baseline and in response to stress. The general principle was validated, as *Nix* null mice exhibit a high level of reticulocyte production that blunted the severity of experimental anemia and permitted more rapid hematocrit recovery, despite lower endogenous EPO levels.

Normal homeostatic erythropoiesis occurs continuously in order to replenish senescent erythrocytes that are removed from the circulation after a lifespan that, in the mouse, has a normal reported half-life of between 12 and 16.5 days^{26,32}. Under these conditions, the number of earlier maturational stage erythroblasts, basophilic and chromatophilic erythroblasts, is held in check by intrinsic apoptosis pathways that are incompletely defined³³. Thus, in the normal mouse spleen, which is more important to stress-mediated erythropoiesis than is bone marrow in this species^{27,28}, examination of the erythroblast maturation sequence reveals a relative paucity of these early erythrocyte precursors, with an abundance of more mature orthochromatic erythroblasts that are readily available for terminal differentiation, enucleation, and release into the circulation during anemic or hypoxic stress. Although there may be multiple mechanisms for EPO-mediated erythropoiesis, EPO stimulation of Epo/Jak2/Stat5³⁴, with or without involvement of GATA-1³⁵, increases expression of the anti-apoptotic factor Bcl-xl, thereby enhancing survival of maturing erythroblasts. Nix is a developmentally regulated pro-apoptotic factor that is co-expressed in differentiating erythroblasts with, and functionally antagonized by, Bcl-xl^{36,37}. Accordingly, ablation of the mouse *Nix* gene alters the erythroblast maturational profile, causing enrichment of early- and mid-maturational sequence erythroblasts and increasing the overall proportion of erythroblasts to non-erythroblasts in spleen.

According to the above conceptual framework, Nix acts in opposition to EPO/Bcl-xl, and absence of Nix should therefore mimic EPO treatment. This was the case for in vitro CFU-E assays performed in cultured *Nix* null splenocytes, which revealed EPO-independent CFU-E formation and increased sensitivity to exogenous EPO¹⁹. The current studies show that *Nix* ablation closely mimics EPO in its effects on in vivo erythroblast maturation. In wild-type mice, EPO dramatically increased the numbers of basophilic erythroblasts in spleen, thus increasing the overall proportion of erythroblasts to non-erythroblasts. However, more mature orthochromatic erythroblasts continued to be present after EPO treatment. We observed that the erythroblast maturation profile of *Nix* null mice was similar to that of wild-type mice recovering from acute anemia (endogenous EPO secretion), or who received exogenous EPO. Thus, *Nix* ablation largely recapitulated the effects of exogenous EPO on erythroblast maturation.

Our results (current study and reference 19), and recent observations implicating a role for Nix in mitochondrial clearance during erythrocyte maturation^{29,30}, suggest that Nix has a dual role in red blood cell formation depending upon the developmental stage. During erythroblast development, Nix functions as a proapoptotic factor which serves as a mechanism to regulate

erythrocyte ‘quantity’. Nix mediated apoptotic cell death in erythroblasts, in opposition to EPO induced survival signaling, may also be an important ‘triage’ mechanism exerting a ‘quality’ control in erythrocyte generation. Indeed, erythroblasts vary in their sensitivity to EPO signaling³⁸, which is likely a mechanism that acts in concert with Nix signaling to orchestrate removal of erythroblast subpopulations. The observed role for Nix in mediating mitochondrial clearance, which apparently can proceed unimpeded in a large proportion (~40-60%) of erythrocytes even in the absence of Nix^{29,30}, is therefore an additional mechanism for quality control in a subpopulation of maturing erythroblasts. During anemic stress, when rapid mobilization of erythroblasts is required, stimulation of EPO production by the hypoxic stimulus in kidneys¹², enhances survival signaling in all erythroblast populations, leading to formation of additional erythrocytes.

Our results show that targeting erythroblast apoptosis downstream of EPO signaling may be a viable strategy to prevent severe anemia in response to an acute anemic stress. Certain aspects of Nix-mediated erythroblast apoptosis are conducive to its therapeutic targeting to promote erythroblast survival: 1. It is an inducible pro-apoptotic protein, which is expressed in a highly regulated fashion during the erythroblast developmental sequence³⁷. 2. *Nix* ablation decreases apoptosis in basophilic and chromophilic erythroblasts¹⁹, which are the predominant population of cells upregulated in wild type mice in response to an acute anemic stress in the spleen (see Figure 3 and Table 1). 3. Importantly targeting Nix does not impact the white blood cell compartment or hematopoietic stem cell population¹⁹, which suggests minimal potential for this approach to create undesirable hematopoietic side-effects.

Rapid and complete reconstitution of the erythrocyte compartment after PHZ induced anemia in *Nix* null mice, suggests enhanced erythroblast survival as the primary mechanism uncovered by *Nix* ablation. Indeed, in mice with enhanced erythroblast apoptosis, such as *Lyn* null mice³⁹, with resultant impairment in STAT5/Bcl-xl mediated survival signaling, PHZ treatment did not result in accelerated recovery from anemia, despite stimulation of extramedullary hematopoiesis. Importantly, survival signaling through a functional EPO receptor (mediated via STAT5 activation) was necessary for recovery from PHZ induced anemic stress⁴⁰. Based on our studies, we postulate that EPO induced Bcl-xl, antagonizes the proapoptotic action of Nix to promote erythroblast survival, both in the basal state¹⁹ and during stress. PHZ induces hemolysis by causing oxidative damage to the erythrocyte membrane⁴¹. Therefore, if the major role for Nix was in mitochondrial clearance from maturing reticulocytes with increased oxidative stress, as postulated by Sandoval et al³⁰, the response to PHZ treatment would be exactly opposite to what we observed, with much poorer recovery from anemia. Indeed, in *p45NF-E2* null mice, with analogously elevated levels of reactive oxygen species in erythrocytes, PHZ treatment provoked a markedly increased severity of anemia²⁶.

There are aspects of this work that suggest major challenges to implementing anti-apoptosis therapy for anemia. The normal hematocrit of unstressed *Nix* null mice does not wholly reflect increased erythroblast production indicated by a reticulocyte count that is five-fold higher than normal. This is likely secondary to increased erythrocyte turn-over supported by the observation of morphologic changes in circulating erythrocytes (Figure 5A), increased osmotic fragility (Figure 5D) and decreased life span of circulating red blood cells³⁰. A similar phenomenon has been observed in polycythemic mice (induced by transgenically elevated circulating levels of EPO⁴²); after EPO treatment, with anemia, and after Friend virus infection in mice^{32,43}, as well as in human polycythemia vera⁴⁴ and a subset of patients having myelofibrosis with myeloid metaplasia, i.e. “spent polycythemia”⁴⁵⁻⁴⁷. In each of these conditions, an abbreviated erythrocyte life span is associated with increased activity of EPO or one of its downstream effectors, just as in *Nix* null mice the stimulus for erythropoiesis is disinhibition of EPO-mediated cell survival signaling in erythroblasts.

The preponderance of evidence supports an important role for Nix as a key regulator of splenic erythroblast maturation. Since *Nix* gene ablation accelerated recovery from PHZ-induced anemia in mice, it is interesting to consider how erythroblast-specific apoptosis inhibition might prove useful to enhance rapid mobilization of erythroblast reserves, in the treatment of anemia.

Acknowledgements

Supported by NHLBI HL59888, HL77101 (to G.W.D.), the American Heart Association (Scientist Development Grant to A.D.), and the U.S. Department of Veterans Affairs. The authors declare no competing financial interests. Author contributions: A.D. designed and performed research, analyzed data and wrote paper, A.G.K. performed research and analyzed data, D.C. performed research and analyzed data, H.G. performed research and analyzed data, T.A.K. performed research and analyzed data; and G.W.D. designed and performed research, analyzed data and wrote paper.

References

1. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* 1995;83:59–67. [PubMed: 7553874]
2. Italian Cooperative Study Group for rHuEpo in Myelodysplastic Syndromes. A randomized double-blind placebo-controlled study with subcutaneous recombinant human erythropoietin in patients with low-risk myelodysplastic syndromes. *Br J Haematol* 1998;103:1070–1074. [PubMed: 9886322]
3. Blumenauer B, Cranney A, Clinch J, Tugwell P. Quality of life in patients with rheumatoid arthritis : which drugs might make a difference? *Pharmacoeconomics* 2003;21:927–940. [PubMed: 12959625]
4. Littlewood TJ, Cella D, Nortier JW. Erythropoietin improves quality of life. *Lancet Oncol* 2002;3:459–460. [PubMed: 12147430]
5. Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. *N Engl J Med* 1987;316:73–78. [PubMed: 3537801]
6. Corwin HL, Gettinger A, Fabian TC, et al. Efficacy and safety of epoetin alfa in critically ill patients. *N Engl J Med* 2007;357:965–976. [PubMed: 17804841]
7. Steinbrook R. Erythropoietin, the FDA, and oncology. *N Engl J Med* 2007;356:2448–2451. [PubMed: 17568025]
8. Macdougall IC. Epoetin-induced pure red cell aplasia: diagnosis and treatment. *Curr Opin Nephrol Hypertens* 2007;16:585–588. [PubMed: 18089975]
9. Fishbane S, Besarab A. Mechanism of increased mortality risk with erythropoietin treatment to higher hemoglobin targets. *Clin J Am Soc Nephrol* 2007;2:1274–1282. [PubMed: 17942772]
10. Phrommintikul A, Haas SJ, Elsik M, Krum H. Mortality and target haemoglobin concentrations in anaemic patients with chronic kidney disease treated with erythropoietin: a meta-analysis. *Lancet* 2007;369:381–388. [PubMed: 17276778]
11. Lacombe C. Resistance to erythropoietin. *N Engl J Med* 1996;334:660–662. [PubMed: 8592534]
12. Van DYKE, Layrisse M, Lawrence JH, Garcia JF, Pollycove M. Relation between severity of anemia and erythropoietin titer in human beings. *Blood* 1961;18:187–201. [PubMed: 13779961]
13. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* 1990;248:378–381. [PubMed: 2326648]
14. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a^{-/-}5b^{-/-} mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* 1999;98:181–191. [PubMed: 10428030]
15. Motoyama N, Wang F, Roth KA, et al. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* 1995;267:1506–1510. [PubMed: 7878471]
16. Kieran MW, Perkins AC, Orkin SH, Zon LI. Thrombopoietin rescues in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. *Proc Natl Acad Sci U S A* 1996;93:9126–9131. [PubMed: 8799165]

17. Lindsten T, Ross AJ, King A, et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* 2000;6:1389–1399. [PubMed: 11163212]
18. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001;292:727–730. [PubMed: 11326099]
19. Diwan A, Koesters AG, Odley AM, et al. Unrestrained erythroblast development in Nix^{-/-} mice reveals a mechanism for apoptotic modulation of erythropoiesis. *Proc Natl Acad Sci U S A* 2007;104:6794–6799. [PubMed: 17420462]
20. Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF. Ineffective erythropoiesis in Stat5a^(-/-)5b^(-/-) mice due to decreased survival of early erythroblasts. *Blood* 2001;98:3261–3273. [PubMed: 11719363]
21. Vannucchi AM, Bianchi L, Cellai C, et al. Accentuated response to phenylhydrazine and erythropoietin in mice genetically impaired for their GATA-1 expression (GATA-1(low) mice). *Blood* 2001;97:3040–3050. [PubMed: 11342429]
22. Giarratana MC, Kobari L, Lapillonne H, et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nat Biotechnol* 2005;23:69–74. [PubMed: 15619619]
23. Ballas SK, Clark MR, Mohandas N, et al. Red cell membrane and cation deficiency in Rh null syndrome. *Blood* 1984;63:1046–1055. [PubMed: 6324926]
24. Kalfa TA, Pushkaran S, Mohandas N, et al. Rac GTPases regulate the morphology and deformability of the erythrocyte cytoskeleton. *Blood* 2006;108:3637–3645. [PubMed: 16882712]
25. Bauer A, Tronche F, Wessely O, et al. The glucocorticoid receptor is required for stress erythropoiesis. *Genes Dev* 1999;13:2996–3002. [PubMed: 10580006]
26. Chan JY, Kwong M, Lo M, Emerson R, Kuypers FA. Reduced oxidative-stress response in red blood cells from p45NFE2-deficient mice. *Blood* 2001;97:2151–2158. [PubMed: 11264184]
27. Peschle C, Magli MC, Cillo C, et al. Kinetics of erythroid and myeloid stem cells in post-hypoxia polycythaemia. *Br J Haematol* 1977;37:345–352. [PubMed: 603766]
28. Ou LC, Kim D, Layton WM Jr, Smith RP. Splenic erythropoiesis in polycythemic response of the rat to high-altitude exposure. *J Appl Physiol* 1980;48:857–861. [PubMed: 7451294]
29. Schweers RL, Zhang J, Randall MS, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci U S A* 2007;104:19500–19505. [PubMed: 18048346]
30. Sandoval H, Thiagarajan P, Dasgupta SK, et al. Essential role for Nix in autophagic maturation of erythroid cells. *Nature*. 2008
31. Walsh M, Lutz RJ, Cotter TG, O'Connor R. Erythrocyte survival is promoted by plasma and suppressed by a Bak-derived BH3 peptide that interacts with membrane-associated Bcl-X(L). *Blood* 2002;99:3439–3448. [PubMed: 11964315]
32. Brodsky I, Dennis LH, Kahn SB. Erythropoiesis in Friend leukemia: red blood cell survival and ferrokinetics. *Cancer Res* 1966;26:1887–1892. [PubMed: 5924954]
33. Weiss MJ, Orkin SH. Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. *Proc Natl Acad Sci U S A* 1995;92:9623–9627. [PubMed: 7568185]
34. Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna JL. Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. *Blood* 1996;88:1576–1582. [PubMed: 8781412]
35. Gregory T, Yu C, Ma A, Orkin SH, Blobel GA, Weiss MJ. GATA-1 and erythropoietin cooperate to promote erythroid cell survival by regulating bcl-xL expression. *Blood* 1999;94:87–96. [PubMed: 10381501]
36. Chen G, Cizeau J, Vande VC, et al. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins. *J Biol Chem* 1999;274:7–10. [PubMed: 9867803]
37. Aerbajinai W, Giattina M, Lee YT, Raffeld M, Miller JL. The proapoptotic factor Nix is coexpressed with Bcl-xL during terminal erythroid differentiation. *Blood* 2003;102:712–717. [PubMed: 12663450]

38. Kelley LL, Koury MJ, Bondurant MC, Koury ST, Sawyer ST, Wickrema A. Survival or death of individual proerythroblasts results from differing erythropoietin sensitivities: a mechanism for controlled rates of erythrocyte production. *Blood* 1993;82:2340–2352. [PubMed: 8400286]
39. Karur VG, Lowell CA, Besmer P, Agosti V, Wojchowski DM. Lyn kinase promotes erythroblast expansion and late-stage development. *Blood* 2006;108:1524–1532. [PubMed: 16705093]
40. Menon MP, Karur V, Bogacheva O, Bogachev O, Cuetara B, Wojchowski DM. Signals for stress erythropoiesis are integrated via an erythropoietin receptor-phosphotyrosine-343-Stat5 axis. *J Clin Invest* 2006;116:683–694. [PubMed: 16511603]
41. Ferrali M, Signorini C, Ciccoli L, Comporti M. Iron release and membrane damage in erythrocytes exposed to oxidizing agents, phenylhydrazine, divicine and isouramil. *Biochem J* 1992;285(Pt 1): 295–301. [PubMed: 1637315]
42. Bogdanova A, Mihov D, Lutz H, Saam B, Gassmann M, Vogel J. Enhanced erythro-phagocytosis in polycythemic mice overexpressing erythropoietin. *Blood* 2007;110:762–769. [PubMed: 17395782]
43. Stohlman F Jr. Humoral regulation of erythropoiesis. VII. Shortened survival of erythrocytes produced by erythropoietine or severe anemia. *Proc Soc Exp Biol Med* 1961;107:884–887. [PubMed: 13917340]
44. Anderson C, Aronson I, Jacobs P. Erythropoiesis: Erythrocyte Deformability is Reduced and Fragility increased by Iron Deficiency. *Hematology* 2000;4:457–460. [PubMed: 11399587]
45. Barosi G, Cazzola M, Frassoni F, Orlandi E, Stefanelli M. Erythropoiesis in myelofibrosis with myeloid metaplasia: recognition of different classes of patients by erythrokinetics. *Br J Haematol* 1981;48:263–272. [PubMed: 7236525]
46. Berlin NI, Lawrence JH, Lee HC. The life span of the red blood cell in chronic leukemia and polycythemia. *Science* 1951;114:385–387. [PubMed: 14883866]
47. Huff RL, Hennessy TG, Austin RE, GARCIA JF, Roberts BM, Lawrence JH. Plasma and red cell iron turnover in normal subjects and in patients having various hematopoietic disorders. *J Clin Invest* 1950;29:1041–1052. [PubMed: 15436873]
48. Syed F, Odley A, Hahn HS, et al. Physiological growth synergizes with pathological genes in experimental cardiomyopathy. *Circ Res* 2004;95:1200–1206. [PubMed: 15539635]

Figure 1. Erythroblast apoptosis is decreased during recovery from anemic stress

Comparative analysis of in vivo apoptosis in splenocytes of WT and *Nix* null mice at baseline and four days after PHZ treatment (baseline data are from Diwan et. al. ¹⁹). The numbers of WT and *Nix*^{-/-} mice are- untreated: 7 and 4; PHZ treated: 5 and 4 respectively. ^{*}=*P*<0.05 vs respective untreated group. [#] *P*=0.092.

Figure 2. *Nix* null mice show accelerated recovery from acute anemia

WT and *Nix*^{-/-} mice received phenylhydrazine (PHZ) on days 0, 1, and 3. Hematocrits (A, B) and corrected reticulocyte counts (% reticulocytes x hematocrit/45; C, D) were serially determined and are shown as raw values (A, C) and as fold change relative to pre-PHZ values (B, D). *= $P < 0.05$ vs WT value; #= $P < 0.05$ vs pre-PHZ value.

Figure 3. Comparative immunoprofiling of splenic erythroblast maturation at baseline, during recovery from PHZ -induced anemia, and after EPO treatment

Freshly isolated cells from spleens of WT and *Nix*^{-/-} mice treated with erythropoietin (EPO) or phenylhydrazine (PHZ) were stained with PE-conjugated anti-Ter119 and FITC-conjugated anti-CD71 for flow cytometry. Density plots are color-coded for erythroblast maturational stage: orange, proerythroblasts; blue, basophilic erythroblasts; pink, chromatophilic erythroblasts; green, orthochromatic erythroblasts. Pooled quantitative data are in Table 1. Studies were performed after 5 days of Epo treatment and the day after the final dose of PHZ.

Figure 4. Comparative erythropoietic responses to exogenous EPO

WT and *Nix*^{-/-} mice received daily EPO on days 0-4. Hematocrits (A, B) and corrected reticulocyte counts (% reticulocytes x hematocrit/45; C, D) were serially determined and are shown as raw values (A, C) and as fold change relative to pre-EPO values (B, D). *= $P < 0.05$ vs WT value; #= $P < 0.05$ vs pre-EPO value.

Figure 5. Effects of *Nix* ablation on terminal erythrocyte formation

(A) Peripheral blood smears from wild type and *Nix* null mice demonstrating polychromasia, poikilocytosis and anisocytosis (at 1000X magnification). (B) Immunophenotyping for nuclei extruded from cultured erythroblasts ex vivo (blue: SYTO-16 high, nucleated erythrocytes, red: SYTO-16 low and negative, enucleated RBCs). (C) Peripheral blood smears showing persistence of mitochondria (red) in circulating red blood cells by MTR CMXRos immunofluorescence (1000X). (D) Increased in vitro sensitivity to osmotic lysis of *Nix* null erythrocytes (n=5/group). (E) Immunoblot showing presence of Nix in Ter119+ (erythroblast lineage) and non-erythroblast Ter119-splenocytes, but not in erythrocytes. Anti-apoptotic Bclxl is shown for comparison. Positive control (+C) is cardiac extract from mice with forced

cardiac expression of Nix⁴⁸. s indicates supernatant, m indicates membrane fraction (see Methods) and E, empty lane.

Characterization of splenic erythroblast maturation after Phenylhydrazine induced anemia and Erythropoietin treatment

Table 1

	Wild Type		Nix ^{-/-}	
	Baseline	Post PHZ	Post EPO	Post PHZ
Total Ter119+ cells, %	51 ± 3	73 ± 7 [*]	78 ± 1 [*]	80 ± 2
Proerythroblasts, %	0.1 ± 0.1	12 ± 4 [*]	2 ± 0.4	16 ± 6 [*]
Baso. erythroblasts, %	2 ± 1	37 ± 4	54 ± 2 [*]	49 ± 6 [*]
Chro. erythroblasts, %	1 ± 0.1	23 ± 5 [*]	2 ± 0.2	15 ± 2
Orth. erythroblasts, %	50 ± 3	1.5 ± 0.5 [*]	20 ± 0.8	0.2 ± 0.1 [*]

All data are Mean ± SEM.

^{*} indicates $P < 0.05$ by post hoc testing for comparison of treatment groups with respective baseline by one way ANOVA. The experimental n for baseline, post EPO and post PHZ, are: (WT)-11, 4 and 5, (Nix^{-/-})- 8, 4 and 5.