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Stem Cell Reports
Report
OPEN ACCESS

Immortalization of Erythroblasts by *c-MYC* and *BCL-XL* Enables Large-Scale Erythrocyte Production from Human Pluripotent Stem Cells

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http://dx.doi.org/10.1016/j.stemcr.2013.10.010

SUMMARY

The lack of knowledge about the mechanism of erythrocyte biogenesis through self-replication makes the in vitro generation of large quantities of cells difficult. We show that transduction of *c-MYC* and *BCL-XL* into multipotent hematopoietic progenitor cells derived from pluripotent stem cells and gene overexpression enable sustained exponential self-replication of glycophorin A^+ erythroblasts, which we term immortalized erythrocyte progenitor cells (imERYPCs). In an inducible expression system, turning off the overexpression of *c-MYC* and *BCL-XL* enabled imERYPCs to mature with chromatin condensation and reduced cell size, hemoglobin synthesis, downregulation of *GCN5*, upregulation of *GATA1*, and endogenous *BCL-XL* and *RAF1*, all of which appeared to recapitulate normal erythropoiesis. imERYPCs mostly displayed fetal-type hemoglobin and normal oxygen dissociation in vitro and circulation in immunodeficient mice following transfusion. Using critical factors to induce imERYPCs provides a model of erythrocyte biogenesis that could potentially contribute to a stable supply of erythrocytes for donor-independent transfusion.

INTRODUCTION

Blood transfusion for patients with severe anemia depends on blood donations. However, factors such as declining birth rates, an aging population, and viral contamination are all associated with reductions in the availability of donor blood. It was once expected that $CD34^+$ cells from cord blood (CB) or bone marrow would someday provide a means for ex vivo expansion or in vitro generation of erythrocytes (red blood cells; RBCs) for transfusion (Giarratana et al., 2011), but the inability to produce sufficient numbers of $CD34^+$ cells remains a bottleneck in this process. It was also thought that the advent of pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), would eliminate the need for blood donation, but a series of differentiation trials to create various blood cells from human ESCs or iPSCs has highlighted the difficulty of obtaining blood cells in quantities sufficient for use in transfusion using this method (Lu et al., 2007, 2011; Takayama et al., 2008, 2010).

In hematopoietic systems, individual blood progenitor cells each appear to have their own proliferation program with "self-replication potential." Identification of the self-replication factors in lineage blood progenitors should enable immortalization of the cells. With that in mind, we have been developing a system based on the concept that immortalized progenitor cells can be created through self-replication. We recently observed that overexpression (O/E) of *c-MYC* in hematopoietic progenitor cells (HPCs) derived from human PSCs induces transient growth of the megakaryocyte-erythrocyte progenitor (MEP) population (Hirata et al., 2013; Takayama et al., 2010). Therefore, with the aim of generating large quantities of mature erythrocytes, we endeavored to determine the conditions for using a combination of *c-MYC* and related gene candidates to induce a self-replicating erythrocyte lineage at an immature stage.

RESULTS AND DISCUSSION

Creation of a Self-Replicable Erythrocyte-Producing Cell Line from Human PSCs

Because MEPs divide into erythrocytes and megakaryocytes (MKs) depending on the actions of specific transcriptional factors and cytokines (Hirata et al., 2013), we suspected that O/E of *c-MYC* plus erythropoietin (EPO) could be specific for erythrocyte self-replication. As expected, in the presence of EPO plus stem cell factor (SCF), O/E of *c-MYC* (but not mock) in HPCs derived from human ESCs (KhES-3) promoted proliferation of glycophorin A

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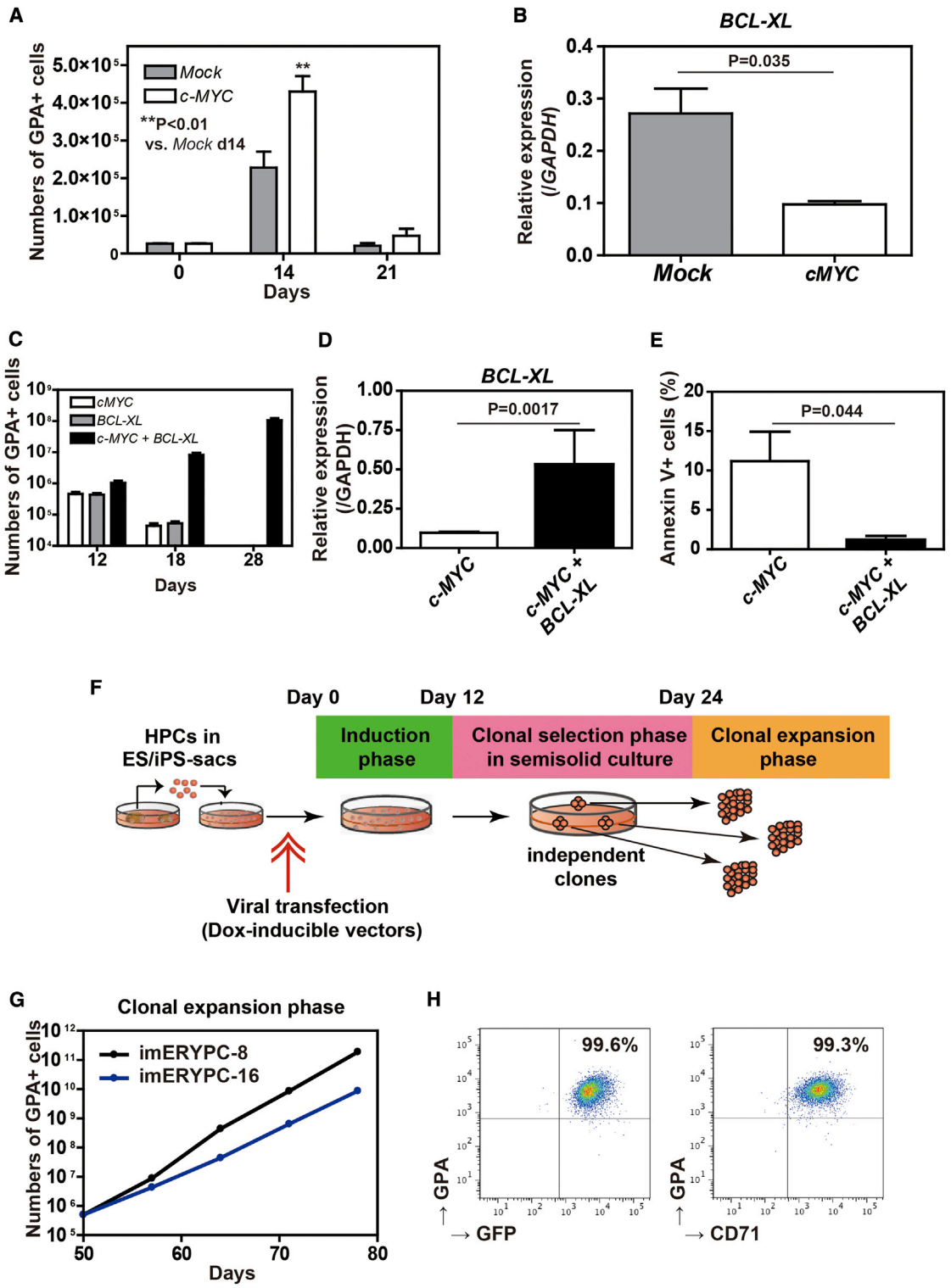

Figure 1. *c-MYC* and *BCL-XL* Are Self-Replication Factors for Erythrocyte Progenitors Derived from Human PSCs

(A) Numbers of glycophorin A (GPA)$^+$ cells transduced with *c-MYC* on days 0, 14, and 21.

(B) Expression of *BCL-XL* in cells transduced with vehicle (mock) or a *c-MYC* overexpression vector.

(C) Numbers of GPA$^+$ cells transduced with *c-MYC* alone, *BCL-XL* alone, or a combination of *c-MYC* plus *BCL-XL* on days 12, 18, and 28.

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(GPA)$^+$ cells. This growth advantage was only transient and disappeared 14 days after transduction (Figure 1A), which was caused by an increment in annexin$^+$ cells in the *c-MYC*-transfected erythrocytes, which we observed previously in *c-MYC*-transfected MKs (Takayama et al., 2010). However, growth was followed by an apoptosis event also caused by the O/E of *c-MYC*. The caspase family negatively regulates *BCL* family genes (Martinou and Youle, 2011). Of these, *Bcl-xl*/*BCL-XL* is reportedly suppressed by elevated *c-MYC* (Jayapal et al., 2010). Consistent with that report, we observed that BCL-XL mRNA levels were reduced in *c-MYC* transductants (Figure 1B). We therefore sought to prevent apoptosis through O/E of *c-MYC* plus *BCL-XL* in KhES-3-derived HPCs.

Transduction of *c-MYC* plus *BCL-XL*, but not *c-MYC* or *BCL-XL* individually, appeared to induce exponential growth that persisted for about a month (Figure 1C). Cells cotransfected with *c-MYC* and *BCL-XL* showed 5.4 times higher *BCL-XL* expression (Figure 1D) and a smaller annexin$^+$ fraction (Figure 1E) than cells transfected with *c-MYC* alone, indicating that *BCL-XL* contributed to an antiapoptotic effect in *c-MYC*-transfected erythroblasts.

To select stably proliferative clones over that period, we isolated cloned colonies using semisolid medium from day 12 to day 24 of culture (Figure 1F). During this clonal selection phase, we confirmed that only cells overexpressing *c-MYC* plus *BCL-XL* generated hematopoietic colonies in semisolid cultures (Figure S1A available online). Figure 1G depicts two independent clones in the clonal expansion phase. Both clones exhibited exponential growth (doubling times: clone 8, 36.8 hr; clone 16, 48.1 hr) for over 6 months. In addition, over 99% of the population expressed GPA and CD71, two phenotypic surface markers of erythroblasts found on erythrocytes derived directly from ESCs or cord blood cells (Figure 1H; unpublished data). We therefore named these cells immortalized erythrocyte progenitor cells (imERYPCs). The selected clones showed a dependency on EPO for growth but did not require SCF (Figure S1B) or feeder cells (Figure S1C), and they exhibited similar growth curves before and after cryopreservation (Figure S1D). Using this gene set, we generated stably proliferating GPA$^+$ erythrocyte progenitors from human iPSCs (Figures S1E and S1F). From these results, we conclude that *c-MYC* and *BCL-XL* are key mediators conferring self-replication potential on erythrocyte progenitors derived from human PSCs.

imERYPCs Are Capable of Differentiating to a Mature State with Heme Synthesis and Oxygen-Carrying Capability

We established two imERYPC clones, clone 8 and 16, that showed exponential cell growth (Figure 2A, DOX+). Interestingly, after turning genes off using a doxycycline (DOX)-inducible system, the imERYPCs stopped growing (Figure 2A, DOX−) and exhibited dramatic changes in morphology within 7 days after genes were turned off, going from basophilic immature erythroblasts to mature polychromatic/orthochromatic erythroblasts with chromatin condensation (Figures 2B and S2A), which was also seen with iPSC-derived imERYPCs (Figure S1G). Seven days after genes were turned off, 47%–52% of imERYPCs were polychromatic and 43%–50% were orthochromatic erythroblasts with 0.36% enucleation. By contrast, over 80% of cells with genes turned on were proerythroblasts (Figure S2A).

In imERYPCs with genes turned on, transmission electron microscopy (TEM) showed a relatively large nucleus with hypocondensed chromatin and mitochondria (Figure 2Ci). Downregulation of the genes induced mitochondrial aggregation, an increment in endosomal vacuoles (Figure 2Cii), and chromatin condensation in more mature imERYPCs (Figure 2Ciii). These changes, along with the morphological changes observed with Giemsa staining, reflect the physiological erythrocyte maturation phase (Simpson and Kling, 1967; Keerthivasan et al., 2010).

The imERYPC cell pellet was red 7 days after genes were turned off, reflecting heme synthesis (Figure 2D). O-dianisidine staining revealed that the fraction of heme$^+$ erythroblasts gradually increased after genes were turned off, ultimately reaching 100% (Figures 2E and S2B). At this stage, the average imERYPC contained 30.0 ± 3.0 (clone 8) or 37.4 ± 4.1 (clone 16) pg of hemoglobin. This is comparable to peripheral-blood-derived RBCs (PB-RBCs),

(D and E) Expression of *BCL-XL* (D) and the annexin$^+$ cell fraction (E) among cells transduced with *c-MYC* alone or *c-MYC* plus *BCL-XL*. Data in (A)–(E) represent the mean ± SE from three independent experiments.

(F) Schematic diagram of immortalized erythrocyte progenitor cell (imERYPC) induction from human PSCs. Hematopoietic progenitor cells (HPCs) within ES/iPS sacs were collected and transduced with *c-MYC* and *BCL-XL* using doxycycline (DOX)-inducible lentiviral vectors. The cells were then expanded for 12 days (induction phase). For clonal selection, on day 12 of culture, the cells were transferred to semisolid culture medium containing DOX and cultivated for an additional 12 days (clonal selection phase). The hematopoietic clones were then picked up and cultured separately for several months (clonal expansion phase).

(G) Numbers of GPA$^+$ cells derived from two highly proliferative imERYPC clones (clones 8 and 16) from day 50 to day 80 of culture. These clones grew exponentially for several months.

(H) Dot plots showing imERYPC clone 8 derived from human ESCs. Left panel: x axis, GFP; y axis, GPA. Right panel: x axis, CD71; y axis, GPA.

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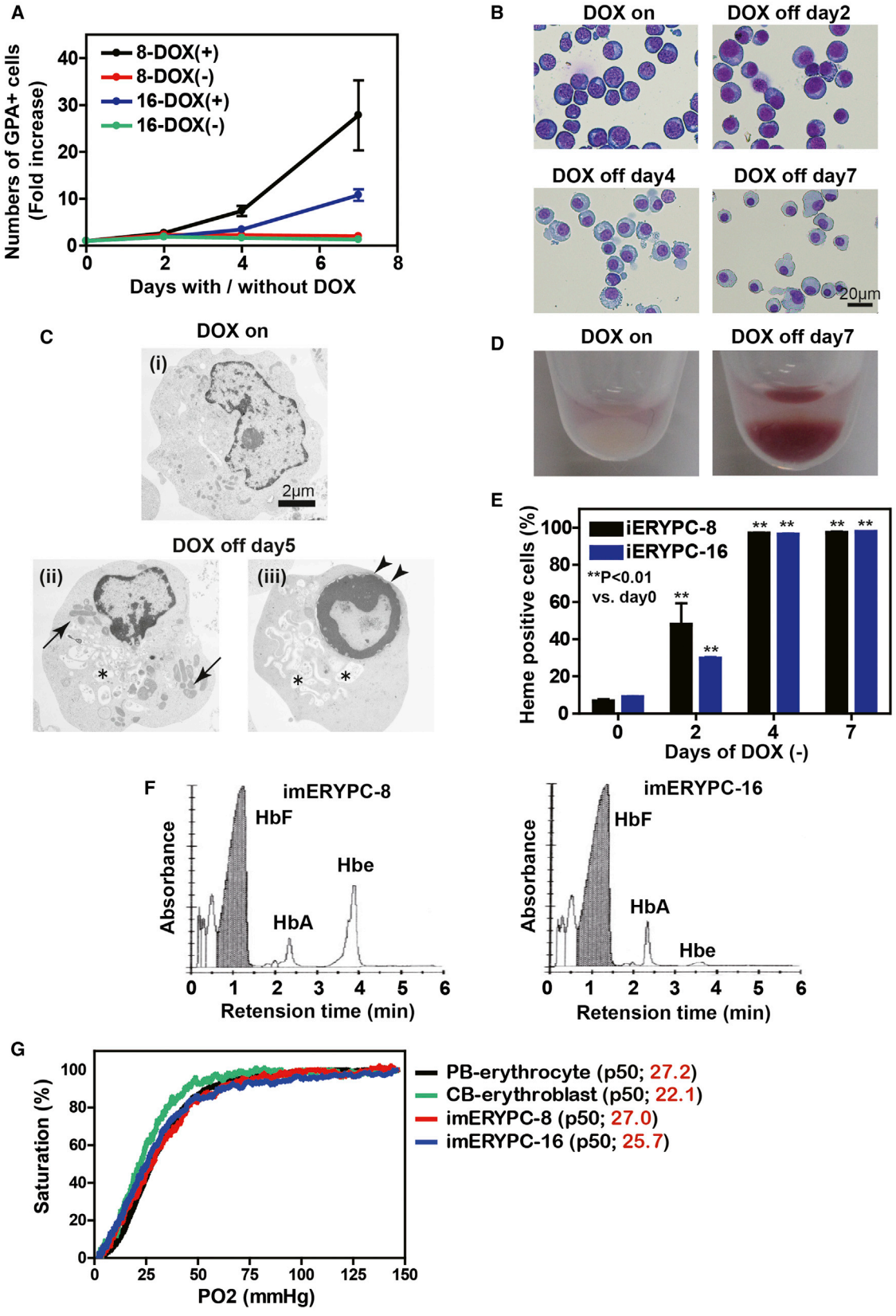

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which contain about 30 pg of hemoglobin per cell (Rappaz et al., 2008).

Human PSC-derived erythroblasts reportedly mainly express γ-globin, but also express small amounts of β- and ε-globin (Ma et al., 2008). To examine the globin types expressed in imERYPCs, we performed cation exchange high-performance liquid chromatography (CE-HPLC) and quantitative PCR (qPCR). In CE-HPLC, the majority of the hemoglobin in imERYPCs (DOX−) was hemoglobin F (HbF; $\alpha 2\gamma 2$), though small amounts of hemoglobin A (HbA; $\alpha 2\gamma 2$) and hemoglobin E (HbE; $\alpha 2\varepsilon 2$) were also present (Figure 2F; imERYPC-8: HbA, 4.5%; HbE, 18.4%; imERYPC-16: HbA, 5.8%, HbE 2.6%). Additional qPCR analysis showed the γ-globin level in imERYPCs to be similar to that in CB-derived erythrocytes (CB-RBCs) (Figure S2C), where HbF ($\alpha 2\gamma 2$) was the major type. Both imERYPC clone 8 and 16 expressed more ε-globin and less β-globin than CB-RBCs, but the levels remained minor fractions. These findings confirmed that imERYPCs produce fetal-type erythroblasts comparable to CB erythroblasts and have oxygen delivery capabilities (Figure 2G). imERYPC-derived erythrocytes (DOX−) showed oxygen dissociation curves (ODCs) similar to those of the CB- or PB-RBCs (Figure 2G; p50: PB, 27.2 mmHg; CB, 22.1 mmHg; imERYPCs, 27.0 mmHg or 25.7 mmHg, n = 3). Thus, HbF composed of α- and γ-globins is the major hemoglobin in imERYPCs.

The Transcriptional Program in ImERYPCs Traces Normal Erythrocyte Differentiation

We confirmed that, after genes were turned off, imERYPCs trace the physiological erythroblast differentiation phases (e.g., chromatin condensation and hemoglobin synthesis). To compare the transcriptional program operating during imERYPC differentiation to that operating in physiological erythroblasts, we used qPCR to assess the time dependence of the expression of *c-MYC* and *BCL-XL* along with *GATA1*, *RAF1*, and *GCN5* in imERYPCs (Figures 3A–3E) and CB-derived erythroblasts (Figures 3F–3J). After turning off exogenous *c-MYC* and *BCL-XL*, total *c-MYC* levels fell to one-tenth of that seen when overexpression was induced (Figure 3A), whereas total *BCL-XL* was upregulated as much as 20-fold, as compared to the overexpression state (Figure 3B), indicating elevated expression of endogenous *BCL-XL*. In fact, endogenous *BCL-XL* expression was ultimately augmented up to 45-fold once the exogenous gene was silenced (Figures S3A and S3B). The quick downregulation of *c-MYC* from the earlier overexpression stage may initiate the upregulation of *GATA1* and endogenous *BCL-XL* and *RAF1* (Figures 3C, 3D, and S3A) for heme synthesis (Hafid-Medheb et al., 2003; Haughn et al., 2003). This scheme might also accelerate the parallel downregulation of *GCN5* (Figure 3E), contributing to chromatin condensation (Jayapal et al., 2010). Intriguingly, these expression patterns were consistent with the temporal changes in gene expression seen in proliferative immature erythroblasts (Figure S3C, days 3–9) and nondividing mature erythroblasts (Figure S3C, days 12–18) derived from human cord blood $CD34^+$ cells (Figures 3F–3J and S3C). We therefore conclude that during differentiation to mature erythrocytes, the transcriptional program operating in imERYPCs is similar to that in their human erythrocyte equivalents (Figure 3).

Enucleated imERYPCs Are Capable of Intact Circulation In Vivo

We next sought to examine the in vivo functionality of circulating imERYPCs, since they seemingly have the capacity to deliver oxygen in vitro. To date, there are no well-established, in vivo models for evaluating transfusion of human erythrocytes. When 1×10^9 human PB-RBCs were intraperitoneally infused into nonobese diabetic (NOD) severe combined immunodeficiency (SCID) interleukin 2Rg (IL-2Rg) null (NOG) mice with irradiation-induced anemia,

Figure 2. Immortalized Erythrocyte Progenitor Cells Can Be Differentiated into Functional Erythroblasts Exhibiting Hemoglobin Synthesis and Chromatin Condensation after Genes Are Turned Off

(A) Numbers of the cells obtained from clones 8 and 16 cultured for 7 days in the presence or absence of DOX. The cells stopped growing when DOX was absent. Results are expressed as means ± SE from four independent experiments.

(B) Photomicrographs showing representative Giemsa staining of imERYPC clone 8 in the presence of DOX or 2, 4, and 7 days after DOX off.

(C) Transmission electron micrographs of imERYPC with DOX on (i) or 5 days after DOX off (ii and iii). Arrows indicate a mitochondrion. Arrowheads indicate chromatin condensation. Asterisks indicate endosomal vacuoles.

(D) Pictures showing representative imERYPC clone 8 pellets obtained in the presence or absence of DOX. In the absence of DOX, cells generate heme and show the characteristic red color.

(E) Percent heme-positive cells obtained 7 days after DOX withdrawal. Results are expressed as means ± SE from four independent experiments.

(F) Chromatograms showing CE-HPLC analyses of hemoglobin generated in imERYPC clones 8 and 16.

(G) Oxygen dissociation curves (ODCs) for adult erythrocytes derived from peripheral blood (PB-erythrocyte) and fetal erythrocytes differentiated from cord blood $CD34^+$ cells (CB-erythrocyte) in imERYPC clone 8 and clone 16.

(F) and (G) show representative data from three independent experiments.

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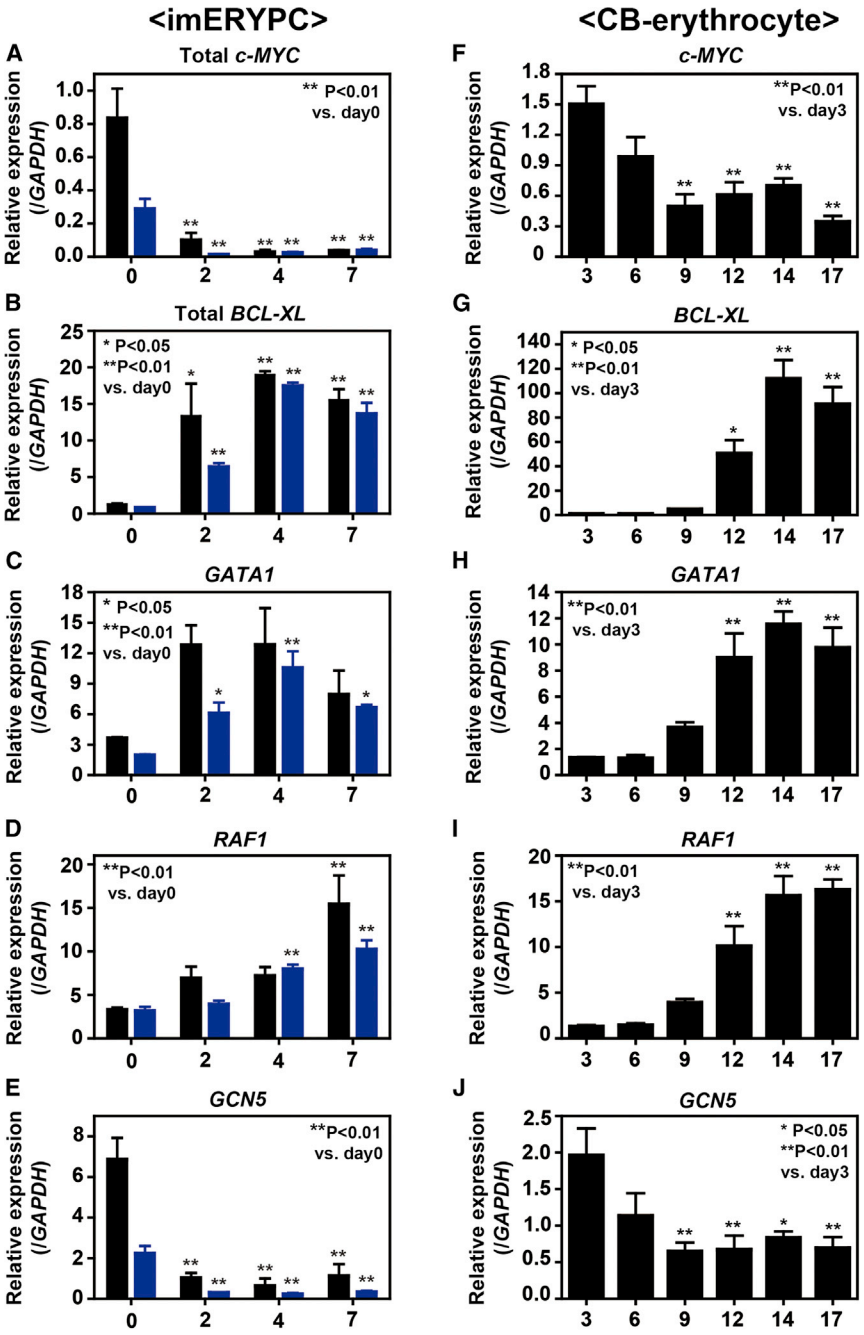

Figure 3. Quantitative PCR Analysis during the Course of imERYPC and CB-Derived Erythroblast Maturation

Time course of changes in the expression of total *c-MYC*, total *BCL-XL*, *GATA1*, *RAF1*, and *GCN5* in imERYPCs after genes were turned off (A–E) or CB-erythroblasts (F–J). Black and blue bars represent clones 8 and 16, respectively, in (A)–(E). Results are expressed as means ± SE from four independent experiments.

circulating levels peaked at less than 1.0% after 24 hr and then quickly declined (Figure 4A). We therefore sought to optimize the mouse model used to assess human erythrocyte transfusion. Earlier reports of erythrocyte transfusion into NOG mice suggested the efficacy of using infused PB-RBCs as a decoy (Giarratana et al., 2005, 2011; Jiménez-Díaz et al., 2009) or injecting clodronate-liposomes to deplete macrophages in vivo (Arnold et al., 2011; Hu et al., 2011). We therefore tested the effect of clodronate-liposomes on imERYPC circulation in mice pretreated with decoy RBCs.

NOG mice were irradiated with 2 Gy to induce anemia and were administered 2×10^9 PB-RBCs 13 days later as a decoy plus clodronate-liposomes (1 mg/body) or PBS. After an additional 24 hr, 1×10^9 imERYPCs stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) were administered intraperitoneally (Figure 4B). Samples collected from the retro-orbital plexus on days 1, 3, 5,

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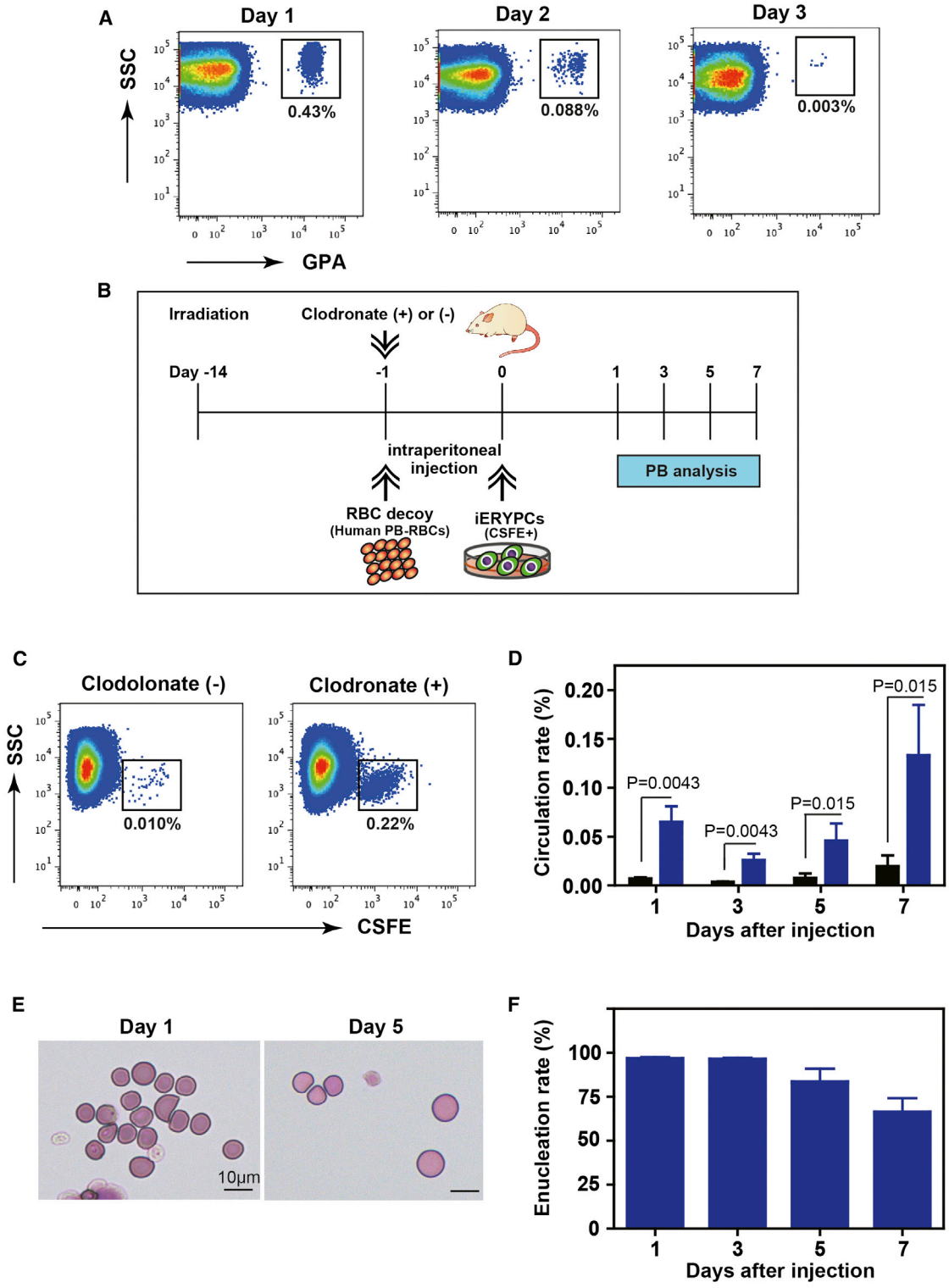

Figure 4. In Vivo Circulation and Enucleation of Immortalized Erythrocyte Progenitor Cells in NOG Mice

(A) Representative dot plots showing the chimerism of human PB-RBCs in NOD/SCID/IL-2Rg null (NOG) mice. A total of 1×10^9 RBCs were transfused intraperitoneally, after which PB samples were collected on days 1, 2, and 3.

(B) Schematic diagram of imERYPC transfusion into NOG mice. NOG mice irradiated with 2 Gy were intravenously administered clodronate-liposomes (1 mg/body, n = 6 from three independent experiments) or PBS (n = 6 from three independent experiments) and transfused

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and 7 after imERYPC administration confirmed the presence of circulating imERYPC erythrocytes, and although the efficiency remained low, it was augmented by clodronate-liposomes (Figures 4C and 4D). We noticed that nearly all of the imERYPCs circulating in the peripheral blood were enucleated (Figures 4E, 4F, and S4A), whereas the enucleation rate before transfusion was only 0.36% ± 0.03%, which suggests that enucleated and deformable erythrocytes might selectively circulate. Moreover, circulation of enucleated imERYPCs was slightly increased at 7 days, suggesting that only enucleated cells have in vivo circulation potential.

Alternatively, the low circulation rate might be caused by injury to imERYPCs in vitro, which would then be removed from the body within 2 hr after transfusion (Hod et al., 2010). To test that possibility, human PB-RBCs (100% enucleated) and carboxyfluorescein succinimidyl ester (CSFE)-labeled imERYPCs (>99% nucleated) were simultaneously infused intraperitoneally to clodronate-pretreated NOG mice, after which the chimerism was examined (Figure S4B). $CSFE^-$ human RBCs and $CSFE^+$ imERYPCs each peaked at around 24 hr, and the circulation was then observed for 120 hr, although the chimerism differed between the two groups (PB-RBCs: 0.1%–0.2%, imERYPCs: 0.01%; Figure S4C). These results show that although the number of PB-RBCs was 10-fold higher than imERYPCs, the kinetics of their chimerism were consistently similar (Figure S4C). This indicates that the lower in vivo rate of imERYPC circulation is due not to rapid removal but possibly to the lower rate of enucleation before transplantation.

In this study, we demonstrated that distinct features of *c-MYC* activation definitely control the proliferation stage at the progenitor level or the subsequent maturation stage, indicating c-MYC is a fate-determinant factor in erythrocytes derived from human PSCs (Acosta et al., 2008; Jayapal et al., 2010; Rylski et al., 2003). We also demonstrated that the distinct pattern of *BCL-XL* expression (i.e., lower during the proliferation phase and higher during the differentiation phase) supports erythrocyte maturation (Figures 3B and S3A), which is consistent with earlier reports (Hafid-Medheb et al., 2003; Haughn et al., 2003; Motoyama et al., 1995; Wagner et al., 2000).

Recent pioneering work produced the successful induction of a human iPSC-derived immortalized erythroid progenitor cell line and a human CB-derived erythroid progenitor cell line through transduction of HPV16-E6/E7 (Kurita et al., 2013). However, HPV16-E6/E7 causes uterocervical cancer and exerts a variety of oncogenic effects (zur Hausen, 2002). Moreover, HPV16-E6/E7 is not normally expressed in human cells and potentially changes the character of the cells. On the other hand, imERYPCs were established through transient activation of c-MYC and BCL-XL, originally expressed in endogenous erythrocyte progenitors, reflecting the physiological machinery of cell proliferation along with physiological characteristics, which contributes to the safety of clinical application.

We have calculated that, theoretically, direct differentiation from undifferentiated hiPSCs would require around 1,000–2,000 l of culture to produce 1 unit of RBCs (1×10^{12} cells), whereas less than 50–100 l of culture might be sufficient using a system of imERYPCs. In addition, imERYPCs are cultured in suspension without feeder cells and are easily manipulated, and a master cell stock of imERYPCs provides sufficient numbers of erythrocytes in a shorter period than hiPSCs. This scale is consistent with the imERYPC growth curve, even after thawing cells from cryopreservation (Figure S1). This strongly suggests that, with more efficient enucleation, master cell and working cell systems using imERYPCs have the potential to achieve a more constant and safer supply of RBCs for transfusion.

EXPERIMENTAL PROCEDURES

Ethics

The human ESC clone KhES-3 was used after approval for hESC use was granted by the Minister of Education, Culture, Sports, Science, and Technology of Japan (MEXT). Collection of peripheral blood from healthy volunteers was approved by the Ethical Committee of the Institute of Medical Science at The University of Tokyo and the Kyoto University Committee for Human Sample-Based Experiments. All studies involving the use of human samples were conducted in accordance with the Declaration of Helsinki. Animal experiments and the use of viral vectors were approved by the committees of University of Tokyo and Kyoto University.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found

intraperitoneally with 2×10^9 human peripheral RBCs 13 days after irradiation. Then 24 hr later they received 1×10^9 imERYPCs with 5 days of DOX off (clone 8) labeled with CFSE. Peripheral blood samples were then collected on days 1, 3, 5, and 7 after imERYPC transfusion.
(C) Representative dot plots showing the circulation chimerism of imERYPCs ($CSFE^+$) in mice treated with or without clodronate-liposomes.
(D) Peripheral blood chimerism of imERYPCs on days 1, 3, 5, and 7 after transfusion. Black and blue bars represent the circulation rates in mice treated with (blue bars) or without (black bars) clodronate-liposomes. Results are expressed as means ± SE.
(E) Representative Giemsa staining of sorted imERYPCs from mice treated with clodronate-liposomes.
(F) Percent enucleation of circulating imERYPCs in mice. The enucleation ratios were calculated as Hoechst-negative cells per total $CSFE^+$ imERYPCs. Results are expressed as means ± SE.

with this article online at http://dx.doi.org/10.1016/j.stemcr.2013.10.010.

AUTHOR CONTRIBUTIONS

S. Hirose, N. Takayama, and K.E. designed the experiments and wrote the manuscript; S. Hirose, N. Takayama, S.N., K.N., K.O., S. Hirata, S.S., S.Y., N. Takahashi, A.S., and K.E. performed experiments and analyzed data; T.Y. provided inducible lentiviral vector; M.O., M.I., T.K., and H.N. provided valuable discussions; and K.E. edited the manuscript.

ACKNOWLEDGMENTS

The authors thank Drs. N. Nakatsuji and H. Suemori (Institute for Frontier Medical Sciences, Kyoto University) for providing the human KhES-3 cell lines; Dr. S. Takeoka and K. Sou (Center for Advanced Biomedical Sciences/TWIns, Waseda University) for providing the Hemox analyzer; Dr. H. Masaki for providing hiPSC; Y. Niwa (Tosoh) for performing CE-HPLC; Y. Ishii, C. Lai, N. Mizuno, and M. Murakami for excellent technical support; and T. Sameshima (Terumo Corporation) for valuable discussion. This work was supported by the MEXT (Japan) Project for Realization of Regenerative Medicine (phase II) (to K.E.) and by a MEXT grant-in-aid (Kiban B to K.E.). This work was also supported in part by Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) from JSPS (to N. Takayama and K.E.) and by Terumo (to H.N.). H.N. and K.E. are founders and nonsalaried members of the Megakaryon scientific advisory board. H.N. is a founder and outside board member of ReproCell. H.N. obtained a research grant from the TERUMO Corporation. S. Hirose and S.S. are employed by the Terumo Corporation. S. Hirose and S.S. performed this research at the University of Tokyo as a visiting researcher. S.N., N. Takayama, H.N., and K.E. submitted the patent related with this manuscript.

Received: October 2, 2013
Revised: October 22, 2013
Accepted: October 22, 2013
Published: December 5, 2013

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