Bmi-1 Regulates Extensive Erythroid Self-Renewal

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SUMMARY

Red blood cells (RBCs), responsible for oxygen delivery and carbon dioxide exchange, are essential for our well-being. Alternative RBC sources are needed to meet the increased demand for RBC transfusions projected to occur as our population ages. We previously have discovered that erythroblasts derived from the early mouse embryo can self-renew extensively ex vivo for many months. To better understand the mechanisms regulating extensive erythroid self-renewal, global gene expression data sets from self-renewing and differentiating erythroblasts were analyzed and revealed the differential expression of Bmi-1. Bmi-1 overexpression conferred extensive self-renewal capacity upon adult bone-marrow-derived self-renewing erythroblasts, which normally have limited proliferative potential. Importantly, Bmi-1 transduction did not interfere with the ability of extensively self-renewing erythroblasts (ESREs) to terminally mature either in vitro or in vivo. Bmi-1-induced ESREs can serve to generate in vitro models of erythroid-intrinsic disorders and ultimately may serve as a source of cultured RBCs for transfusion therapy.

INTRODUCTION

Every year in the United States, 14 million units of red blood cells (RBCs) are used to treat more than 3.5 million severely anemic patients (Ansari and Szallasi, 2012). This great clinical need for RBCs is expected to increase as our population ages. The current reliance on blood donors is associated with infectious risks, high costs of screening, and supply bottlenecks for rare blood types and for alloimmunized patients requiring chronic transfusions. The in vitro production of RBCs is one potential solution to meet this growing need for blood (Migliaccio et al., 2012).

Adult humans synthesize more than two million RBCs every second to maintain steady-state circulating levels. These RBCs are derived from lineage-committed progenitors that give rise to maturing erythroblasts that ultimately enucleate. Erythropoiesis is regulated by several cytokines, particularly erythropoietin (EPO) and stem cell factor (SCF), which provide survival and proliferation signals to erythroid progenitors (Koury and Bondurant, 1990; Chui and Russell, 1974). Additionally, glucocorticoid signaling facilitates the rapid expansion of the erythron that occurs in response to acute anemia (Bauer et al., 1999).

Studies of viral-induced avian erythroleukemia led to the discovery that signaling pathways downstream of EPO, SCF, and glucocorticoids synergize to drive the in vitro self-renewal of mammalian erythroid cells (Beug et al., 1979; Panzenböck et al., 1998). Self-renewing erythroblasts (SREs) derived from postnatal sources undergo limited ex vivo proliferation when cultured with EPO, SCF, and the synthetic glucocorticoid dexamethasone (DEX) (von Lindern et al., 1999; England et al., 2011). We previously reported that erythroblasts derived from the yolk sac and early fetal liver of the mouse embryo or from differentiating mouse embryonic stem cells undergo an initial phase of limited self-renewal, which is followed by a subset of cells that undergo an extensive phase of self-renewal (England et al., 2011). Even after 10⁶-fold expansion in vitro, extensively self-renewing erythroblasts (ESREs) maintain the capacity to terminally mature into reticulocytes. While the continuous presence of EPO, SCF, and DEX is necessary both for the restricted and for the extensive phases of erythroblast proliferation, the regulation of SRE and ESRE self-renewal remains poorly understood.

To better understand the mechanisms underlying the self-renewal of erythroid lineage cells, we generated and compared global gene expression data sets from SREs and ESREs as well as primary proerythroblasts (ProEs). This analysis revealed that Bmi-1 and other polycomb repressive complex 1 (PRC1) components are upregulated in self-renewing erythroblasts. Importantly, overexpression of Bmi-1 conferred extensive self-renewal capacity upon erythroblasts derived from adult bone marrow without interfering with the ability of these Bmi-1-induced ESREs (iESREs) to terminally differentiate into enucleated RBCs both in vitro and in vivo. iESREs may ultimately provide an alternative
Figure 1. Bmi-1 Is Highly Expressed in ESREs and Is Required for Erythroblast Self-Renewal

(A) SREs and ESREs were isolated from restricted and extensive phases of self-renewal, respectively. One representative growth curve of four independent ESRE cultures is shown with timing of SRE and ESRE isolation boxed.

(B) Analysis of Affymetrix data sets revealed upregulation of genes associated with the PRC1 in ESREs and SREs compared to primary ProEs. Several known targets of Bmi-1 are significantly downregulated in ESREs/SREs compared to ProEs (mean ± SEM; N = 4 independent replicates for ESREs/SREs; N = 5 independent replicates for ProEs). p value was calculated using one-tailed Student’s t test. *p < 0.05; **p < 0.01.

(C) Bmi-1 transcripts are expressed at significantly higher levels in ESREs compared to primary CFU-Es, ProEs, and maturing erythroblasts (EryBs) (mean ± SEM; N = 3 independent replicates). p value was calculated using one-tailed Student’s t test. *p < 0.05.

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source of cultured RBCs to meet the growing need for blood transfusions.

RESULTS

Bmi-1 Expression Is Upregulated in ESREs and Is Required for Erythroblast Self-Renewal

We previously determined that ESREs and primary ProEs share similar morphological and immunophenotypic characteristics, including high surface expression of KIT (CD117) and transferrin receptor (CD71) (England et al., 2011). Like ProEs, ESREs lie only 3–4 cell divisions upstream of reticulocytes; however, ESREs are blocked from maturing in vitro by DEX (England et al., 2011). To better understand the mechanisms regulating erythroid self-renewal, we compared global gene expression of self-renewing erythroblasts in the restricted and extensive phases of self-renewal (Figure 1A) with primary ProEs derived from adult bone marrow.

Analysis of differentially expressed genes (Figure S1A) revealed that several PRC1 components, including Bmi-1, Ring1, and Phc1, were expressed at significantly higher levels in self-renewing erythroblasts (Figure 1B). Bmi-1 functions primarily as a repressor of multiple downstream target genes, including Hoxa9 (Abdouh et al., 2009; Zacharek et al., 2011; Biehs et al., 2013). Consistent with the differential expression of Bmi-1, the potential downstream target Hoxa9 was not expressed in ESREs/SREs but was highly expressed in primary ProEs (Figures 1B and S1C). Bmi-1 also represses several cell-cycle inhibitors, including Cdkn1b (p27) and Cdkn2c (p18) (Leung et al., 2004; Abdouh et al., 2009; Zhang et al., 2010), which were also reduced in ESREs/SREs compared to ProEs (Figures 1B and S1C). The differential expression of Bmi-1 was validated in ESREs compared with primary late-stage erythroid progenitors (CFU-Es), ProEs, and maturing erythroblasts isolated from mouse bone marrow (Figures 1C and S1B).

We used a loss-of-function approach to test the hypothesis that Bmi-1 regulates erythroid self-renewal. ESREs transduced with shRNA targeting Bmi-1 rapidly died following puromycin selection, while ESREs transduced with a scrambled control vector continued to proliferate (Figure 1D). Furthermore, the BMI-1 inhibitor PTC-209 reduced ESRE proliferation in a dose-dependent manner (Figure 1E). Taken together, these data support the concept that Bmi-1 is required for in vitro erythroblast self-renewal.

Bmi-1 Is Sufficient to Induce Extensive Ex Vivo Self-Renewal of Adult Erythroblasts Cultured with EPO, SCF, and DEX

We next used a gain-of-function approach to determine whether Bmi-1 can extend the proliferative capacity of adult marrow-derived SREs, which normally proliferate for only 1–2 weeks ex vivo (England et al., 2011). Empty lentiviral vector-transduced adult SREs ceased proliferating within 2 weeks, consistent with their limited ex vivo self-renewal capacity. In contrast, erythroblasts from 10 of 11 bone-marrow-derived SRE cultures transduced with Bmi-1 proliferated at least 25 days (Figure 2A), while 3 cultures of Bmi-1-induced ESREs (iESREs) were maintained for more than 100 days (Figure 2B). iESREs were also generated from adult mice with hereditary hemolytic anemia due to Protein 4.1R deficiency (Figures S2A–S2D), providing a proof of principle that this experimental approach can be used to generate large numbers of mutant erythroblasts that can facilitate the study of red cell-intrinsic disorders.

Since the Bmi-1 expression vector contains GFP, we analyzed the percentage of Bmi-1-transduced erythroblasts over time in culture. While 20%–30% of erythroblasts were initially GFP+ following transduction, almost all of the erythroblasts in the extensive phase of proliferation were GFP+ (Figure 2C), consistent with the notion that Bmi-1 expression facilitates erythroblast self-renewal. Indeed, Bmi-1 is overexpressed in these transduced iESREs compared to ESREs derived from fetal sources (Figure 2D).

SREs transduced with Bmi-1 display a high nuclear to cytoplasmic ratio and a basophilic cytoplasm, similar to the morphology of primary ProEs and to ESREs derived from embryonic sources (Figure 2E). Like their embryo-derived counterparts, iESREs expressed KIT and transferrin receptor (Figure 2F). Similar to embryo-derived ESREs, which remain highly dependent on EPO, SCF, and DEX (England et al., 2011), Bmi-1-induced adult iESREs are also dependent on the presence of each of these cytokines to maintain ex vivo self-renewal (Figure 2G).

iESREs Maintain the Potential to Terminally Mature into Reticulocytes In Vitro

A mandatory feature of self-renewal is the generation of daughter cells with the same identity as the parent cell, which for ESREs include the maintenance of their capacity to terminally differentiate into reticulocytes. Transfer of iESREs and ESREs into maturation media resulted in similar
Figure 2. Bmi-1 Is Sufficient to Induce the Extensive Ex Vivo Self-Renewal of Adult Erythroblasts
(A) Lentiviral transduction of mouse Bmi-1 led to prolonged proliferation of bone-marrow-derived SREs grown in erythroid expansion media. Erythroid cells transduced with an empty vector proliferated for 2 weeks, while erythroid cells transduced with a Bmi-1 over-expression vector stably proliferated for more than a month (representative date from one of ten independent experiments). The dotted line represents expected cell proliferation if cells divide daily.
(B) Bmi-1-induced ESRE (iESRE) was maintained for 100 days, exhibiting more than $10^{30}$-fold total erythroblast expansion.

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kinetics of differentiation into reticulocytes as evidenced by the rapid accumulation of hemoglobin (Figure 3A) and by their transition at day 3 into enucleated reticulocytes (Figure 3B; England et al., 2011). Consistent with normal erythroblast maturation, iESREs also progressively downregulated the cell surface expression of KIT and transferrin receptor (Figure 3C). Taken together, these data indicate that iESREs are immature erythroid precursors poised to terminally mature in vitro.

iESREs Are Capable of Terminal Erythroid Maturation In Vivo
We next asked whether iESREs also have the capacity to mature in vivo. To track erythrocytes after transfusion, iESREs were generated from the marrow of adult ubiquitin C (UBC)-GFP mice that express GFP driven by the ubiquitin C promoter. 5–10 \( \times \) 10^7 iESREs were injected intravenously into NOD scid gamma (NSG) or C57BL/6J mice treated with 1.5 Gy total body irradiation (TBI) to transiently suppress

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(C) The percentage of erythroblasts transduced with Bmi-1 (GFP+ cells) when analyzed 3 days and 18 days after infection. Representative data from one of two independent experiments are shown.

(D) BMI-1 protein expression is increased in erythroblasts transduced with FUGW-Bmi1. Actin served as an internal control. Representative data from one of three for ESREs and one of four for iESREs independent experiments are shown.

(E) Bmi-1-induced iESREs exhibited a high nuclear to cytoplasmic ratio and basophilic cytoplasm resembling proerythroblasts.

(F) iESREs express both KIT (CD117) and transferrin receptor (CD71) on their cell surface. Representative data from one of three independent experiments are shown.

(G) iESREs remain dependent on the continued presence of EPO, SCF, and DEX for ex vivo self-renewal. Withdrawal of individual factors resulted in the rapid loss of cell proliferation. One representative culture of five independent experiments is shown.

See also Figure S2.
endogenous erythropoiesis. While most ESREs were localized to the spleen and bone marrow 1 day after transfusion (Figure S3B), a small percentage of GFP+ reticulocytes were detected in the circulation, likely derived from the small number of spontaneously maturing (benzidine-) cells present in the self-renewing cultures (Figure 3A). A large, transient wave of reticulocytes entered the bloodstream between 4 and 8 days after iESRE transfusion into irradiated mice (Figures 4A and S3C). At day 6, 40%–90% of the reticulocytes in the bloodstream were GFP+ (Figures 4A and S3C).

This robust wave of GFP+ reticulocytes was followed by the circulation of GFP+ mature RBCs. iESRE-derived RBCs were significantly larger than endogenous RBCs, consistent with their recent maturation as a neocyte cohort on day 6 posttransfusion (Figure 4C). The circulation of this large cohort of GFP+ RBCs for several weeks suggests that they can deform normally to survive the microcirculation. Indeed, pipette aspiration measurements revealed that iESRE-derived RBCs had normal surface area to volume ratios (sphericity) (Figures 4D and 4E). Furthermore, RBCs derived from iESREs had similar hemoglobin levels compared with endogenous RBCs (Figure S3D). Taken together, these data indicate that iESREs mature synchronously into reticulocytes both in vitro and in vivo and can become fully mature RBCs in vivo with a functional cytoskeleton that facilitates their persistent circulation for weeks in the bloodstream.

**DISCUSSION**

The self-renewal capacity of erythroid progenitors is an important component of current protocols to generate cultured RBCs for potential therapeutic purposes. However, a major obstacle in generating the 2.5 × 10^{12} RBCs that constitutes a single unit of blood is the limited capacity of erythroblasts derived from neonatal and adult sources to self-renew in vitro. Understanding the mechanisms promoting extensive erythroid self-renewal could lead to improved generation of cultured RBCs. Here, we have discovered that *Bmi-1* is preferentially expressed in self-renewing compared to differentiating erythroblasts. Importantly, we determined that overexpression of *Bmi-1* is sufficient to induce the extensive ex vivo self-renewal of adult erythroblasts that normally are only capable of limited self-renewal. These iESREs have a similar morphology, cell surface phenotype, and cytokine dependence compared to ESREs derived from embryonic sources.

*Bmi-1* regulates the self-renewal of several adult stem cell populations, including hematopoietic, neural, and cancer stem cells, often through repression of *p16^{ink4a}/p19^{Arf}* (Jacobs et al., 1999; Park et al., 2003; Molofsky et al., 2003; Kreso et al., 2014). While we did not detect *Cdkn2a* in erythroblasts (Kingsley et al., 2013), we did identify several other potential BMI-1 target genes that are differentially expressed in ESREs/iESREs, studies suggesting that BMI-1 may function independently of *p16^{ink4a}/p19^{Arf}* repression in ESREs (Bruggeman et al., 2007; Abdouh et al., 2009). The function of *Bmi-1* in ESRE self-renewal may be independent of its role in erythroid maturation, where it was recently shown to positively regulate ribosomal protein genes (Gao et al., 2015).

*Bmi-1* transduction of self-renewing erythroblasts did not interfere with terminal in vitro maturation of iESREs into reticulocytes. The generation of iESREs from adult Protein 4.1R null mice indicates that this experimental approach can be used to generate large numbers of mutant erythroblasts for the study of red-cell-intrinsic disorders, particularly diseases that affect terminal stages of erythroid maturation such as disorders of the membrane cytoskeleton or of globin gene expression.

Intravenous transfusion of iESREs resulted in the emergence of a large wave of reticulocytes 4–8 days later. The timing and the transient nature of this reticulocyte emergence indicate that iESREs rapidly switched in vivo from a self-renewal to a maturation program. This transient wave of iESRE-derived reticulocytes generated a population of mature RBCs that constituted 10%–15% of the total circulating RBC mass from week 2 to week 6 following iESRE transfusion. Consistent with a near normal life span, iESRE-derived RBCs displayed normal shape and deformability when compared to co-circulating endogenous RBCs. Transfusion of 10 × 10^{12} iESREs resulted in the stable circulation of approximately 1.6 × 10^{9} RBCs, suggesting that each iESRE generated 16–32 RBCs, consistent with the differentiation potential of normal ProE/CFU-E. Taken together, these findings indicate that *Bmi-1*-induced ESREs are not immortalized but rather constitute committed erythroid precursors that rapidly mature, not only in vitro but also in vivo. These findings also distinguish iESREs from immortalized erythroid cell lines generated by genetic perturbation of multiple transcription regulators, including C-MYC, *TP53*, *E6/E7*, and *SOX2*, some of which must be exogenously extinguished before terminal maturation can proceed (Huang et al., 2014; Hirose et al., 2013; Kurita et al., 2013).

Our data indicate that *Bmi-1* promotes the extensive erythroid self-renewal of adult erythroblasts cultured ex vivo with EPO, SCF, and DEX. iESREs may ultimately provide an alternative source of cultured RBCs to meet
Figure 4. Intravenous Transfusion of iESREs Results in a Transient Wave of Reticulocytes and the Circulation of iESRE-Derived RBCs for Several Weeks

(A) Kinetics of GFP+/CD71+ reticulocytes (left panel) and GFP+/CD71− mature RBCs derived from 10 × 10^7 GFP+ iESREs transfused on day 0 into recipient mice. The percentages of iESRE-derived reticulocytes and RBCs were calculated as the number of GFP+ cells divided by the total cell number in the parent gate (mean ± SEM; N = 3 mice).

(B) The morphology of circulating mature RBCs (Ter119+/CD71+ /DRAQ5−), was visualized by imaging flow cytometry on day 6 post-GFP+ iESRE transfusion. Reticulocytes and RBCs derived from iESREs (GFP+, upper panel) showed similar biconcave shape as endogenous (GFP−, lower panel) RBCs. Representative data from one of three mice are shown (size bar represents 10 μm).

(C) The size of iESRE-derived (GFP+) and endogenous (GFP−) circulating reticulocytes and RBCs on day 6 post-transfusion were compared by imaging flow cytometry. Reticulocytes were similar in size (mean ± SEM; N = 3 independent experiments; p = 0.22; left panel), while iESRE-derived RBCs were significantly larger than endogenous RBCs (mean ± SEM; N = 3 independent experiments; right panel). p value was calculated using two-tailed Student’s t test. *p < 0.05.

(D) Fluorescence-imaged microdeformation analysis of iESRE-derived (GFP+, upper panel) and endogenous (GFP−, lower panel) RBCs.

(E) Sphericity of iESRE-derived (GFP+) and endogenous (GFP−) RBCs on day 26 post-transfusion, based on micropipette measurements of surface area and volume measurements (individual value ± SD; p value = 0.177). p value was calculated using two-tailed Student’s t test. Representative data from one of three mice are shown.

See also Figure S3.
the challenge of generating the extremely large numbers of RBCs needed for transfusion therapy. Interestingly, iESREs transfused in vivo result in large numbers of fully mature RBCs, obviating the need to generate and fully mature RBCs in vitro. Importantly, no GFP+ cells in peripheral blood or tumors were detected in recipients 6 months after transfusion (data not shown). However, host conditioning and safety concerns must be evaluated before iESREs can be considered as a potential transfusion product.

EXPERIMENTAL PROCEDURES

Mice and Tissues
All experiments were approved by the University of Rochester’s Committee on Animal Resources. Mouse strains included outbred ICR mice (Taconic Farms), C57BL/6j mice (Jackson Laboratory), C57BL/6-Tg(UBC-GFP)30Scha/J (Jackson Laboratory), and Protein 4.1R knockout mice (kindly provided by Dr. John Conboy). Tissues from adult male bone marrow were processed as previously described to derive iESREs (England et al., 2011). iESREs were transfused into NOD.Cg-PkdcreERT2/lox H2rxtm1ajw/SzJ mice (NSG; Jackson Laboratory) or C57BL/6j mice.

Erythroid Cell Evaluation
Cultured cells were stained with Wright-Giemsa or benzidine as previously described (Palis et al., 1995). Images were acquired with live DAPI-, Ter119hi(PE-Cy7), CD71+/−(PE) cells, and reticulocytes were gated as Ter119+(APC), CD71+(PE) cells. Cells derived from transfused iESREs were identified as GFP+ within each gate (Figure S3A). RBCs collected from iESRE-transfused NSG recipient mice were analyzed for surface area and volume (sphericity) as described (Waugh et al., 2013).

Statistical Analysis
Statistical significance was calculated by Student’s t test. All data are presented with mean ± SEM, except sphericity and relative hemoglobin levels of RBCs, which are shown as value ± SD.

ACCESSION NUMBERS
Microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3112.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.05.003.

AUTHOR CONTRIBUTIONS
A.R.K. designed and performed experiments, analyzed data, and wrote the manuscript. J.L.O, S.J.E., Y.-S.H., K.H.F., and L.F.D. performed experiments. P.D.K. performed experiments and analyzed data. K.E.M. and R.E.W. analyzed data. J.P. designed experiments, analyzed data, and wrote the manuscript.

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REFERENCES
Abdouh, M., Facchino, S., Chatoo, W., Balasingam, V., Ferreira, J., and Bernier, G. (2009). BMI1 sustains human glioblastoma multi-forme stem cell renewal. J. Neurosci. 29, 8884–8896.
phenotypes of differentiation. Cell strains of defective avian leukemia viruses display three distinct
Graf, T. (1979). Chicken hematopoietic cells transformed by seven
ment in fetal liver. Dev. Biol.
Biels, B., Hu, J.K., Strauli, N.B., Sangiorgi, E., Jung, H., Heber, R.P.,
Ho, S., Goodwin, A.F., Dasen, J.S., Capeccchi, M.R., and Klein, O.D.
(2013). BMI1 represses Ink4a/Arf and Hox genes to regulate stem
cells in the rodent incisor. Nat. Cell Biol.
Bruggerman, S.W., Hulsman, D., Tanger, E., Buckle, T., Blom, M., Ze-
venhoven, J., van Tellingen, O., and van Lohuizen, M. (2007).
Bmi1 controls tumor development in an Ink4a/Arf-independent manner in a mouse model for glioma. Cancer Cell 12, 328–341.
Chui, D.H., and Russell, E.S. (1974). Fetal erythropoiesis in steel
mutant mice. I. A morphological study of erythroid cell develop-
ment in fetal liver. Dev. Biol. 40, 256–269.
England, S.J., McGrath, K.E., Frame, J.M., and Palis, J. (2011).
Immature erythroblasts with extensive ex vivo self-renewal capac-
ity emerge from the early mammalian fetus. Blood 117, 2708–
2717.
Gao, R., Chen, S., Kobayashi, M., Yu, H., Zhang, Y., Wan, Y., Young,
S.K., Soltis, A., Yu, M., Vemula, S., et al. (2015). Bmi1 promotes
erthroid development through regulating ribosome biogenesis.
Stem Cells 33, 925–938. Published online November 11, 2014.
http://dx.doi.org/10.1002/stem.1986.
Hirose, S., Takayama, N., Nakamura, S., Nagasawa, K., Ochi, K., Hir-
ata, S., Yamazaki, S., Yamaguchi, T., Otsu, M., Sano, S., et al. (2013).
Immortalization of erythroblasts by c-MYC and BCL-XL enables
large-scale erythrocyte production from human pluripotent stem
cells. Stem Cell Reports 1, 499–508.
Huang, X., Shah, S., Wang, J., Ye, Z., Dowey, S.N., Tsang, K.M.,
Mendelsohn, L.G., Kato, G.J., Kickerl, T.S., and Cheng, L. (2014).
Extensive ex vivo expansion of functional human erythroid precursors established from umbilical cord blood cells by defined fac-
tors. Mol. Ther. 22, 451–463.
Jacobs, J.J., Kieboom, K., Marino, S., DePinho, R.A., and van Lohui-
zen, M. (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a lo-
cus. Nature 397, 164–168.
Kingsley, P.D., Greenfist-Allen, E., Frame, J.M., Bushnell, T.P., Ma-
l, J., McGrath, K.E., Stockert, C.J., and Palis, J. (2013). Ontogeny of
erythroid gene expression. Blood 121, e5–e13.
Koury, M.J., and Bondurant, M.C. (1990). Erythropoietin retards
dNA breakdown and prevents programmed death in erythroid
progenitor cells. Science 248, 378–381.
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Supplemental Information

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Supplemental Information

Supplemental Figures

Figure S1, related to Figure 1.

A

![Figure S1A](image)

B

![Figure S1B](image)

C

![Figure S1C](image)
Figure S2, related to Figure 2

A. Protein 4.1R KO Adult BM

- Empty vector
- Bmi-1 transduction

Fold Expansion of Live Cells vs. Expansion Days

B. Western Blot
- WT iESRE
- EPB41 KO iESRE
- Protein 4.1R
- Actin

C. Self-renewing

D. Micrographs
- WT iESRE-derived Maturation Day 2
- EBP41 KO iESRE-derived Maturation Day 2

Scale bar: 10um
Figure S3, related to Figure 4.

A

B

C

D

Irradiated recipients

Unirradiated recipient

% GFP+ RBCs (mean ± SEM)

% GFP+ reticulocytes (mean ± SEM)

Relative hemoglobin absorbance

Proportion of tissues for ESRE homing

Day 1

GFP− GFP+
Supplemental Figure Legends

Figure S1, related to Figure 1. Analysis of genes differentially expressed by self-renewing and differentiating erythroblasts.

(A) Genes differentially expressed between ESRE and primary ProE. Members of PRC1 complex were significantly upregulated in ESRE compared with primary ProE.

(B) Primary erythroid cells at different maturational stages from adult mouse bone marrow were isolated FACS using live, lineage-, Ter119, CD117 and CD105 expression.

(C) Potential BMI-1 downstream targets, *Hoxa9*, *Cdkn1b*, and *Cdkn2c*, expression levels in ESRE and iESRE compared to primary ProE (mean±SEM; N=2).

Figure S2, related to Figure 2. iESRE can be derived from adult mice with hemolytic anemia.

(A) Lentiviral transduction of mouse *Bmi-1* led to extensive proliferation of EPB41-null bone marrow-derived iESRE.

(B) Western blots indicate that iESRE derived from wild-type bone marrow, but not from EPB41-null, bone marrow express Protein 4.1R. Actin served as an internal control.

(C) Similar to iESRE derived from wild-type bone marrow, iESRE derived from EPB41-null mice reveal an immature erythroid morphology.

(D) *In vitro* maturation of wild-type (left panel) and EPB41-null (right panel) erythroblasts reveals abnormal morphology of mutant late-stage erythroblasts.

Figure S3, related to Figure 4. Quantitation of the percentage of circulating reticulocytes and mature RBCs derived from transfused GFP⁺ iESRE.
(A) Left panel- mature RBCs and reticulocytes are defined by expression of Ter119 and CD71. Right panels- iESRE-derived reticulocytes (upper panel) and mature RBCs (lower panel) are distinguished from endogenous cells by GFP expression. Representative analysis from day 8 post-transfusion is shown.

(B) Transfused iESRE rapidly home primarily to spleen on day 1 post-transfusion (mean±SEM; N=2 independent experiments).

(C) Transfusion of 5x10^7 iESRE into sublethally irradiated (1.5 Gy TBI) C57BL/6J resulted in a transient wave of reticulocytes and circulation of mature RBCs for at least 4 weeks (mean±SEM; N=2 mice; top panel). Transfusion of 5x10^7 iESRE into an unirradiated C57BL/6J mouse resulted in extremely low reconstitution of reticulocytes and mature RBCs (bottom panel).

(D) Endogenous (GFP⁻) and iESRE-derived (GFP⁺) RBCs on day 28 post-transfusion showed similar relative hemoglobin absorbance (mean±SD; p=0.228). p-value was calculated using two-tailed Student t-test (N=2 independent experiments; 3 technical replicates).

**Supplemental Experimental Procedures**

**Erythroid expansion and maturation cultures**

Murine erythroblasts were cultured in erythroid expansion media and in maturation media as previously published (England et al., 2011), except that the lipid supplement was substituted with EXCYTE (Millipore).

**Lentivirus production and transduction**

To produce lentiviral particles for scrambled vector, HEK293T were transfected with PAX2, VSV-G, pLKO.1 (TRC1 or 1.5; Sigma), DMEM/F12, and FuGENE6 (Promega). For lentiviral particles containing shRNA targeting murine Bmi-1, pre-made lentiviral particles of 4
clones (TRCN0000012563, TRCN0000012567, TRCN0000235387, and TRCN0000235388 referred as shRNA#1, shRNA#2, shRNA#3, shRNA#4, respectively; Sigma) were used. To produce lentiviral particles with Bmi-1 overexpression, HEK293T were transfected with PAX2, VSV-G, pFUGW-empty vector (Addgene) or pFUGW-Bmi1 overexpression (Fasano et al., 2007; Addgene), DMEM/F12 (Gibco), and FuGENE6. After 24-hours, supernatant was removed and replenished with fresh media. 72-hours post-transfection, lentiviral particles was harvested and filtered (Pall Corporation).

Erythroblasts were combined with lentiviral particles and centrifuged at 1,600 g for 90 minutes, incubated at 37°C overnight, and replenished with fresh erythroid expansion media. For cells transduced with pLKO.1 or shRNA targeting Bmi-1, antibiotic selection with 1 µg/mL of puromycin (Sigma) was performed 72-hours post-transduction. Live cells after puromycin selection were counted to plot the erythroid cell proliferation. For cells transduced with pFUGW-empty vector or Bmi1 overexpression, expression of green fluorescent protein (GFP) was monitored by LSR-II flow cytometer (BD Bioscience) and analyzed with FlowJo software (Version 8.8.7; TreeStar).

**BMI-1 inhibitor treatments**

ESRE were treated with PTC-209 at 0.1, 0.5, 2.5, 12.5, and 62.5 µM (Xcessbio), with DMSO (same volume as PTC-209 at 62.5 µM) served as vehicle control. Live cells were counted by trypan blue exclusion.

**Isolation of primary erythroid cells**

Primary erythroid lineage cells were isolated from adult bone marrow using FACS. Cells were first gated as live (DAPI) and lineage-negative (Biotin-CD3e, CD45R, CD11b, Ly6g, Sca-1, CD41, CD16/32 conjugated with APC-Cy7). CFU-E were defined as CD117hi(PE-Cy7),
Ter119^lo (FITC), CD105^+ (PE), CD150^− (APC) cells. Proerythroblasts were defined as CD117^hi (PE-Cy7), Ter119^+ (FITC) cells. Mature erythroblasts were defined as CD117^+ (PE-Cy7), Ter119^+ (FITC) cells. Proerythroblasts used in Figure S1C were defined as live (DAPI−), lineage-negative (Biotin-CD3e, CD45R, CD11b, Ly6g with or without Sca-1, CD41, and CD16/32 conjugated with PE Cy5.5 or APC), CD117^+ (PE Cy7), CD71^+ (FITC), Ter119^+ (PE or APC) cells.

**Analysis of gene and protein expression**

Quantitative reverse-transcription polymerase chain reaction (qPCR) was performed as described (Kingsley et al., 2006). Taqman Gene Expression Assays of *Bmi-1* (Mm03053308_g1), *Hoxa9* (Mm00439364_m1), *Cdkn1b* (Mm00438168_m1), *Cdkn2c* (Mm00483243_m1), and *18s* (Hs99999901_s1) were used with an annealing temperature of 60°C. For analysis of *Hoxa9*, *Cdkn1b*, and *Cdkn2c*, ESRE and iESRE were isolated by FACS for CD117^+ (PE-Cy7) and CD71^+ (PE) cells.

For the analysis of BMI-1 expression, iESRE transduced with pFUGW-Bmi1 and ESRE derived from E12.5 fetal livers were isolated by FACS for CD117^+ (PE-Cy7) and CD71^+ (PE) cells. For analysis of Protein 4.1R expression, iESRE derived from wild-type and EPB41-null bone marrow were harvested. Whole-cell lysates were prepared with RIPA buffer (150 mM NaCl, 1% Nonidet P-40/Igepal CA-630, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1mM ethylenediaminetetraacetic acid (Fisher Scientific), 1mM dithiothreitol (Sigma), 1mM Na3VO4, 25 µg/mL phenylmethanesulfonyl fluoride (Calbiochem), and 50 mM Tris, pH 8.0) in the presence of protease inhibitor cocktails (Roche), and analyzed with 4-20% SDS-PAGE (Biorad) with BMI-1 (Abcam) and actin (Sigma) antibodies. Protein 4.1R antibody was kindly provided by Drs. Mohandas Narla and Xiuli An (New York Blood Center, New York, NY).
Analysis of erythroblast homing

10x10^7 iESRE, generated from UBC-GFP mice, were transfused intravenously into irradiated (1.5 Gy TBI) NSG mice. Bone marrow, spleen, lung, and peripheral blood were collected on day 1 post-transfusion, and the total cell numbers of dissociated spleen, lungs, and one femur (estimated as 17% of total marrow), were counted. The number of cells per 1 µL in peripheral blood was calculated using a CountBright™ absolute counting beads (Life technologies). iESRE-derived erythroblasts were gated as GFP+/Ter119+.

Analysis of hemoglobin level

400 endogenous (GFP) and iESRE-derived (GFP+) RBCs on day 28 post-transfusion were isolated by FACS for live (DAPI), Ter119+(APC), and CD71(PE). RBCs were lysed and stained for hemoglobin using the Mouse Hemoglobin ELISA kit (Kamiya Biomedical Company). Hemoglobin absorbance at 450 nm was measured using ELx800™ Absorbance Microplate Readers (BioTek).
List of Antibodies

| Antibody               | Company     | Catalog #    | Clone  |
|------------------------|-------------|--------------|--------|
| CD117 (c-kit) PE Cy7   | eBioscience | 25-1171-82   | 2B8    |
| CD117 (c-kit) PE Cy5   | eBioscience | 15-1171-81   | 2B8    |
| CD71 PE                | eBioscience | 12-0711-82   | R17217 |
| CD71 FITC              | eBioscience | 11-0711-82   | R17217 |
| Ter119 PE              | eBioscience | 12-5921-83   | Ter-119|
| Ter119 PE Cy7          | eBioscience | 25-5921-81   | Ter-119|
| Ter119 APC             | eBioscience | 17-5921-83   | Ter-119|
| CD105 PE               | eBioscience | 12-1051-81   | MJ7/18 |
| CD150 APC              | BioLegend   | 115910       | TC15-12F12.2 |
| CD3e Biotin            | eBioscience | 13-0033-85   | eBio500A2 |
| CD45R (B220) Biotin    | eBioscience | 13-0452-82   | RA3-6B2 |
| CD11b Biotin           | eBioscience | 13-0112-82   | M1/70  |
| Ly6G (Gr1) Biotin      | eBioscience | 13-5931-82   | RB6-8C5|
| Ly6A/E (Sca1) Biotin   | eBioscience | 13-5981-82   | D7     |
| CD41 Biotin            | eBioscience | 13-0411-82   | eBioMWReg30 |
| CD16/32 Biotin         | eBioscience | 13-0161-82   | 93     |
| PE Cy5.5 conjugated Streptavidin | eBioscience | 35-4317-82   |        |
| APC conjugated Streptavidin | eBioscience | 17-4317-82   |        |
| DRAQ5                  | eBioscience | 65-0880-92   |        |
| Propidium Iodide       | Invitrogen  | P3566        |        |
| DAPI                   | Invitrogen  | D1306        |        |
| BMI-1                  | AbCam       | Ab38295      |        |
| Actin                  | Sigma       | A1978        | AC-15  |

Supplemental Reference

England S.J., McGrath K.E., Frame J.M., Palis J. (2011). Immature erythroblasts with extensive ex vivo self-renewal capacity emerge from the early mammalian fetus. Blood 117, 2708-2717.

Fasano C.A., Dimos, J.T., Ivanova, N.B., Lowry, L., Lemischka, I.R., Temple S. (2007). shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. Cell Stem Cell 1, 87-99.
Kingsley P.D., Malik J., Emerson R.L., Bushnell T.P., McGrath K.E., Bloedorn L.A., Bulger M., Palis J. (2006). “Maturational” globin switching in primary primitive erythroid cells. Blood 107, 1665-1672.