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

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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 glycoporphin 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 (KilnES-3) promoted proliferation of glycoporphin A
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.

Figure 1. (legend continued on next page)
clones grew exponentially for several months. 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 expressed by elevated c-MYC (Jayapal et al., 2010). Consistent with that report, we observed that BCL-XL mRNA levels were reduced in c-MYC-transfected MKs (Takayama et al., 2010). 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 polychromatocytochromeic erythroblasts with chromatins 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 polychromatocytochromeic and 43%–50% were orthochromatocytochromeic erythroblasts with 3.6% enucleation. By contrast, over 80% of cells with genes turned on were proerythroblasts (Figure S2A).

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 polychromatocytochromeic erythroblasts with chromatins 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 polychromatocytochromeic and 43%–50% were orthochromatocytochromeic erythroblasts with 3.6% 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 chromatins 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),
Erythrocytes from Immortalized Erythroblasts

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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; α2γ2), though small amounts of hemoglobin A (HbA; α2γ2) and hemoglobin E (HbE; α2ε2) 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 (α2γ2) 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 (Jaya pal 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 2Rγ (IL-2Rγ) null (NOG) mice with irradiation-induced anemia,
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 $2 \times 10^9$ PB-RBCs 13 days later as a decoy plus clodronate-liposomes (1 mg/body) or PBS. After an additional 24 hr, $1 \times 10^9$ 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,
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 $1 \times 10^9$ 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 (legend continued on next page).
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 (CFSE)-labeled imERYPCs (>99% nucleated) were simultaneously infused intraperitoneally to clodronate-pretreated NOG mice, after which the chimera was examined (Figure S4B). CFSE- human RBCs and CFSE+ imERYPCs each peaked at around 24 hr, and the circulation was then observed for 120 hr, although the chimera 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 chimera 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 iPSCs (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 × 1012 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
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., Y.N., T. Sameshima (Terumo Corporation) for valuable discussion. H.N. and T. Sameshima (Terumo Corporation) for valuable technical support; 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 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.

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