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ORIGINAL ARTICLE

Ex vivo large-scale generation of human red blood cells from cord blood CD34$^+$ cells by co-culturing with macrophages

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Abstract We generated red blood cells (RBC) from cord blood (CB) CD34$^+$ cells using a four-phase culture system. We first cultured CB CD34$^+$ cells on *telomerase* gene-transduced human stromal cells in serum-free medium containing stem cell factor (SCF), Flt-3/Flk-2 ligand, and thrombopoietin to expand CD34$^+$ cells (980-fold) and the total cells (10,400-fold) (first phase). Expanded cells from the first phase were liquid-cultured with SCF, interleukin-3 (IL-3), and erythropoietin (EPO) to expand (113-fold) and differentiate them into erythroblasts (second phase). To obtain macrophages for the next phase, we expanded CD34$^+$ cells from a different donor using the same co-culture system. Expanded cells from the first phase were liquid-cultured with granulocyte-macrophage colony stimulating factor, macrophage-colony stimulating factor (M-CSF), IL-3, and SCF to generate monocytes/macrophages (75-fold), which were incubated with type AB serum and M-CSF to fully differentiate them into macrophages. Erythroblasts were then co-cultured with macrophages in the presence of EPO to expand (threefold) and fully differentiate them (61% orthochromatic erythroblasts plus 39% RBC) (third phase). RBC were purified from erythroblasts and debris through a deleukocyting filter to generate 6.0×10^{12} RBC from 1.0 unit of CB (3.0 transfusable units). Qualitatively, these RBC showed a hemoglobin content, oxygenation of hemoglobin, and in vivo clearance similar to those of adult peripheral RBC. Finally, an almost complete enucleation of orthochromatic erythroblasts (99.4%) was achieved by the cultivation method recently described by Miharada et al. in the absence of macrophages and cytokines (fourth phase). RBC were purified from remnant erythroblasts and debris by passage through a deleukocyting filter to generate 1.76×10^{13} RBC from 1.0 unit of CB (8.8 transfusable units), the highest yield ever reported. Thus, this method may be useful for generating an alternative RBC supply for transfusions, investigating infectious agents that target erythroid cells, and as a general in vitro hematopoietic model system.

Keywords Red blood cell · Cord blood · CD34$^+$ cell · Stromal cell

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1 Introduction

Developing methods for the large-scale production of red blood cells (RBC) from hematopoietic stem cells (HSC) in vitro is important for generating an alternative source of

RBC. A new supply of RBC would be useful for blood transfusion and for investigating infectious agents that target erythroid cells [1, 2]. Giarratana et al. [3] reported that hematopoietic cells could be substantially expanded from human cord blood (CB) $CD34^+$ cells in liquid culture and completely differentiated into RBC by cultivation on a murine stromal cell line. The yield of RBC by their method was equivalent to 4.9 transfusable RBC units of random donor-derived packed RBC. However, the expansion rate of the CB $CD34^+$ cells in their liquid culture system was apparently lower than that in the co-culture system employing immortalized human stromal cells (*human telomerase catalytic subunit* gene-transduced stromal cells; *hTERT* stroma), which we previously established and used for the large-scale generation of platelets [4, 5]. Thus, there may be room for improving the yield of $CD34^+$ cells by employing our method. In addition, an advantage of using the *hTERT* stroma of human origin is that it should be more conducive to developing safer human therapies than the murine stromal cells employed in other studies [3]. Furthermore, we may be able to modify the immortalized *hTERT* stroma by transducing genes for hematopoietic factors, such as vascular endothelial growth factor (VEGF) [6], indian hedgehog [7], and HOXB4 [8], thereby increasing their ability to support HSC expansion. Recently, Miharada et al. [6] developed a method to produce RBC from CB $CD34^+$ cells in the absence of feeder cells, claiming that their method might be easier and less expensive than cultures with feeder cells. However, with our method, *hTERT* stroma could be stored frozen after expansion and ready for use, overcoming the drawbacks encountered with freshly preparing cells each time for use. Furthermore, the expansion rate of CB $CD34^+$ cells in their system was again significantly lower than that of the *hTERT* stroma system. The maximum enucleation rate of erythroblasts in their culture system was 77.5%, even when they promoted enucleation with mifepristone, an antagonist of glucocorticoids.

It has recently been suggested that under in vivo physiological conditions, macrophages are responsible for the terminal differentiation of erythroblasts to produce mature RBC [9, 10]. In retinoblastoma-deficient ($Rb^{-/-}$) embryos, macrophages are unable to interact with erythroblasts, and RBC production is impaired [9]. DNase-null mice [10] were not viable due to severe anemia, and fetal liver macrophages abundantly contained erythroid nuclei, suggesting that macrophage DNase II plays an essential role in destroying nuclear DNA in erythroblasts. Further, Hanspal et al. [11–13] reported that not only terminal differentiation, but also the proliferation of erythroblasts was significantly reduced in macrophage-depleted erythroid cultures.

Hence, in the present study, we attempted to generate RBC from human CB $CD34^+$ cells employing *hTERT* stroma, which can be easily expanded and stocked for multiple-use, and to generate drug-free macrophages by differentiating CB $CD34^+$ cells from another donor in a four-phase culture system. We succeeded in generating 8.8 transfusable RBC units from 1.0 unit of CB (5×10^6 $CD34^+$ cells), which is the highest yield ever reported with a 100% enucleation rate. In addition, our system may be used to establish excellent in vitro models of infectious diseases targeting erythroid cells, with which the pathobiology of these disorders may be intensively studied. Further, because each phase of the four-phase culture system represents a distinct proliferation and differentiation step of the erythroid lineage, our culture system may be used to study the molecular mechanisms of these steps.

2 Materials and methods

2.1 Sources of human CB $CD34^+$ cells and peripheral RBC

We collected CB from normal full-term deliveries and fresh RBC from healthy adult volunteers after obtaining written informed consent approved by the Sapporo Medical University Institutional Review Board, responsible for ensuring that the Declaration of Helsinki is abided by. To obtain CB $CD34^+$ cells, we first separated low-density mononuclear cells from CB by Histopaque-1077 (Sigma, St Louis, MO, USA) centrifugation, and then purified CB $CD34^+$ cells from mononuclear cells via positive selection using a MACS Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions. More than 90% of the selected cells were CD34-positive, as confirmed by fluorescence-activated cell sorting (FACS) analysis.

2.2 Cultivation of CB $CD34^+$ cells on *human telomerase catalytic subunit* gene-transduced stromal cells (*hTERT* stroma) for expansion of hematopoietic progenitor/stem cells: first-phase culture

We expanded hematopoietic progenitor/stem cells from CB $CD34^+$ cells using first-phase culture (days 0–14), as described previously [4, 5]. In brief, we seeded 500 CB $CD34^+$ cells on a monolayer of *hTERT* stroma that had been plated in a 75-cm^2 flask (Greiner Bio-One, Frickenhausen, Germany) in 10 mL serum-free X-VIVO10 medium (Bio Whittakar, Walkersville, MD, USA) supplemented with 10 ng/mL recombinant human stem cell factor

(SCF) (a gift from Kirin Brewery, Tokyo, Japan), 50 ng/mL recombinant human thrombopoietin (TPO) (a gift from Kirin Brewery), and 50 ng/mL recombinant human Flt-3/Flk-2 ligand (FL) (R&D Systems, Minneapolis, MN, USA) at 37°C in 5% CO_2. After 7 days (day 7), we added 10 mL of fresh complete medium containing the same concentration of cytokines and continued cultivation for another 7 days. At the end of this cultivation period, we assessed the numbers of total cells, erythroid cells, $CD34^+$ cells, glycophorin-A-positive cells (Gly-A^+ cells), and Rh-D^+ cells using the method described below.

2.3 Cultivation of hematopoietic progenitor/stem cells obtained from the first-phase culture for erythroid cell expansion: second-phase culture

In the second phase (days 15–28), we cultured 1×10^5 cells obtained from the first-phase culture in a six-well tissue-culture plate (NUNC, Roskilde, Denmark) in 3 mL of X-VIVO10 supplemented with 1% deionized BSA (PASEL + LOREI GmbH & Co., Hanau, Germany), 2% human AB serum (Sigma), 500 μg/mL diferric human transferrin (Sigma), 100 ng/mL SCF, 10 ng/mL recombinant human interleukin-3 (IL-3) (a gift from Kirin Brewery), and 4 IU/mL recombinant human erythropoietin (EPO) (a gift from Kirin Brewery) in the presence or absence of *hTERT* stroma at 37°C in 5% CO_2. After 7 days (day 21), we resuspended 1×10^6 cells in 3 mL of X-VIVO10 and continued culturing in a six-well tissue-culture plate for another 7 days. At the end of this cultivation period, we assessed the numbers of total cells, erythroid cells, Gly-A^+ cells, and Rh-D^+ cells using the method described below.

2.4 Preparation of human macrophages from CB $CD34^+$ cells

To obtain a sufficient number of macrophages, CB $CD34^+$ cells from another donor were cultured using the first-phase culture as above described. Cells (3×10^5) obtained from the first-phase culture were cultured in a six-well tissue-culture plate in 3 mL of X-VIVO10 at 37°C in 5% CO_2 for 10 days in the presence of 50 ng/mL recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) (R&D systems), 100 ng/mL macrophage-colony stimulating factor (M-CSF) (a gift from Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan), and 10 ng/mL IL-3 with or without 100 ng/mL SCF. After 10 days, non-adherent and adherent cells attached to the plate were harvested by vigorous pipetting, and monocytes/macrophages were purified by positive selection using MACS CD14 Micro Beads (Miltenyi Biotec) according to the manufacturer's instructions. To fully differentiate monocytes/macrophages into macrophages, they were incubated in a six-well tissue-culture plate with X-VIVO10 containing 2% AB serum and 100 ng/mL M-CSF at 37°C in 5% CO_2 for 4 days, as described by Hashimoto et al. [14] After removing non-adherent cells, adherent cells were used as macrophages for third-phase culture, as described below.

2.5 Co-cultivation of erythroid cells obtained from the second-phase culture with macrophages for further expansion and differentiation: third-phase culture

In the third phase (days 29–34), to further expand and differentiate erythroblasts from the second phase, we cultured cells (1×10^6) with various concentrations of macrophages that had been plated from day 25 in a six-well tissue culture plate in 3 mL of X-VIVO10 supplemented with 1% deionized BSA, 2% human AB serum, 500 μg/mL diferric human transferrin, and 4 IU/mL EPO at 37°C in 5% CO_2. For the control, erythroblasts were cultured without macrophages under the same conditions. At the end of this cultivation period, we assessed the numbers of total cells, erythroid cells, Gly-A^+ cells, and Rh-D^+ cells using the method described below.

2.6 Cultivation of erythroblasts obtained from the third-phase culture for enucleation: fourth-phase culture

At the end of the third-phase, erythroblasts were detached from macrophages and collected by gentle pipetting. Some macrophages had detached from the culture plate and contaminated the erythroblast preparation, but they could be removed due to their adherence to tissue-culture plastic. Then, the erythroblasts (5×10^6) were cultured for 4 days in 10 mL of enucleation medium [6] in a 25 cm^2 flask containing IMDM supplemented with 0.5% Plasmanate® Cutter (Bayer Japan, Yodogawa, Osaka, Japan), 14.57 mg/mL D-mannitol (Sigma), 0.14 mg/mL adenine (Sigma), and 0.94 mg/mL disodium hydrogenphosphate dodecahydrate (Sigma) with or without 1 μM mifepristone (an antagonist of the glucocorticoid receptor; Sigma) at 37°C in 5% CO_2. At the end of this cultivation period, we assessed the numbers of total cells, erythroid cells, Gly-A^+ cells, and Rh-D^+ cells using the method described below.

2.7 Cell counts and morphological analysis of the cultured cells

Cell numbers and morphology were assessed by the automated cell counter Z1 (Beckman Coulter, Fullerton, CA, USA) and May–Grünwald–Giemsa staining, respectively.

2.8 FACS analysis

Cultured cells were labeled with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies, washed, and resuspended in PBS. Antibodies to glycophorin-A and Rh-D (Chemicon Australia, Melbourne, Australia) were used for phenotyping. Controls were isotype-matched FITC- and PE-conjugated antibodies (Immunotech). Analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using Cell Quest software.

2.9 Purification of RBC

RBC from day 34 of culture were separated from remnant erythroblasts and debris by passage through a deleukocyting filter (Immuguard III-RC, Terumo, Tokyo, Japan). These cells were designated as $CD34^+$-RBC.

2.10 Hemoglobin (Hb) analysis

The proportion of Hb A and F in lysates of erythroblasts, $CD34^+$-RBC, and CB from a healthy volunteer were determined by high-performance liquid chromatography (HPLC) [15] on a cation-exchange TSK gel G7 HSi column (Tosoh Corporation, Tokyo, Japan). The Hb content of erythroblasts, $CD34^+$-RBC, and RBC from a healthy volunteer was determined photometrically at 610 nm [16]. Oxygen dissociation curves for Hb in RBC were measured using a Hemox-Analyzer (Technical Consulting Service, Medical Products Division, Southampton, PA, USA).

2.11 ABO blood type analysis of $CD34^+$-RBC

The blood type of $CD34^+$-RBC was analyzed using the Ortho Bioclone kit (Ortho-Clinical Diagnostics Inc., Tokyo, Japan) according to the manufacturer's instructions.

2.12 Detection of human RBC circulating in NOD/SCID mice

Six- to ten-week-old NOD/LtSz-scid/scid (NOD/SCID) mice purchased from Jackson Laboratory (Bar Harbor, ME, USA) were used. All animals were handled under sterile conditions and maintained in a microisolator. All animal experiments were performed in accordance with institutional guidelines approved by the Animal Care Committee of the Sapporo Medical University School of Medicine. Before RBC injection, mice were injected intraperitoneally with CL2MDP-liposome [17] three times (days −5, −3, and −1) to deplete macrophages and then sublethally irradiated with 3 Gy (day 0) to impair lymphocytes. $CD34^+$-RBC (1.5×10^8) or adult peripheral RBC were injected into the tail vein of mice, and the proportion of human RBC in the peripheral blood of mice was monitored by FACS. Heparinized blood was drawn by retro-orbital vein puncture at each time point and double-stained with anti-human Gly-A and the vital nucleic acid dye LDS-751 (LDS) [18, 19] to identify enucleated cells (LDS^- cells) in the human Gly-A-positive population.

3 Results

3.1 Expansion of $CD34^+$ cells in the first-phase

In the first-phase, we expanded hematopoietic progenitor/stem cells from 500 CB $CD34^+$ cells in 10 mL cultures in 75-cm^2 flasks according to our previous reports [5] ("Materials and methods", Fig. 1). At the end of the first-phase, total cell and $CD34^+$ cell numbers increased 10,400 ± 750-fold and 980 ± 97-fold, respectively (Table 1). Morphologically, 98% of the expanded cells were blast-like and the remaining 2% were monocytes/macrophages (Table 1; Fig. 2a). These data are consistent with those we previously reported [5]. Less than 1.0% of the cells expressed Gly-A and Rh-D (Table 1).

3.2 Expansion and differentiation of erythroblasts from $CD34^+$ cells in the second-phase

In the second-phase, we switched cultivation conditions to the liquid system in the presence of SCF, IL-3, and EPO because the number of erythroid cells that expanded during continuous cultivation on *hTERT* stroma was significantly lower than that in liquid cultivation in our preliminary experiments ("Materials and methods", Figs. 1, 3A). At the end of the second phase (day 28), the total cell number increased to $1.17 \times 10^6 \pm 9.2 \times 10^5$-fold of the input $CD34^+$ cells (Table 1). Morphologically, 95.6% of the cultured cells were erythroblasts and the remaining 4.4% were monocytes/macrophages (Table 1, Fig. 2b). There was a rapid increase in the expression of Gly-A and Rh-D, and the percentage of Gly-A- and Rh-D-positive cells was 92.1 and 74.7, respectively.

3.3 Expansion and differentiation of macrophages from $CD34^+$ cells

To obtain macrophages from $CD34^+$ cells, cells obtained from the first-phase culture were cultured for 10 days in the presence of (1) GM-CSF, M-CSF, IL-3, and SCF, or (2) GM-CSF, M-CSF, and IL-3. On day 25, the number of sorted $CD14^+$ cells obtained by culturing with GM-CSF, M-CSF, IL-3, and SCF (75.2 ± 4.6-fold of the input cells) was higher than that of $CD14^+$ cells obtained by culturing

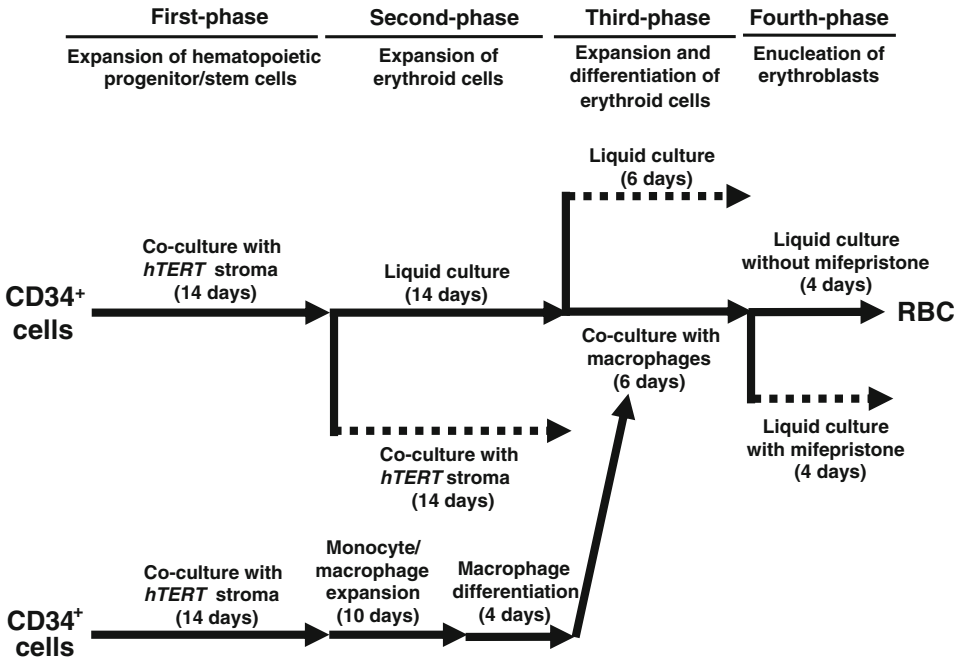

Fig. 1 Culture protocols of this study. Cord blood (*CB*) $CD34^+$ cells were co-cultured with *hTERT* stroma in the presence of SCF, TPO, and FL for 14 days in order to expand hematopoietic progenitor/stem cells (first-phase). Cells obtained from the first-phase were cultured with (*dotted line*) or without (*dashed line*) *hTERT* stroma in the presence of SCF, IL-3, and EPO for 14 days in order to expand erythroid cells (second-phase). Cells obtained from the second-phase without *hTERT* stroma were cultured with (*dashed line*) or without (*dotted line*) macrophages in the presence of EPO for 6 days in order to expand and differentiate erythroid cells (third-phase). Cells obtained from the third-phase with macrophages were cultured in the absence of any cytokines for 4 days with (*dotted line*) or without (*dashed line*) mifepristone to enucleate erythroblasts for the formation of RBC (fourth-phase). To obtain a sufficient amount of macrophages, CB $CD34^+$ cells from another donor were cultured using the first-phase culture as described. Cells obtained from the first-phase culture were cultured for 10 days in the presence of GM-CSF, M-CSF, IL-3, and SCF to expand monocytes/macrophages. Then, they were incubated with AB serum and M-CSF for 4 days to fully differentiate them into macrophages

with GM-CSF, M-CSF, and IL-3 (28.4 ± 3.8-fold) (Fig. 3B-a). To fully differentiate monocytes/macrophages into macrophages, cells were incubated with AB serum and M-CSF for 4 days. On day 28 after removing non-adherent cells, the number of adherent cells was almost the same as that of input $CD14^+$ cells on day 25 (Fig. 3B-b). Thus, we used the adherent cells on day 28 obtained by culturing first-phase culture cells with GM-CSF, M-CSF, IL-3, and SCF as macrophages for the third-phase culture, as described below.

3.4 Further expansion and maturation of erythroblasts in the third-phase

In the third-phase, we cultivated cells from the second-phase with or without macrophages obtained from differentiated CB $CD34^+$ cells of another donor ("Materials and methods", Fig. 1). At the end of the third-phase, when we collected erythroblasts by pipetting, some macrophages had detached from the culture plate and contaminated the erythroblast preparation. Therefore, we removed these macrophages from the preparation by adherence to tissue-culture plastic, and analyzed the number and proportion of erythroblasts. In preliminary experiments with macrophages, we altered the ratio of macrophages to erythroblasts from 1:10 to 10:10. As shown in Fig. 3C, preparation with macrophages resulted in a much more mature pattern, consisting of orthochromatic erythroblasts (61–84%) and RBC (16–39%) (Fig. 2c), than when prepared without macrophages, consisting of polychromatic (17%) and orthochromatic erythroblasts (77%) and RBC (6%) (Fig. 2d). Furthermore, the number of erythroid cells (erythroblasts plus RBC) was higher in preparations with (approximately threefold) than without (Fig. 3C) macrophages. When the numbers of erythroid cells and RBC were compared among the three preparations with macrophages, those cultured with a ratio of macrophages to erythroblasts of 1:10 were lower than those with ratios of 3:10 or 10:10 (there was no significant difference in the latter two preparations) (Fig. 3C). Thus, culture conditions with a macrophage to erythroblast ratio of 3:10 were used for the third-phase culture. At the end of the third-phase culture (day 34), the total cell number cultured with macrophages increased to $3.63 \times 10^6 \pm 1.84 \times 10^5$-fold

Table 1 Characteristics of cultured cells

Protocol	Day 14	Day 28	Day 34		Day 36				Day 38				Fresh RBC
			Mac(+)	Mac(−)	Mac(+) Mf(+)	Mac(+) Mf(−)	Mac(−) Mf(+)	Mac(−) Mf(−)	Mac(+) Mf(+)	Mac(+) Mf(−)	Mac(−) Mf(+)	Mac(−) Mf(−)	
Fold expansion of $CD34^+$ cells	980 ± 97	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Fold expansion of total cells	10400 ± 750	113 ± 12	3.1 ± 0.2	1.1 ± 0.1	1.2 ± 0.3	1.2 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	
Accumulated fold expansion cells ($\times 10^4$)	1.04 ± 0.08	117 ± 92	363 ± 18.4	117 ± 9.2	435 ± 5.0	435 ± 3.0	128 ± 0.9	140 ± 1.8	435 ± 0.5	435 ± 0.3	128 ± 0.1	128 ± 0.2	
Total cell number ($\times 10^7$)	0.52 ± 0.04	58.5 ± 46	182 ± 9.2	58.5 ± 4.6	218 ± 2.5	218 ± 1.5	64 ± 0.5	70 ± 0.9	218 ± 0.3	218 ± 0.2	64 ± 0.1	64 ± 0.1	
% Gly-A^+ cells[a]	<1	92.1 ± 7.2	94.1 ± 3.3	92.0 ± 4.1	94.0 ± 3.9	94.2 ± 3.1	93.8 ± 3.7	94.0 ± 2.3	96.1 ± 3.8	96.2 ± 3.4	94.9 ± 4.9	94.2 ± 3.3	95.1 ± 3.5
%Rh-D^+ cells[a]	<1	74.7 ± 2.9	95.8 ± 4.7	93.0 ± 3.9	95.6 ± 2.7	96.3 ± 2.5	94.5 ± 3.9	95.7 ± 2.9	96.5 ± 2.8	96.7 ± 2.3	96.1 ± 3.2	95.5 ± 3.3	95.6 ± 3.4
% Ortho cells[a]	<1	52.2 ± 4.7	56.3 ± 4.4	63.4 ± 5.5	<1	<1	43.2 ± 3.5	52.0 ± 4.9	<1	<1	23.1 ± 4.3	43.8 ± 5.3	
% RBC[a]	0	0	39.4 ± 2.9	6.2 ± 1.9	99.3 ± 5.6	99.4 ± 4.5	50.8 ± 4.6	42.0 ± 3.6	99.5 ± 5.8	99.5 ± 3.5	72.9 ± 5.3	50.2 ± 4.5	
RBC number ($\times 10^5$)	0	0	7100 ± 26	360 ± 8	21600 ± 14	21600 ± 6	3250 ± 2	2940 ± 3	21600 ± 2	21600 ± 1	4670 ± 1	4670 ± 1	
Filtrated RBS number ($\times 10^8$)[b]	ND	ND	6.0 ± 0.4	ND	ND	ND	ND	ND	ND	17.6 ± 2.6	ND	ND	

Days 14, 28, 34, and 36 (38) represent cells at first-phase, second-phase, third-phase and fourth-phase, respectively. Five hundred cord blood $CD34^+$ cells were co-cultured with *hTERT* stroma in the presence of SCF, TPO, and FL in the first phase. 1×10^5 cells obtained from the first-phase were cultured without *hTERT* stroma in the presence of SCF, IL-3, and EPO in the second-phase. 1×10^6 cells obtained from the second-phase were cultured with or without macrophages (*Mac*) in the presence of EPO in the third-phase. 5×10^6 cells obtained from the third-phase were liquid-cultured with enucleation medium (see "Materials and methods") in the presence or absence of mifepristone (*Mf*) in the fourth-phase. Data shown are mean ± SD of triplicate cultures and are representative of five experiments

Mac(+) cultivation with macrophages in the third-phase, *Mac*(−) cultivation without macrophages in the third-phase, *Mf*(+) cultivation in the presence of mifepristone, *Mf*(−), cultivation in the absence of mifepristone, Ortho cells orthochromatic erythroblasts, *Gly-A* glycophorin A, *ND* not determined, *Fresh RBC* adult peripheral RBC

[a] Percent of total cells

[b] After passage through a deleukocyting filter

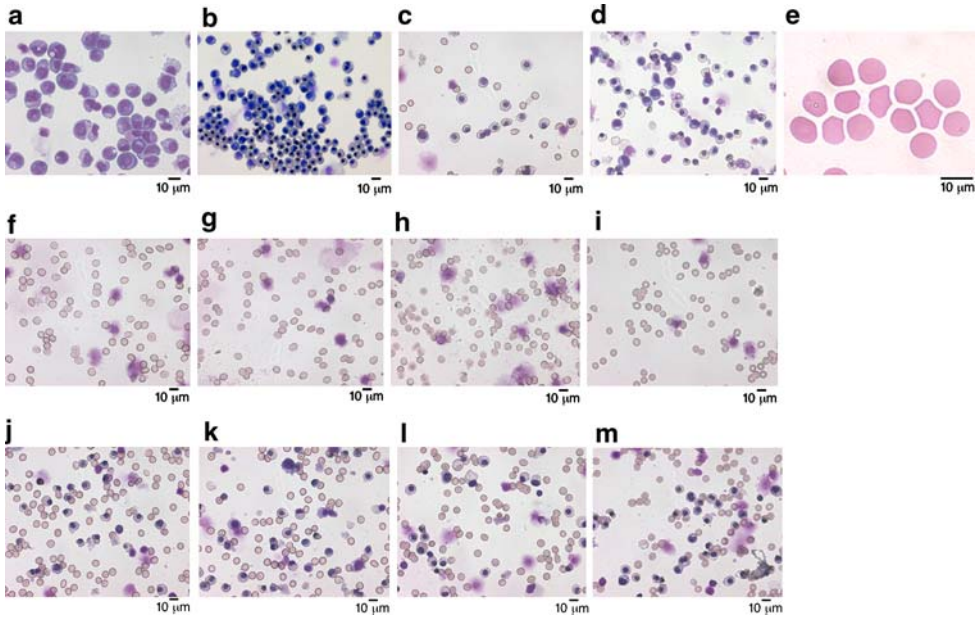

Fig. 2 Morphologic appearance of cultured cells. **a** Day 14 cells, **b** day 28 cells, **c** day 34 cells cultured with macrophages in the third-phase, **d** day 34 cells cultured without macrophages in the third-phase, **e** RBC sorted by deleukocyting filters for transfusion from day 34 cells, which were cultured with macrophages in the third-phase and without mifepristone in the fourth-phase, **f** day 36 cells cultured with macrophages in the third-phase and with mifepristone in the fourth-phase, **g** day 36 cells cultured with macrophages in the third-phase and without mifepristone in the fourth-phase, **h** day 38 cells cultured with macrophages in the third-phase and with mifepristone in the fourth-phase, **i** day 38 cells cultured with macrophages in the third-phase and without mifepristone in the fourth-phase, **j** day 36 cells cultured without macrophages in the third-phase and with mifepristone in the fourth-phase, **k** day 36 cells cultured without macrophages in the third-phase and without mifepristone in the fourth-phase, **l** day 38 cells cultured without macrophages in the third-phase and with mifepristone in the fourth-phase, **m** day 38 cells cultured without macrophages in the third-phase and without mifepristone in the fourth-phase

of the input $CD34^+$ cells (Table 1). Morphologically, 39.4 and 6.2% of the cultured cells with or without macrophages were RBC, respectively. There was an increase in the expression of Rh-D. The expression of Gly-A was still present in more than 90%. These data indicate that the cultured cells matured to erythroblasts. RBC from the day 34 culture ($CD34^+$-RBC) were separated from remnant erythroblasts and debris by passage through a deleukocyting filter (Fig. 2e) and counted. The recovery rate from filtration was 84.6 ± 4.1% (mean ± SD of five experiments). Therefore, our calculations indicate that 6.0×10^8 RBC were obtained from 500 $CD34^+$ cells by the three-phase culture system (Table 1). This in vitro yield of RBC is equivalent to 3.0 transfusable units of random donor-derived packed RBC from 1.0 unit of CB (5×10^6 $CD34^+$ cells).

3.5 RBC generation in the fourth-phase

Miharada et al. [6] recently developed a method to efficiently produce enucleated RBC from erythroblasts using mifepristone, an antagonist of glucocorticoid [20], in the absence of feeder cells such as macrophages. Thus, in the fourth phase, we cultured the cells from the third-phase by the cultivation method reported by Miharada et al. [6] with or without mifepristone ("Materials and methods", Fig. 1) for 4 days to obtain a larger amount of RBC. Nearly 100% of the erythroblasts obtained from third-phase culturing with macrophages were enucleated irrespective of the presence of mifepristone in the medium both on day 36 (Table 1, Fig. 2f, g) and day 38 (Table 1, Fig. 2h, i). These results suggest that mifepristone is not essential for the complete enucleation of erythroblasts in our culture system. On the other hand, 100% of erythroblasts obtained from third-phase culturing without macrophages were not enucleated on both days 36 and 38 of the fourth-phase (Table 1, Fig. 2j–m). Enucleation rates of erythroblasts obtained from third-phase culturing without macrophages were higher in the presence of mifepristone (Table 1, Fig. 2j, l) as compared to controls (Table 1, Fig. 2k, m), and higher on day 38 (Fig. 2l, m) than on day 36 (Fig. 2j, k). On days 36 and 38, the total cell number was almost the same as that on day 34. RBC from the day 38 culture were separated from remnant erythroblasts and debris by passage through a deleukocyting filter and were counted. The recovery rate from filtration was 80.8 ± 3.1% (mean ± SD of five experiments). Therefore, our calculations indicate that 1.76×10^9 RBC were obtained from

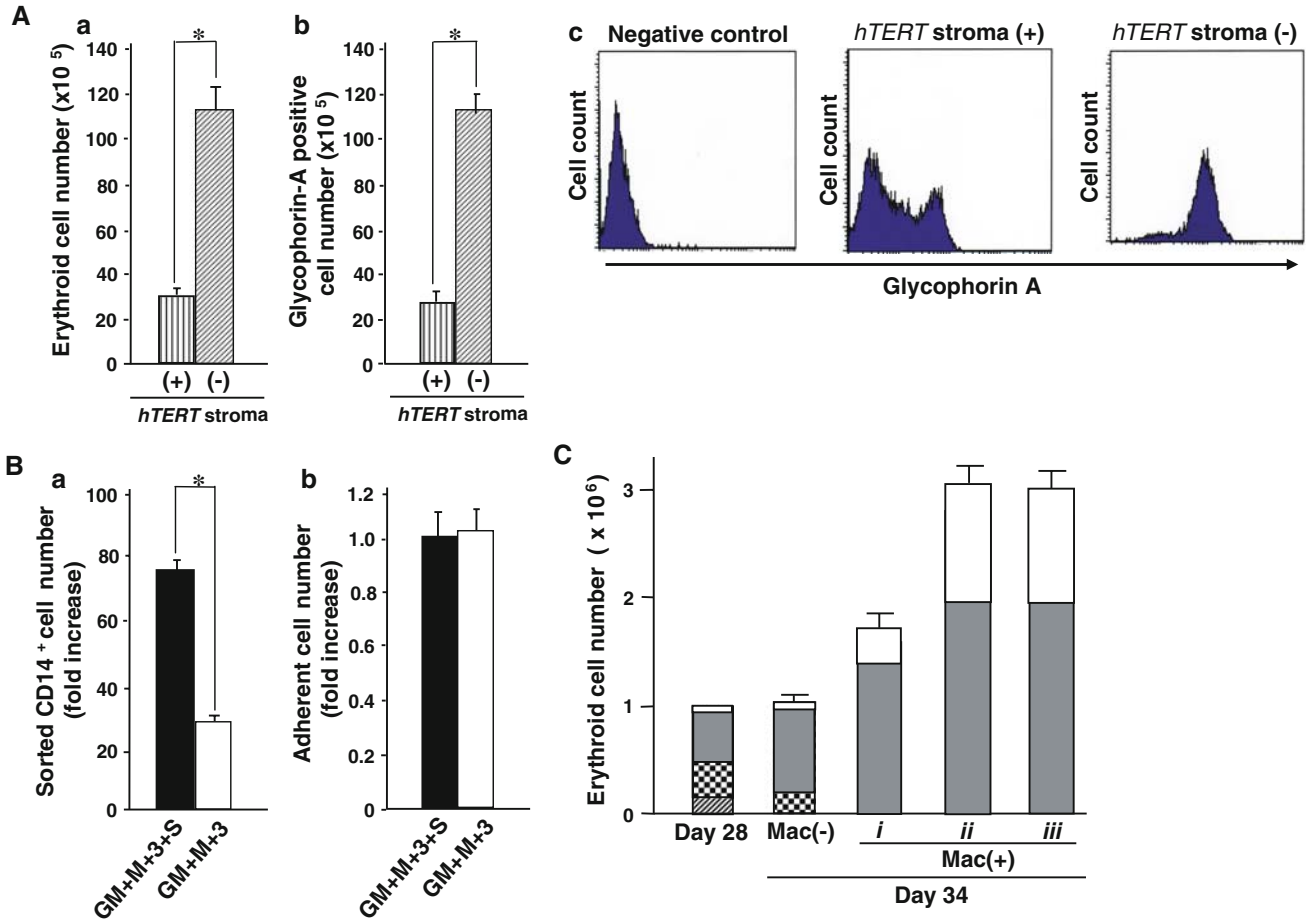

Fig. 3 Expansion of erythroid cells by second-phase culture and further proliferation and differentiation of erythroblasts by co-culturing with macrophages. **A** Cells (1×10^5) obtained from the first-phase were cultured in 3 mL X-VIVO10 supplemented with BSA, human transferrin, human AB serum, SCF, IL-3 and EPO with (*vertical bar*) or without (*light diagonal bar*) *hTERT* stroma for 7 days. Then, cells were resuspended to a concentration of 1×10^6/3 mL in fresh complete medium and cultured for another 7 days. Erythroid cell (erythroblasts plus RBC) numbers (*a*) and glycophorin-A-positive cell numbers (*b*) were analyzed on day 28 by measuring the glycophorin-A positive percentage by FACS (*c*). Data shown are mean ± SD of triplicate cultures and are representative of five experiments. *hTERT* stroma (+), cultivation with *hTERT* stroma; *hTERT* stroma (−), cultivation without *hTERT* stroma; *, $P < 0.05$. **B** Cells (3×10^5) obtained from the first-phase were cultured in 3 mL X-VIVO10 supplemented with GM-CSF, M-CSF, IL-3, and SCF (*filled bar*) or GM-CSF, M-CSF, and IL-3 (*open bar*) for 10 days. After the 10 days, non-adherent and adherent cells attached to the plate were harvested, and monocytes/macrophages were selected as $CD14^+$ cells (Materials and methods). To fully differentiate monocytes/macrophages into macrophages, they were incubated in X-VIVO 10 supplemented with AB serum and M-CSF for 4 days. On day 28 after removing non-adherent cells, adherent cells were used as macrophages. The numbers of sorted $CD14^+$ cells on day 25 (*a*) and adherent cells on day 28 (*b*) were counted. Data shown are mean ± SD of triplicate cultures and are representative of five experiments. GM + M + 3 + S, cultivation in the presence of GM-CSF, M-CSF, IL-3, and SCF; GM + M + 3, cultivation in the presence of GM-CSF, M-CSF, and IL-3; *, $P < 0.05$. **C** Erythroblasts (1×10^6) obtained from the second-phase were co-cultured with 0, 1×10^5 (*i*), 3×10^5 (*ii*), or 10×10^5 (*iii*) macrophages plated from day 25 in the presence of EPO. Aliquots of the cultures were taken at the indicated times for cell counts and morphologic analysis of the cells. Proerythroblasts or basophilic erythroblasts (*dark diagonal bar*); polychromatic erythroblasts (*checked bar*); orthochromatic erythroblasts (*gray bar*); RBC (*white bar*). Data shown are the mean percentages of the total cells from triplicate cultures and are representative of five experiments. Mac(−), cultivation without macrophages; Mac(+), cultivation with macrophages

500 $CD34^+$ cells by the four-phase "stroma-supported macrophage co-culturing system" on day 38 (Table 1). To the best of our knowledge, this in vitro yield of RBC is the highest ever reported and is equivalent to 8.8 transfusable units of random donor-derived packed RBC from 1.0 unit of CB (5×10^6 $CD34^+$ cells).

3.6 Characterization of $CD34^+$-RBC

The surface markers (Table 1) and Hb content (Fig. 4a-*i*) of the $CD34^+$-RBC were identical to those of adult peripheral RBC. However, the Hb A/F ratio of $CD34^+$-RBC was somewhat low compared to that of adult

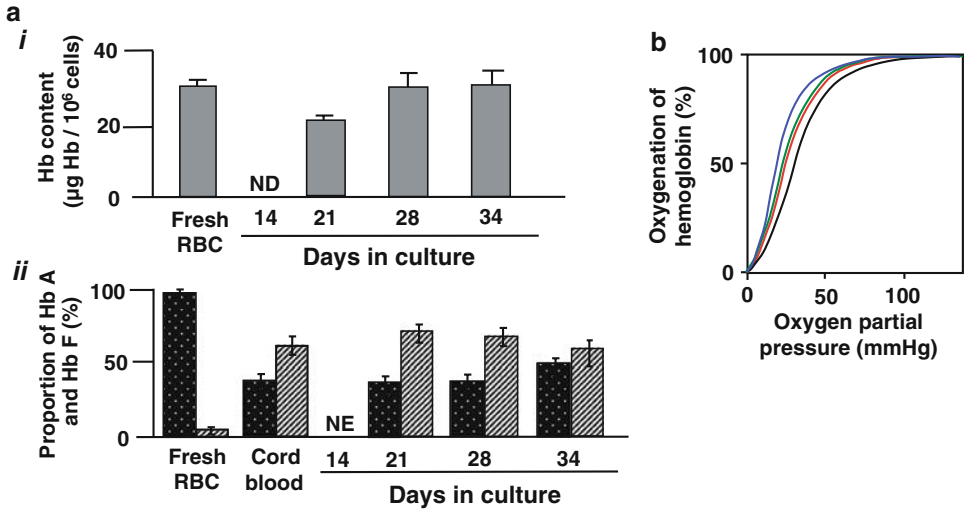

Fig. 4 Functional study of the manufactured RBC. **a**-*i* The hemoglobin (*Hb*) content in cord blood (*CB*)-derived erythroblasts and RBC obtained on day 34 ($CD34^+$-RBC) by culturing with macrophages in the third-phase, expressed as μg $Hb/10^6$ cells, was determined on the indicated days of cultivation. Fresh RBC obtained from the peripheral blood of three healthy adult volunteers were used as the control. Data shown are mean ± SD of triplicate cultures and are representative of five experiments. *ND* not detected. **a**-*ii* HPLC analysis of Hb on the indicated day of culture. Fresh RBC obtained from the peripheral blood of three healthy adult volunteers and CB obtained from three healthy donors were used as the controls. *Dotted bar*, HbA; *dark diagonal bar*, HbF; data shown are mean ± SD of triplicate cultures and are representative of five experiments. *NE* not examined. **b** The oxygen dissociation curve of RBC obtained on day 34 ($CD34^+$-RBC) by culturing with macrophages in the third phase was measured with a Hemox-Analyzer. Fresh RBC obtained from a healthy adult volunteer, peripheral blood, and red cell concentrate in mannitol–adenine–phosphate solution (RC-MAP) were used as controls. Fresh RBC (*balck curve*); Fresh CB RBC (*green curve*); RC-MAP (*blue curve*); RBC obtained on day 34(*red curve*). Data shown are representative of five experiments

peripheral RBC but similar to that of fresh CB (Fig. 4a-*ii*). The blood type of $CD34^+$-RBC was also identical to that of CB (data not shown). The O_2 dissociation curve (Fig. 4b) of $CD34^+$-RBC was nearly identical to those of fresh CB RBC and adult peripheral RBC. The fact that $CD34^+$-RBC did not express LDS at levels similar to adult peripheral RBC also indicated that $CD34^+$-RBC were mature RBC (Fig. 5a-*i*,*ii*). Although the half-life of adult peripheral RBC in the NOD/SCID mice was much shorter than when in human circulation, the in vivo clearance of $CD34^+$-RBC was nearly identical to that of adult peripheral RBC (Fig. 5a-*i*,*ii* b-*i*,*ii*). To examine the feasibility of using erythroblasts as an alternative source of RBC in transfusion, as reported by Neildez-Nguyen et al. [21], we also intravenously injected erythroblasts from the second-phase culture (day 28) into NOD/SCID mice treated with 3 Gy irradiation without CL2MDP-liposome. We observed no erythroblasts nor RBC in circulation (Fig. 5a-*iii* b-*iii*).

3.7 *hTERT* stroma-free and macrophage-free preparation of RBC

We determined via FACS analysis that $CD34^+$-RBC did not express the stromal cell antigens CD73, CD105, and CD166 and the monocyte/macrophage antigens CD11b, CD14, and CD15 (data not shown).

4 Discussion

Large scale, ex vivo-differentiated RBC from HSC provide transfusion source, desperately needed for severe acute bleeding as most medical facilities frequently suffer from a shortage of RBC products. New types of blood substitutes based on cell-free Hb solutions initially generated a significant amount of optimism [22]. However, serious adverse effects, including hypertension, bradycardia, and gastrointestinal symptoms as a consequence of arteriolar vasoconstriction, were encountered during clinical trials [23].

To overcome the drawbacks of cell-free Hb, Giarratana et al. [3] and Miharada et al. [6] produced RBC from CB $CD34^+$ cells by employing an in vitro-culturing system. However, the yields of RBC by the method of Giarratana et al. [3] and Miharada et al. [6] were at most 4.9 and 1.8 transfusable RBC units from one CB unit (5×10^6 $CD34^+$ cells), respectively. In the present study, we succeeded in producing 8.8 transfusable RBC units, two-times as many RBC as Giarratana et al. [3] and five-times that of Miharada et al. [6] Furthermore, our RBC exhibited features such as a surface marker expression, Hb content, oxygenation of Hb, and in vivo clearance similar to those of adult peripheral blood RBC. Thus, our RBC preparation appears to hold promise for future

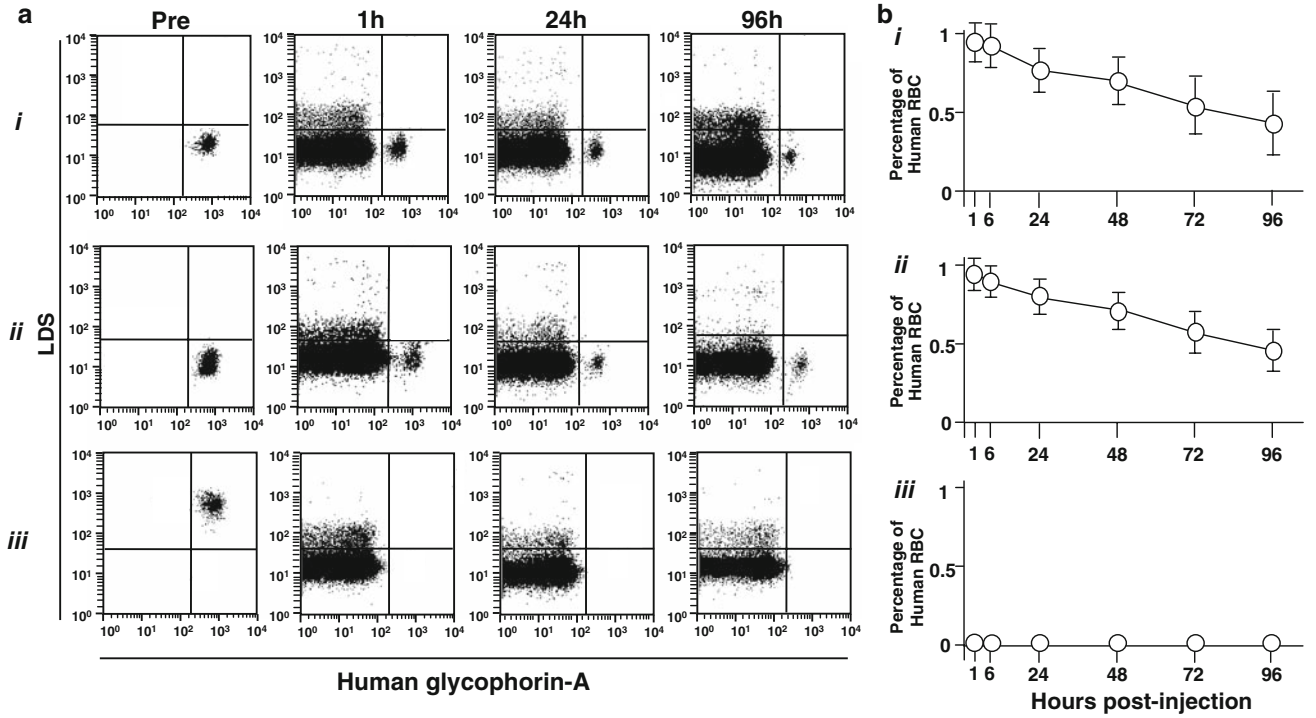

Fig. 5 In vivo clearance of cord blood (*CB*)-derived RBC in NOD/SCID mice. **a** FACS analysis of human erythroid cells in the peripheral blood of NOD/SCID mice injected with the cultured cells. Aliquots of the cells taken at the indicated times were stained with anti-human glycophorin-A (*GPA*) and LDS, and the percentage of human RBC (GPA^+LDS^- cells) was analyzed. **a**-*i* Blood cells from mice injected with $CD34^+$-RBC; **a**-*ii* blood cells from mice injected with healthy adult human peripheral blood; **a**-*iii* blood cells from mice injected with human erythroblasts obtained on day 28 of the second-phase. Data shown are representative of three mice. *LDS* laser dye styryl, *Pre* pre-injection. **b** The percentage of human RBC in the peripheral blood of NOD/SCID mice injected with $CD34^+$-RBC (**b**-*i*), healthy adult human peripheral blood (**b**-*ii*), and human erythroblasts obtained on day 28 of the second-phase (**b**-*iii*). Data shown are mean ± SD of three mice and are representative of three experiments

transfusion applications, both in terms of quality and quantity.

One of the techniques of particular emphasis in the present study was the use of *hTERT* stroma for the first-phase of cultivation. In addition to the obvious safety advantage of using the same species of stromal cells [3, 8, 24], employing *hTERT* stroma for the large-scale cultivation of HSC is made amenable by multiple uses of stocked preparations. In fact, in the current study, and consistent with our previous results [6], we were able to expand $CD34^+$ cells approximately 1,000-fold, a considerable expansion rate that has never been achieved by the non-stromal methods hitherto reported [25–27]. We have previously shown that Indian hedgehog gene-transduced *hTERT stroma* were indeed more potent in supporting burst-forming unit-erythroid (BFU-E) and in NOD/SCID-$\beta 2m^{-/-}$ mice repopulating cells than mock transduced stromal cells [7]. Thus, developing more potent *hTERT* stroma via the introduction of any hematopoietic factor genes may be the next step.

In the second-phase culture, we chose a liquid culture system, eliminating *hTERT* stroma support, based upon our findings that the efficiency of the differentiation of first-phase culture cells into erythroblasts by co-culture with *hTERT* stroma was inferior to that of cells cultured without *hTERT* stroma (Fig. 3A). The most likely explanation is that there is a mechanism inhibiting the differentiation of HSC into erythroblasts in the presence of *hTERT* stroma. Elucidation of this mechanism is also a crucial future task.

As for the cytokine combination (SCF, IL-3, and EPO) for the second-phase culture, we essentially followed a report by Oda et al. [28], who produced pure human erythroid cells from peripheral blood $CD34^+$ cells, with the exception that we added concentrated transferrin [3, 6] to the described medium.

The second technical advance emphasized in the present study is the use of macrophages. Although previous circumstantial evidence suggested the crucial role of macrophages in erythroblast maturation and the enucleation process [9–13], no attempts had been made to utilize macrophages for the in vitro generation of RBC. Our data indicate that macrophages can be expanded sufficiently from CB $CD34^+$ cells for future clinical application (Fig. 3B), and were indeed potent stimulators of erythroblast proliferation, of erythroblast maturation from

basophilic erythroblasts to orthochromatic erythroblasts, and of orthochromatic erythroblast enucleation. Numerous adhesion molecules and their receptors have been reported to be involved in the formation of erythroblastic islands consisting of macrophages and surrounding erythroblasts [29–31], phagocytosis of apoptotic cells by macrophages [32, 33], and enucleation of erythroblasts by macrophages [11–13]. Thus, we investigated the molecules responsible for erythroblast differentiation, expansion, and enucleation by macrophages. Though the expressions of VLA-4 [29] and phosphatidylserine (PS) [32, 33] on erythroblasts from day 28 and VCAM-1 [29], αv-integrin (CD51) [30, 31], and β3-inetgrin (CD61) [30, 31] on macrophages were evident by FACS, none of the antibodies to or inhibitors of these molecules suppressed the expansion and maturation of erythroblasts or enucleation in the third-phase culture (data not shown). On the other hand, the incubation of erythroblasts with heparin dose-dependently inhibited their differentiation, expansion, and enucleation (data not shown), suggesting that the heparin-binding protein erythroblast macrophage protein (Emp) [11–13] is involved in the differentiation, expansion, and enucleation of erythroblasts into RBC in our culture system. A rather unexpected result of the present study was that the enucleation rate in the third phase was at most 39%. The fact that the enucleation rate increased when the proportion of macrophages to erythroblasts was increased from 1:10 to 10:10, plateauing at 3:10, suggests that, as far as the enucleation process is concerned, the number of macrophages is not the sole critical factor.

Although the half-life of adult peripheral RBC in the NOD/SCID mice was much shorter than when in human circulation, $CD34^+$-RBC showed clearance curves similar to adult peripheral RBC, also supporting the applicability of our RBC for transfusion. Incidentally, when we intravenously injected CB $CD34^+$ cell-derived erythroblasts from the second-phase culture (day 28) into NOD/SCID mice without macrophage depletion, these erythroblasts were not detected in circulation within 4 days (Fig. 5). This indicates that the previous proposal to use erythroblasts as an alternative source of RBC for transfusion [21] is not feasible. Although the reason as to why our result differed from that of Neildez-Nguyen et al. [21] is uncertain, we postulate that erythroblasts obtained on day 28 in our culture system adhered to vascular endothelial cells via interaction with VLA-4 to VCAM-1 immediately after intravenous injection.

While we were establishing the three-phase culture system as described above, Miharada et al. [6] reported a method by which they could obtain high-yield RBC in a liquid cultivation system supplemented with mifepristone, an antagonist to glucocorticoid [20], in the absence of macrophages and EPO. Therefore, we attempted to modify our three-phase cultivation system by applying their method during fourth-phase culture. The results obtained from the four-phase culture system clearly showed a higher enucleation rate (99.4%) than that (77.5%) of Miharada et al. [6], indicating that co-culturing erythroblasts with macrophages more efficiently facilitates enucleation in the absence of EPO stimulation than treating erythroblasts with mifepristone, although the precise mechanism is unknown. The overall yield of RBC was also substantially higher (8.8 transfusable units) in our system compared to theirs (1.8 transfusable units). Furthermore, and most importantly, we could eliminate the need for a non-physiological drug, mifepristone, which may cause some unfavorable adverse effects when intravenously infused, and yet we were able to achieve nearly 100% enucleation.

In conclusion, our method has been proven to be the most efficient for producing RBC from CB in vitro among those reported, and may have the most potential for diverse applications.

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References

1. Cooke BM, Mohandas N, Coppel RL. Malaria, the red blood cell membrane. Semi Hematol. 2004;41:173–88.
2. Penzhorn BL, Schoeman T, Jacobson LS. Feline babesiosis in South Africa: a review. Ann NY Acad Sci. 2004;1026:183–6.
3. 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.
4. Kawano Y, Kobune M, Yamaguchi M, et al. Ex vivo expansion of human umbilical cord hematopoietic progenitor cells using a coculture system with human telomerase catalytic subunit (hTERT)-transfected human stromal cells. Blood. 2003;101:532–40.
5. Matsunaga T, Tanaka I, Kobune M, et al. Ex vivo large-scale generation of human platelets from cord blood $CD34^+$ cells. Stem Cells. 2006;24:2877–87.
6. Miharada K, Hiroyama T, Sudo K, Nagasawa T, Nakamura Y. Efficient enucleation of erythroblasts differentiated in vitro from hematopoietic stem and progenitor cells. Nat Biotechnol. 2006;24:1255–6.
7. Kobune M, Ito Y, Kawano Y, Sasaki K, Uchida H, Nakamura K. Indian hedgehog gene transfer augments hematopoietic support of human stromal cells including NOD/SCID-$\beta 2m^{-/-}$ repopulating cells. Blood. 2004;104:1002–9.
8. Amsellem S, Pflumio F, Bardinet D, Izac B, Charneau P, Romeo PH. Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. Nat Med. 2003;9:1423–7.
9. Iavarone A, King ER, Dai XM, Leone G, Stanley ER, Lasorella A. Retinoblastoma promotes definitive erythropoiesis by repressing Id2 in fetal liver macrophages. Nature. 2004;432:1040–5.
10. Kawane K, Fukuyama H, Kondoh G, et al. Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver. Science. 2001;292:1546–9.
11. Hanspal M, Hanspal JS. The association of erythroblasts with macrophages promotes erythroid proliferation and maturation:

a 30-kD heparin-binding protein is involved in this contact. Blood. 1994;84:3494–504.
12. Hanspal M, Smockova Y, Uong Q. Molecular identification and functional characterization of a novel protein that mediates the attachment of erythroblasts to macrophages. Blood. 1998;92: 2940–50.
13. Soni S, Bala S, Gwynn B, Sahr KE, Peters LL, Hanspal M. Absence of erythroblast macrophage protein (Emp) leads to failure of erythroblast nuclear extrusion. J Biol Chem. 2006;281:20181–9.
14. Hashimoto S, Yamada M, Motoyoshi K, et al. Enhancement of macrophage colony-stimulating factor-induced growth and differentiation of human monocytes by interleukin-10. Blood. 1997;89:315–21.
15. Pic P, Ducrocq R, Girot R. Separation des hemoglobines F, Fac, S, C, A1c et dosage de I'hemoglobine F par chromatographie liquide haute performance. Ann Bio Clin. 1994;52:129–32.
16. Worthington RE, Bossie-Codreanu J, Zant G. Quantitation of erythroid differentiation in vitro using a sensitive colorimetric assay for hemoglobin. Exp Hematol. 1995;15:85–92.
17. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Meth. 1994;174:83–93.
18. Hentzen ER, Neelamengham S, Kansas GS, et al. Sequential binding of CD11a/CD18 and CD11b/CD18 defines neutrophil capture and stable adhesion to intercellular adhesion molecule-1. Blood. 2000;95:911–20.
19. Lewis DE, Schober W, Murrell S, et al. Rare event selection of fetal nucleated erythrocytes in maternal blood by flow cytometry. Cytometry. 1996;23:218–27.
20. Kolbus A, Blazquez-Domingo M, Carotta S, et al. Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: molecular analysis by expression profiling. Blood. 2003;102:3136–46.
21. Neildez-Nguyen TM, Wajcman H, Marden MC, et al. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. Nat Med. 2002;20:467–72.
22. Stowell CP, Levin J, Spiess BD, Winslow RM. Progress in the development of RBC substitutes. Transfusion. 2001;41:287–99.
23. Winslow RM. Current status of blood substitute research: towards a new paradigm. J Intern Med. 2003;253:508–17.
24. Issaad C, Croisille L, Katz A, Vainchenker W, Coulombel L. A murine stromal cell line allows the proliferation of very primitive human $CD34^{+}/CD38^{-}$ progenitor cells in long-term cultures and semisolid assays. Blood. 1993;81:2916–24.
25. Conneally E, Cashman J, Petzer A, Eaves C. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc Natl Acad Sci USA. 1997;94:9836–41.
26. McNiece I, Kubegov D, Kerzic P, Shpall EJ, Gross S. Increased expansion and differentiation of cord blood products using a two-step expansion culture. Exp Hematol. 2000;28:1181–6.
27. Ueda T, Tsuji K, Yoshino H, et al. Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. J Clin Invest. 2000;105:1013–21.
28. Oda A, Sawada K, Druker B, et al. Erythropoietin induces tyrosine phosphorylation of Jak2, STAT5A, and STAT5B in primary cultured human erythroid precursors. Blood. 1998;92:443–51.
29. Sadahira Y, Yoshino T, Monobe Y. Very late activation antigen 4-Vascular cell adhesion molecule 1 interaction is involved in the formation of erythroblastic island. J Exp Med. 1995;181:411–5.
30. Lee G, Spring FA, Parsons SF, et al. Novel secreted isoform of adhesion molecule ICAM-4: potential regulator of membrane-associated ICAM-4 interactions. Blood. 2003;101:1790–979.
31. Lee G, Lo A, Short SA, et al. Targeted gene deletion demonstrates that the cell adhesion molecule ICAM-4 is critical for erythroblastic island formation. Blood. 2006;108:2064–71.
32. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. Nature. 2002;417:182–6.
33. Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature. 2000;405:85–90.