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Development of a high-resolution purification method for precise functional characterization of primitive human cord blood–derived CD34–negative SCID-repopulating cells

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Objective. **We have successfully identified human cord blood (CB)–derived CD34-negative ($CD34^-$) severe combined immunodeficiency (SCID)-repopulating cells (SRCs) with extensive lymphomyeloid repopulating ability using the intrabone marrow injection method. In our previous study, a limiting dilution analysis demonstrated the frequency of $CD34^-$ SRCs in CB-derived 13lineage-negative (Lin^-) $CD34^-$ cells to be approximately 1/25,000. In this study, we intended to develop a high-resolution purification method to obtain highly purified $CD34^-$ SRCs.**

Materials and Methods. **The pooled CB-derived Lin^- cells were stained with 13 reported Lin monoclonal antibodies (mAbs) and 5 more Lin mAb, against CD11b, CD33, CD66c, CD45RA, and CD127. Then $18Lin^-CD34^{high}$, $18Lin^-CD34^-$, and $13Lin^-CD34^{high}CD38^-$ cells were sorted by fluorescence-activated cell sorting. Stem cell characteristics of these three fractions of cells were analyzed by in vitro cultures and in vivo repopulation assays for evaluation of this new purification method.**

Results. **A limiting dilution analysis demonstrated the frequency of $CD34^-$ SRCs in these $18Lin^-CD34^-$ cells to be approximately 1/1,000, which is associated with a seeding efficiency 25 times greater than the previous method. All primary recipient nonobese diabetic/Shi-scid/IL-2Rγc^{null} mice that received transplants of only two $CD34^-$ SRCs were highly engrafted with human lymphomyeloid cells at 24 weeks after primary transplantation and showed secondary multilineage repopulating abilities.**

Conclusions. **We succeeded to highly purify the $CD34^-$ SRCs using 18Lin mAbs and the intrabone marrow injection technique. This newly developed high-resolution purification method is indispensable to precisely characterize a distinct class of primitive human CB-derived $CD34^-$ hematopoietic stem cells.** **© 2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Hematopoietic stem cells (HSCs) are a self-renewing population with the developmental potential to give rise to all of the mature blood cell types [1–3]. Recent advances in fluorescence-activated cell sorting (FACS) technology make it possible to prospectively isolate HSCs to a high purity using various cell surface markers. Among them, the CD34 antigen (Ag) has long been believed to be a reliable HSC marker in mammals, including mice and humans [4]. However, it was reported that murine bone marrow (BM)–derived long-term lymphomyeloid reconstituting HSCs are lineage marker–negative (Lin^-), c-kit–positive ($c\text{-}kit^+$), Sca-1–positive ($Sca\text{-}1^+$), and CD34-negative ($CD34^-$) cells, also known as $CD34^-$ KSL cells [5].

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The measurement of the repopulation and differentiation capabilities of primitive human HSCs has been greatly facilitated by development of a xenotransplantation assay system using severe combined immunodeficient (SCID) mice, the SCID-repopulating cell (SRC) assay [6–8]. We developed a highly sensitive SRC assay system using intrabone marrow injection (IBMI) technique [9]. Using this technique, we have successfully identified human cord blood (CB)–derived $CD34^-$ SRCs with extensive lymphoid and myeloid repopulating abilities [9]. These $CD34^-$ SRCs seemed to be more primitive HSCs than previously reported $CD34^+$ SRCs [9–13]. They could home into the BM niche by IBMI only, partly because they expressed lower levels of homing receptors, including CXCR4 [9–13]. Flow cytometry (FCM) revealed that the $CD34^-$ SRCs did not express detectable levels of c-kit tyrosine kinase receptor [11], in contrast to the expression observed in murine $CD34^-$ KSL cells [5]. In our previous studies [9–12], 13 lineage (Lin)-specific monoclonal antibodies (mAbs) against CD2, CD3, CD4, CD7, CD10, CD14, CD16, CD19, CD20, CD24, CD41, CD56, and CD235a, were used to purify $CD34^-$ SRCs. An in vivo limiting dilution analysis using the IBMI demonstrated the frequency of the $CD34^-$ SRCs in the $13Lin^-CD34^-$ cells to be approximately 1/25,000 [12]. Conversely, the frequency of the SRCs in CB-derived $13Lin^-CD34^+CD38^-$ cells was 1/40 cells [12]. When these $Lin^-CD34^+CD38^-$ cells were transplanted by tail vein injection, the SRC frequency decreased to approximately 1/600 cells [14,15]. These results demonstrated that the IBMI technique is associated with a seeding efficiency 15 times greater than the tail vein injection. Therefore, the SRC assay system using the IBMI technique is very useful for investigating the HSC activity of certain target populations. However, the SRC frequency in $13Lin^-CD34^-$ cells was still very low in comparison to the frequency of SRC in $13Lin^-CD34^+CD38^-$ cells. Therefore, it is crucial to further purify the $CD34^-$ SRCs in order to more precisely characterize the $CD34^-$ SRCs.

Here we report the development of a high-resolution purification method for the rare class of primitive human $CD34^-$ SRCs (HSCs) using mAbs to 18Lin-specific Ags, including the 13 Ags and 5 additional Ags mentioned previously (CD11b, CD33, CD45RA, CD66c, and CD127). Using these 18Lin mAbs, we have succeeded to highly purify the human CB-derived $CD34^-$ SRCs.

Materials and methods

Collection of CB samples and processing of lineage-negative cells

CB samples were obtained from normal full-term deliveries with signed informed consent. This study was approved by the Institutional Review Board of Kansai Medical University. The CB-derived Lin^- mononuclear cells were separated using a StemSep device (StemCell Technologies, Vancouver, BC, Canada) [9–12].

High-resolution purification of Lin^-CD34^- cells

The pooled Lin^- cells from multiple donors were stained with the following: 7-amino-actinomycin D (7-AAD; Beckman Coulter, Fullerton, CA, USA); fluorescein isothiocyanate (FITC)–conjugated 13Lin mAbs, against CD2, CD16, CD24, and CD235a (DAKO, Kyoto, Japan), CD3, CD10, CD20, and CD41 (Beckman Coulter), CD7, CD19, and CD56 (BD Biosciences, San Jose, CA, USA), CD4 and CD14 (eBioscience, San Diego, CA, USA); phycoerythrin (PE)-conjugated 5 Lin mAbs, against CD11b (Serotec, Oxford, UK), CD33 and CD66c (Beckman Coulter), CD45RA (Southern Biotech, Birmingham, AL, USA), and CD127 (R&D Systems, Minneapolis, MN, USA); Pacific Blue–conjugated anti-CD45 mAb (BioLegend, San Diego, CA, USA); apophycocyanin (APC)-conjugated anti-CD34 mAb (BD Biosciences); and phycoerythrin-cyanin 7 (PE-Cy7)-conjugated anti-CD38 mAb (BioLegend), as reported previously [9–12]. The stained cells were then sorted into three fractions ($18Lin^-CD34^{high}$, $18Lin^-CD34^-$, and $13Lin^-CD34^{high}CD38^-$ cells) using an FACS Aria (BD Biosciences) (Fig. 1). A portion of the cells were cytospun and then stained with May-Grünwald/Giemsa for the morphological analysis.

RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNAs were isolated from 10,000 CB-derived $13Lin^-CD34^+CD38^-$ or $18Lin^-CD34^-$ cells using RNeasy plus micro kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The complementary DNAs were synthesized by using the iScript DNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). The complementary DNAs were amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, Tokyo, Japan). PCR cycling protocol consisted of the following: 95°C for 30 seconds; 58°C for 30 seconds; 72°C for 45 seconds; repeat protocol for 38 cycles. The PCR products were separated by electrophoresis on 1% agarose gels. The primer sets are listed in Supplementary Table E1 (online only, available at www.exphem.org).

Clonal cell culture

Colony-forming cells were assayed using our standard methylcellulose cultures as reported [11,16,17].

Coculture with HESS-5 cells and analysis of culture-generated cells by FCM

A total of 1×10^3 purified $18Lin^-CD34^{high}$ and 1 to 3×10^3 purified $18Lin^-CD34^-$ cells per well of 24-well plate (BD Labware, Franklin Lakes, NJ, USA) were plated onto pre-established irradiated HESS-5 layers in StemPro-34 medium (Gibco Laboratories, Grand Island, NY, USA); a cocktail of cytokines comprising 50 ng/mL stem cell factor (BioScience Autogen, Wiltshire, UK), 50 ng/mL flt3 ligand (R & D Systems), 100 ng/mL thrombopoietin (TPO), 10 ng/mL interleukin (IL)-3 (R&D Systems), 100 U/mL IL-6 (kindly provided by Ajinomoto Co. Inc., Tokyo, Japan), 10 ng/mL granulocyte colony-stimulating factor (G-CSF), and 5% fetal calf serum (HyClone, Laboratories, Logan, UT, USA). G-CSF and TPO were generous gifts from Kyowa Kirin Company (Tokyo, Japan). After 1 week, all of the cells were collected by vigorous pipetting and a portion was used for cell counting using

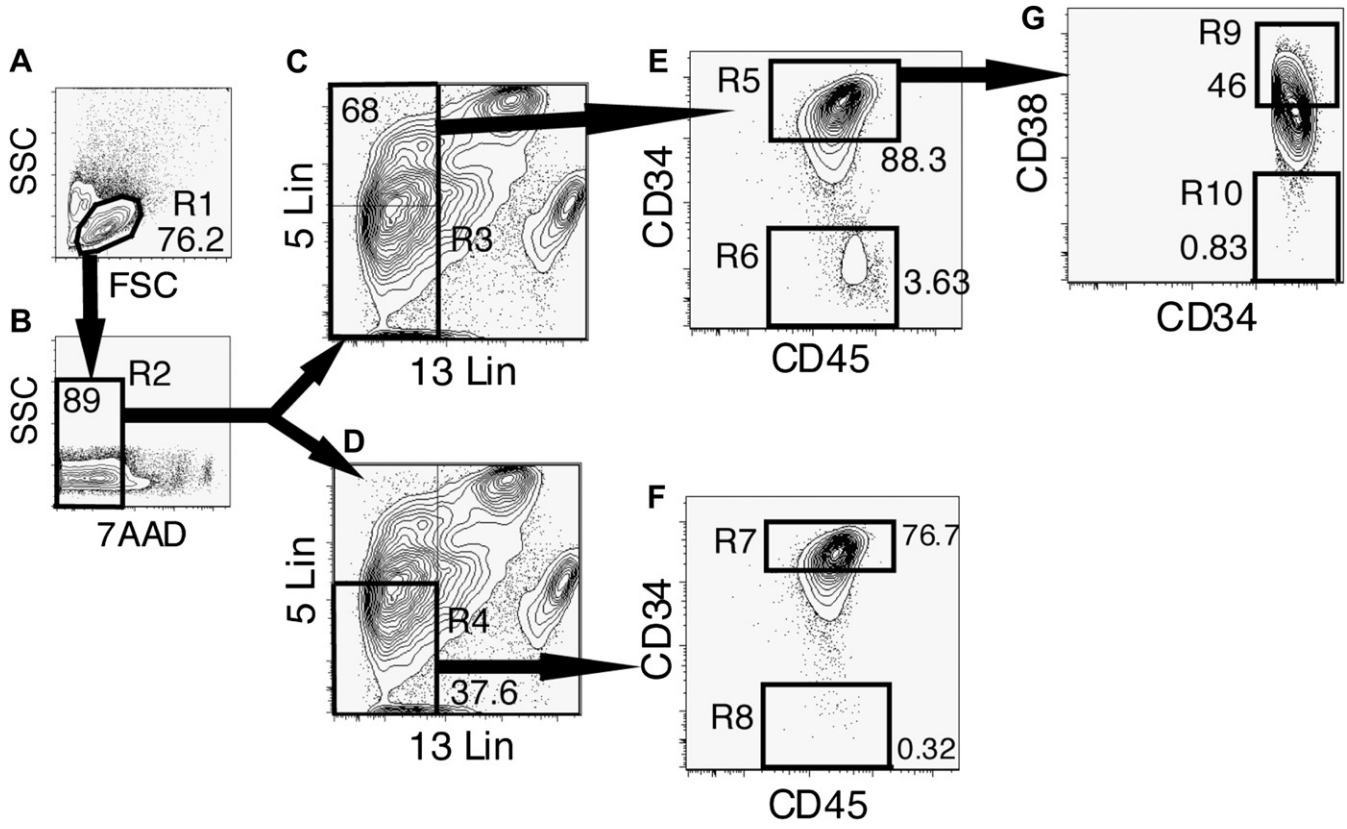

Figure 1. Representative FACS profiles of CB-derived 13Lin$^-$CD45$^+$CD34^{high}CD38$^-$ and 18Lin$^-$CD45$^+$CD34$^{high/-}$ cells. (**A**) The forward scatter/side scatter (FSC/SSC) profile of immunomagnetically separated Lin$^-$ cells. The R1 gate was set on the blast-lymphocyte window. (**B**) The R2 gate was set on the living cells. (**C**, **D**) Expression of FITC-conjugated 13Lin markers, including CD2, CD3, CD4, CD7, CD10, CD14, CD16, CD19, CD20, CD24, CD41, CD56, and CD235a, and the PE-conjugated 5 Lin markers, including CD11b, CD33, CD45RA, CD66c and CD127, are presented. (**E**) The 13Lin$^-$ (R3) cells were subdivided into 2 fractions: 13Lin$^-$CD45$^+$CD34^{high} (R5) and CD34$^-$ (R6) cells, according to their expression levels of CD34. The definitions of CD34$^{high/-}$ cells are as follows: the CD34^{high} fraction contains cells expressing maximum APC fluorescence intensity (FI) to 5% level of FI and the CD34$^-$ fraction contains cells expressing $<$0.5% level of FI. (**F**) The 18Lin$^-$ cells residing in the R4 gate were further subdivided into two fractions: 18Lin$^-$CD45$^+$CD34^{high} (R7) and CD34$^-$ (R8) cells, according to their expression levels of CD34. The definitions of CD34$^{high/-}$ cells are as follows: the CD34^{high} fraction contains cells expressing maximum APC FI to 15% level of FI and the CD34$^-$ fraction contains cells expressing $<$ 0.2% level of FI. (**G**) The R5-gated cells were further subdivided into two fractions: 13Lin$^-$CD45$^+$CD34^{high}CD38$^+$ (R9) and CD38$^-$ (R10) cells. The definitions of CD38$^{+/-}$ cells are as follows: the CD38$^+$ fraction contains cells expressing maximum PE-Cy7 FI to 5% level of FI and the CD38$^-$ fraction contains cells expressing $<$0.5% level of FI.

a counting chamber. The cells were then stained with 7-AAD, PE-conjugated anti-CD34 mAb (Beckman Coulter) and APC-conjugated anti-CD45 mAb (BD Biosciences). The number of CD34$^+$ cells was analyzed by FCM as described here.

NOD/SCID and NOG mice

Female 5-week-old nonobese diabetic (NOD)/Shi-*scid*/*scid* (NOD/SCID) mice were purchased from Clea Japan (Tokyo, Japan). Female 6-week-old NOD/Shi-scid/IL-2Rγ_c^{null} (NOG) mice [18] were purchased from the Central Institute of Experimental Animals (Kawasaki, Japan). All mice were handled under sterile conditions and were maintained in germ-free isolators located in the Central Laboratory Animal Facilities of Kansai Medical University. The animal experiments were approved by the Animal Care Committees of Kansai Medical University.

IBMI of purified cells

IBMI was carried out as reported previously [9–12].

SRC assay and serial analysis of human CD45$^+$ cell engraftment in NOD/SCID or NOG mice by FCM

SRC assays were performed using a previously reported method [9–12]. In this study, 400 to 2 $\times$ 10^3 purified CB-derived 18Lin$^-$ CD34$^-$ or 200 purified CB-derived 13Lin$^-$CD34^{high}CD38$^-$ cells were transplanted by IBMI into sublethally irradiated (250 cGy using a ^{137}Cs-γ irradiator) 7- to 8-week-old NOD/SCID or NOG mice. The repopulation of human CD45$^+$ hematopoietic cells in NOD/SCID or NOG mouse BM was analyzed by FCM. The mice were sacrificed 10 to 12 weeks after transplantation and the BM cells were collected by crushing the pairs of femurs and tibiae of each mouse in a mortar. Cells were suspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 2% fetal calf serum and the remaining bone tissues were removed using a cell strainer (BD Falcon). In long-term human hematopoietic cell reconstitution experiments using NOG mice, 1 to 2 μL BM fluid were serially aspirated from the contralateral sites (right tibia) of each mouse at 5, 8, 12, 18, and 24 weeks after

transplantation and the BM fluid was then diluted with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 2% fetal calf serum.

The repopulation of human hematopoietic cells in murine BM was determined by detecting the number of 7-AAD$^-$ cells positively stained with Pacific Blue–conjugated anti-human CD45 mAb by six-color FCM (FACS CantoII; BD Biosciences). The mice were scored as positive if $>0.1\%$ of the total murine BM cells were human CD45$^+$ cells. Cells were also stained with PE-Cy7–conjugated anti-mouse CD45.1 mAb (Beckman Coulter); PE-conjugated anti-human CD19 (eBioscience) and CD41 (Beckman Coulter) mAbs; FITC-conjugated anti-human CD33 and CD14 (eBiosciences), and anti-human CD235a mAbs (DAKO); and APC-conjugated anti-human CD34 mAb (BD Biosciences) for the detection of human stem/progenitor, lymphoid, and myeloid hematopoietic cells.

For the precise analysis of human cell repopulation in NOG mice at 24 weeks after transplantation, the NOG mice were sacrificed and then murine BM, peripheral blood (PB), and spleens were collected. Cells obtained from these organs were stained with the previously mentioned anti-human mAbs, FITC-conjugated anti-human CD3 and PE-conjugated anti-human CD4 mAbs (Beckman Coulter), and APC-conjugated anti-human CD8 mAb (eBioscience) to analyze the T-cell differentiation potentials.

In vivo limiting dilution analysis

To assess the frequency of SRCs in the CB-derived 18Lin$^-$CD34$^-$ cells, various numbers of 18Lin$^-$CD34$^-$ cells (400, 1000, and 2000; n = 20) were transplanted to NOD/SCID mice by IBMI as reported [9–12]. The mice were sacrificed 10 to 12 weeks after transplantation and the human cell repopulation in mouse BM was analyzed by FCM as described here.

Secondary transplantation

For secondary transplantations, murine BM cells were obtained 24 weeks after transplantation from the pairs of femurs and tibiae of moderately to highly engrafted primary recipient NOG mice receiving 2×10^3 18Lin$^-$CD34$^-$ or 200 13Lin$^-$CD34^{high}CD38$^-$ cells. Three of the four portions of the whole BM cells were transplanted by IBMI into sublethally (250 cGy) irradiated secondary recipient NOD/SCID mice. Twelve weeks after transplantation, the presence of human CD45$^+$ cells in the secondary recipients' BM was analyzed by FCM, as described for primary transplantation.

Statistical analysis

In the limiting dilution analyses, the frequencies of SRCs were calculated using the Poisson statistics as reported previously [9,10,12,14]. The significance of the differences in human cell repopulation in NOD/SCID or NOG mice was determined using the Mann-Whitney *U*-test.

Results

High-resolution purification of human CB–derived CD34$^-$ cells

First, the R1 gate was set on the blast-lymphocyte window (Fig. 1A). Next, the 7-AAD$^-$ cells were gated as R-2 in Figure 1B. The 13Lin$^-$CD45$^+$ and 18Lin$^-$CD45$^+$ cells

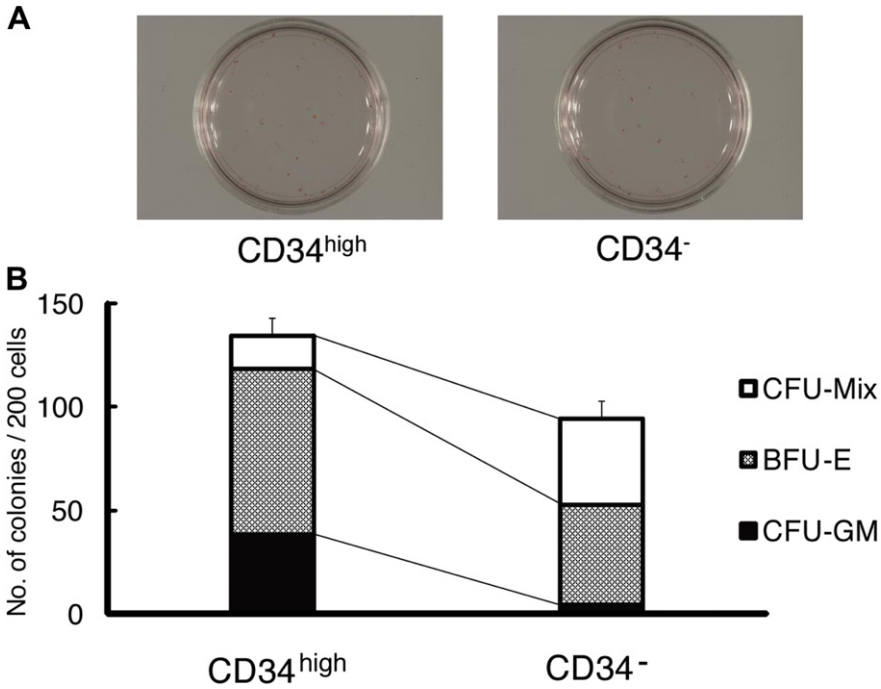

Figure 2. Colony-forming capacities of sorted 18Lin$^-$CD45$^+$CD34^{high} and CD34$^-$ cells.(**A**) Two hundred sorted CD34^{high} and CD34$^-$ cells were cultured in methylcellulose at 37°C with 5% CO_2, 5% O_2 and 90% N_2 in the presence of stem cell factor, TPO, IL-3, GM-CSF, G-CSF, and erythropoietin for 12 to 14 days. Many macroscopic colonies containing erythroid cells are seen in both dishes. (**B**) The number and composition of various colonies derived from CD34^{high} and CD34$^-$ cells are shown. Open, shaded, and closed bars represent the number of CFU-Mix, BFU-E, and CFU-GM (including CFU-G, CFU-M, and CFU-GM), respectively. The types of colonies identified in situ were granulocyte (CFU-G), macrophage (CFU-M), granulocyte/macrophage (CFU-GM), erythroid burst (BFU-E), and erythrocyte-containing mixed (CFU-Mix). The numbers of all the types of hematopoietic colonies were determined as the mean of quintuple cultures.

were then gated as R3 and R4 (Fig. 1C, D), respectively. The 18Lin$^-$CD45$^+$ cells were further subdivided into two distinct populations gated as R7 (CD34^{high}) and R8 (CD34$^-$) based on their surface CD34 expression (Fig. 1F). On the other hand, the 13Lin$^-$CD45$^+$CD34^{high} (R5) cells (Fig. 1E) were further subdivided into two distinct populations gated as R9 (CD34^{high}CD38$^+$) and R10 (CD34^{high}CD38$^-$) based on their surface CD38 expression (Fig. 1G). Then 18Lin$^-$CD34^{high} (R7), 18Lin$^-$CD34$^-$ (R8), and 13Lin$^-$CD34^{high}CD38$^-$ (R10) fractions were sorted for the subsequent in vitro cultures and in vivo SRC assays.

As shown in Figures 1E and F, the 18Lin$^-$CD34$^-$ (R8) fraction was highly purified (0.32% of R4 gated cells) in comparison to the 13Lin$^-$CD34$^-$ (R6) fraction (3.63% of R3 gated cells). The 13Lin$^-$CD34^{high}CD38$^-$ (R10) fraction was 0.83% of R5 gated cells (Fig. 1G). Morphological analysis showed that almost all of the 18Lin$^-$CD34$^-$ cells were immature blast-like in appearance, with high nuclear-to-cytoplasmic ratio, fine chromatin network, a few nucleoli, and a scant basophilic cytoplasm (data not shown).

Next, we analyzed the expression of various messenger RNAs (mRNAs), including c-kit, flt3, c-Mpl, and IL-3Rα,

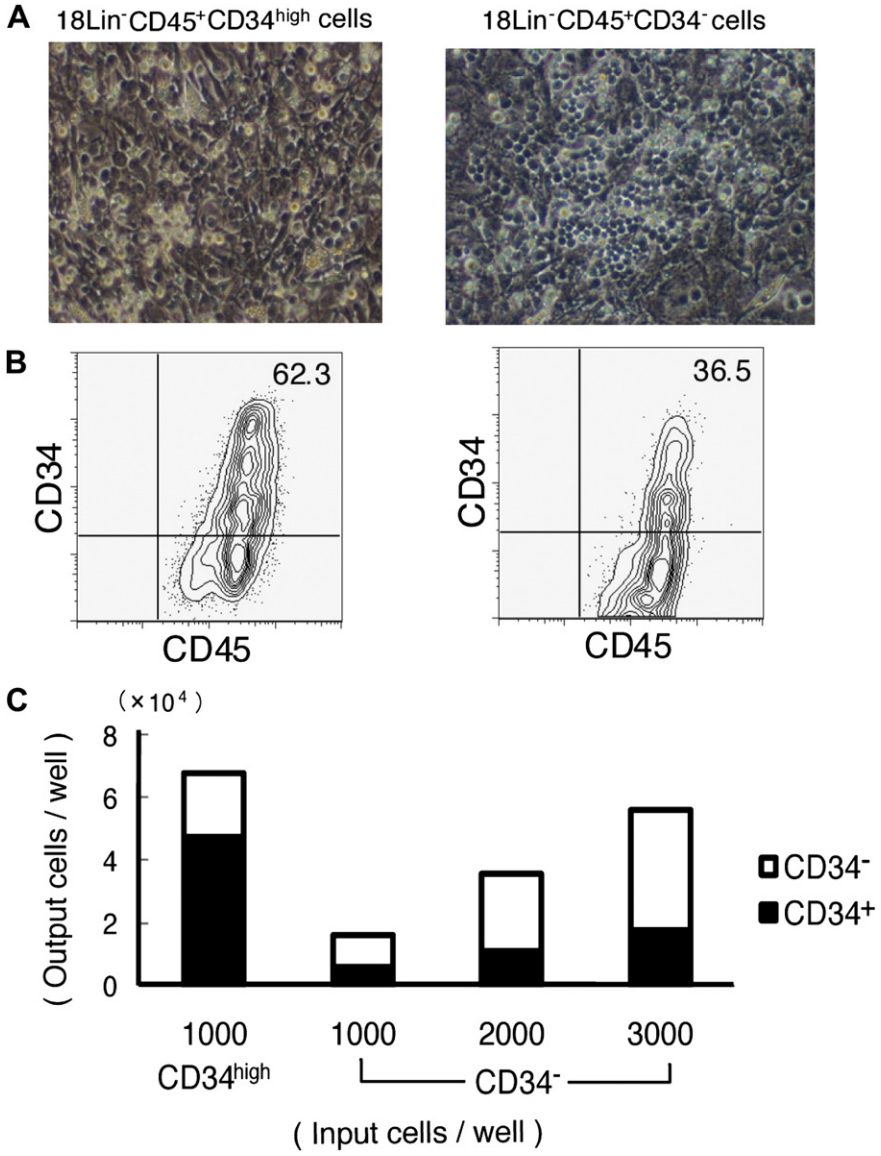

Figure 3. Coculture of 18Lin$^-$CD34^{high} and CD34$^-$ cells with HESS-5 cells. (**A**) 18Lin$^-$CD34^{high} and CD34$^-$ cells were cocultured with HESS-5 cells in the presence of six cytokines (stem cell factor + flt3 ligand +TPO + IL-3 + IL-6 + G-CSF) for 1 week. Representative photomicrographs taken on day 7 are presented. 18Lin$^-$CD34$^-$ cells formed many large cobblestone areas (right panel). In contrast, 18Lin$^-$CD34^{high} cells mainly formed small cobblestone areas (left panel). (**B**) Expression patterns of CD34 antigen on the culture-generated CD45$^+$ cells are shown. The percentages of CD34$^+$ cells are presented in right upper corner. (**C**) The number of CD34$^+$ cells produced in coculture with HESS-5 cells is shown. The total number of cells derived from 1 to 3 × 10^3 18Lin$^-$CD34$^-$ cells linearly expanded by 20-fold, and 30% to 40% of them consisted of CD34$^+$ cells. On the other hand, the total number of cells derived from 1 × 10^3 18Lin$^-$CD34^{high} cells expanded by 70-fold, and 60% to 70% of them consisted of CD34$^+$ cells.

of 18Lin$^-$CD34$^-$ (R8) and 13Lin$^-$CD34^{high}CD38$^-$ (R10) cells by RT-PCR. As shown in Supplementary Figure E1 (online only, available at www.exphem.org), both the 18Lin$^-$CD34$^-$ and 13Lin$^-$CD34^{high}CD38$^-$ cells expressed all of these mRNAs. However, the 18Lin$^-$CD34$^-$ cells weakly expressed the mRNAs of flt3, c-Mpl, and IL-3Rα in comparison to those of 13Lin$^-$CD34^{high}CD38$^-$ cells.

Characteristics of hematopoietic colony-forming capacity of CB-derived 18Lin$^-$CD34^{high} and CD34$^-$ cells

The colony-forming cell capacities of the CB-derived 18Lin$^-$CD34^{high} and CD34$^-$ cells were quite unique and many macroscopic colonies were seen (Fig. 2A). The plating efficiency of 18Lin$^-$CD34^{high} cell fraction was approximately 60% to 70%, which contained approximately 30% colony-forming unit (CFU) granulocyte-macrophage (GM), 60% burst-forming unit erythroid (BFU-E), and <10% CFU-Mix (Fig. 2B). As previously reported [9], the 13Lin$^-$CD34$^-$ cell fraction showed almost no colony formation. In contrast, the plating efficiency of the 18Lin$^-$CD34$^-$ cell fraction was 40% to 50%, which contained approximately <10% CFU−GM, 60% BFU-E, and 40% CFU-Mix (Fig. 2B). These results demonstrated that the highly purified 18Lin$^-$CD34$^-$ cell fraction contains a large number of BFU-E, as well as CFU-Mix, and also indicated for the first time that the colony-forming cell capacities are detected in the CB-derived CD45$^+$CD34$^-$ cell fraction.

Coculture with HESS-5 cells

The CD34$^-$ SRCs could produce CD34$^+$ SRCs in vitro, as reported [9,11]. Therefore, 1 to 3 × 10^3 18Lin$^-$CD34$^-$ and 1 × 10^3 18Lin$^-$CD34^{high} cells were cocultured with the murine stromal cell line HESS-5 in the presence of cytokines (i.e., stem cell factor, flt3 ligand, TPO, IL-3, IL-6, and G-CSF) for 1 week. The 18Lin$^-$CD34$^-$ cells actively proliferated and formed many large cobblestone areas (Fig. 3A, right panel). In contrast, the 18Lin$^-$CD34^{high} cells mainly formed small cobblestone areas and many cells were floating over the HESS-5 cells (Fig. 3A, left panel). After 1 week, the cells in each well were collected by vigorous pipetting, and were then counted and analyzed for the CD34$^+$ cell content by FCM. The total number of cells derived from 18Lin$^-$CD34$^-$ cells expanded by 20-fold, 30% to 40% of which consisted of CD34$^+$ cells (Fig. 3B, C). A linear proliferation response was seen with cells plated at 1 to 3 × 10^3 cells per well. On the other hand, the total number of cells derived from 1 × 10^3 18Lin$^-$CD34^{high} cells expanded by about 70-fold, 60% to 70% of which consisted of CD34$^+$ cells (Fig. 3B, C). These results indicated that the 18Lin$^-$CD34$^-$ cell fraction contained a substantial number of CD34$^+$ cell-producing hematopoietic progenitor cells/HSCs.

SRC activity and limiting dilution analysis of CB-derived 18Lin$^-$CD34$^-$ cells by IBMI

Thereafter, we investigated the SRC activity of the 18Lin$^-$CD34$^-$ cells using NOD/SCID mice. As expected, a limiting dilution analysis using the IBMI demonstrated the frequency of the CD34$^-$ SRCs in the 18Lin$^-$CD34$^-$ cells to be approximately 1/1000 (Fig. 4). The frequency of the CD34$^-$ SRCs in the 13Lin$^-$CD34$^-$ cells was 1/25,000, as we reported previously [12]. These results clearly demonstrated that application of the 18Lin mAbs plus the IBMI technique is associated with a seeding efficiency 25 times greater than the previously described method.

Long-term repopulation patterns of CD34$^-$ SRCs and CD34$^+$CD38$^-$ SRCs in NOG mice

The next approach to characterize the self-renewal potential and the long-term repopulating potential of the CD34$^-$ SRCs was to serially analyze the kinetics of BM engraftment for 24 weeks in NOG mice that received transplants of 2 × 10^3 18Lin$^-$CD34$^-$ cells (containing two SRCs). This was compared with the repopulating patterns in NOG mice that received 200 13Lin$^-$CD34^{high}CD38$^-$ cells (containing 5 SRCs) (Fig. 5). The numbers of SRCs transplanted were estimated based on the data obtained from the limiting dilution analyses presented in Figure 4 and recently reported data [12].

In these experiments, both the mice that received transplants of CD34$^-$ and CD34$^+$CD38$^-$ SRCs showed

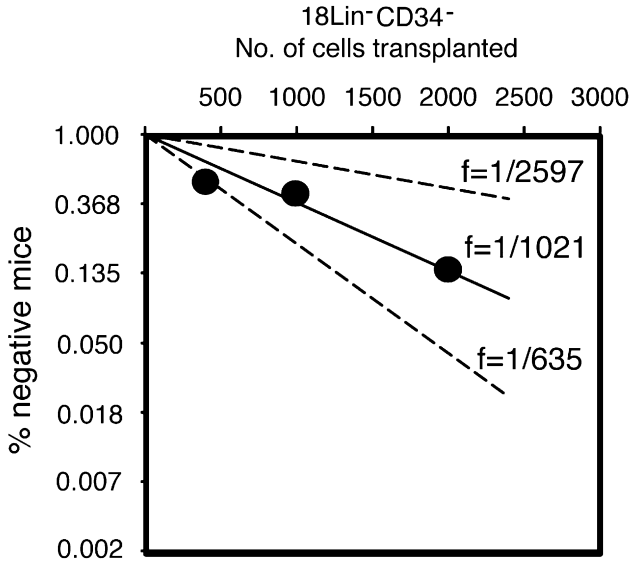

Figure 4. The frequency of SRC in 18Lin$^-$CD34$^-$ cells. Various numbers of 18Lin$^-$CD34$^-$ cells (400, 1000, and 2000) were transplanted to NOD/SCID mice (n = 20). The human CD45$^+$ cell repopulation in the mouse BM was analyzed by FCM 10 weeks after transplantation. The frequency of SRC was 1 per 1021 18Lin$^-$CD34$^-$ cells. For the frequency determination, the middle solid line represents the estimated weighted mean frequency (f_{WM}). The lower and upper dotted lines represent 95% confidence interval of f_{WM}.

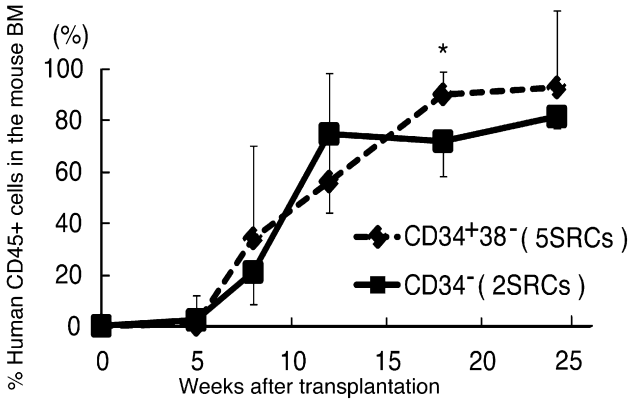

Figure 5. Long-term human hematopoietic cell reconstitution in NOG mice. Five $CD34^+CD38^-$ SRCs (dotted line) or 2 $CD34^-$ SRCs (solid line) were injected into the left tibiae of NOG mice by IBMI. The number of SRCs transplanted was estimated based on the limiting dilution analysis data. The human $CD45^+$ cell rates in the contralateral sites of recipient mice were serially analyzed by the BM aspiration method 5, 8, 12, 18, and 24 weeks after transplantation by six-color FCM. Data represent the median ± standard deviation of results from six mice at each time point except 8 weeks (n = 3). $*p < 0.05$.

signs of human cell repopulation at 5 weeks after transplantation (Fig. 5). The repopulation level of all six mice that received transplants of two $CD34^-$ SRCs was 0.4% to 28.9% (median: 2.5%). Five of six mice that received transplants of five $CD34^+CD38^-$ SRCs also showed a comparable level of human cell repopulation (0.2% to 28.7%; median: 0.4%). The mice that received transplants of $CD34^+CD38^-$ SRCs showed a peak level of repopulation at 18 weeks after transplantation (74.2% to 98.5%; median: 90.0%), and their repopulation rates were maintained at this same level until 24 weeks after transplantation. On the other hand, the repopulation rates of the mice that received transplants of $CD34^-$ SRCs gradually increased over 5 to 12 weeks, then reached high levels and maintained the same levels (74.9% to 88.0%; median: 81.6% at 24 weeks after transplantation) during the observation period. These results demonstrated for the first time that the $CD34^-$ SRCs had potent human cell repopulation potentials, which was almost equivalent to that of the $CD34^+CD38^-$ SRCs.

Secondary repopulating ability of $CD34^-$ SRCs and $CD34^+CD38^-$ SRCs by IBMI

All of the secondary recipient mice that received the transplants from the primary recipient mice (receiving the transplants of two $CD34^-$ SRCs or five $CD34^+CD38^-$ SRCs) showed signs of human cell repopulation at 12 weeks after transplantation (Table 1). The human $CD45^+$ cell rates in all the secondary recipient mice showed comparable levels of human cell repopulation (0.1% to 10.8% vs. 0.1% to 10%). We confirmed that all of the secondary recipient mice that received transplants of $CD34^-$ as well as $CD34^+CD38^-$ SRCs showed multilineage human hematopoiesis, including $CD34^+$, $CD19^+$, and $CD33^+$ cells (data not shown).

These results demonstrated that the $CD34^-$ and $CD34^+CD38^-$ SRCs both had comparable levels of secondary reconstituting abilities and could sustain long-term (up to 36 weeks) human hematopoiesis in NOG and NOD/SCID mice, suggesting that both SRCs had significant self-renewing potential.

Comparison of the differentiation potentials of CB-derived $CD34^-$ SRCs and $CD34^+CD38^-$ SRCs

To further evaluate the functional differences between the $CD34^-$ and $CD34^+CD38^-$ SRCs, we studied their multilineage reconstitution abilities in various organs, including the BM, PB, and spleen, in NOG mice that were transplanted with either 2×10^3 $18Lin^-CD34^-$ cells (2 SRCs) or 200 $13Lin^-CD34^{high}CD38^-$ cells (5 SRCs) by IBMI. The results of the BM repopulation patterns are shown in Figure 6. Analyses of the two representative mice that were transplanted with either $CD34^-$ SRCs (Fig. 6, right column) or $CD34^+CD38^-$ SRCs (Fig. 6, left column) demonstrated that both of the $CD34^-$ and $CD34^+CD38^-$ SRCs generated $CD34^+$ stem/progenitor cells and both SRCs had a comparable in vivo differentiation capacity to the B-lymphoid, myeloid, monocytic, megakaryocytic, and erythroid lineages 24 weeks after the transplantation.

Next, the percentages of T-lymphoid cells in various organs were analyzed (Fig. 7). The $CD4^+$ and $CD8^+$ single positive T-lymphoid cells were detected in the $CD3^+$ cell gates of the BM, PB, and spleen in both NOG mice that were transplanted with either $CD34^-$ SRCs or $CD34^+CD38^-$ SRCs. These results clearly demonstrated that the $CD34^-$ SRCs could supply comparable levels of mature

Table 1. Secondary repopulating ability of $CD34^+CD38^-$ and $CD34^-$ SRCs

Type and no. of cells transplanted into PR	No. of SRCs*	% Human cells in PR†	No. of cells transplanted into SR ($\times 10^7$)	Incidence of engraftment in SR	% Human cells in SR‡
$CD34^{high}CD38^-$, 200	5	26.6–72.6 (67.0)	4.2–5.2 (4.5)	4/4	0.1–10.8 (0.76)
$CD34^-$, 2000	2	14.8–65.1 (50.6)	4.0–5.5 (5.2)	5/5	0.1–10 (1.01)

Median values are presented in parentheses.
PR = primary recipient; SR = secondary recipient.
*No. of SRCs transplanted is estimated by limiting dilution analysis.
†Human cell repopulation of BM in PR NOG mice was analyzed 24 weeks after transplantation.
‡Human cell repopulation of BM in SR NOD/SCID mice was analyzed 12 weeks after secondary transplantation.

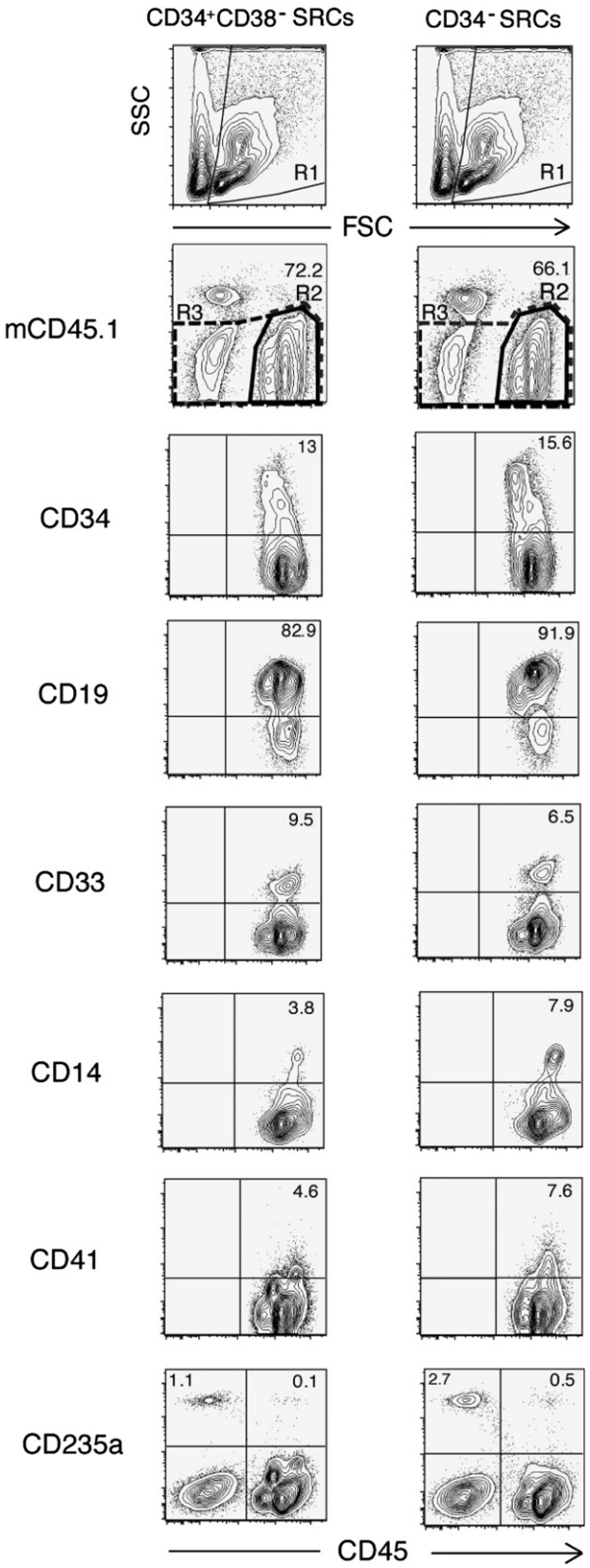

Figure 6. Long-term multilineage reconstitution abilities of $CD34^{+}38^{-}$ and $CD34^{-}$ SRCs. First, the R1 gate was set on the total murine BM cells obtained from these two representative NOG mice that received five $CD34^{+}CD38^{-}$ SRCs (left column) or two $CD34^{-}$ SRCs (right column) 24 weeks after transplantation. Thereafter, the human $CD45^{+}$ cells were gated as R2 (solid line). The expression of surface markers, including CD34, CD19, CD33, CD14, and CD41, on the R2 gated cells was analyzed by six-color FCM. Only the expression of CD235a was analyzed using R3 gate (dotted line) containing $CD45^{+/-}$ cells. The percentages of positive cells in each scattergram (CD45, CD34, CD19, CD33, CD14, and CD41) are presented in the right upper corner. The percentages of CD235a-positive cells are presented in bilateral upper corners.

T-lymphoid cells at 24 weeks after transplantation similar to the $CD34^{+}CD38^{-}$ SRCs.

Discussion

A number of studies, including ours, have demonstrated that long-term– and short-term–repopulating SRCs are present in human CB and BM cells [6–15,19–22] and also provided evidence that CB- or BM-derived $CD34^{+}CD38^{-}$ SRCs are very primitive human HSCs [7,8,12,14,20–22]. As reported, we first identified human CB-derived $CD34^{-}$ SRCs using a sensitive IBMI method, which can detect these $CD34^{-}$ SRCs very efficiently [9]. Because these $CD34^{-}$ SRCs expressed lower levels of homing receptors, including CXCR4, they could not home into the BM niche by conventional tail vein injection methods [9]. In a series of experiments, we extensively studied the functional characteristics of the $CD34^{-}$ SRCs using in vitro cultures with murine stromal HESS-5 cells as well as in vivo repopulation assays using NOD/SCID or NOG mice [9–12]. Importantly, the $CD34^{-}$ SRCs could produce $CD34^{+}$ SRCs in vitro as well as in vivo [9,11]. Moreover, the CB-derived $CD34^{-}$ SRCs did not express c-kit and flt3 tyrosine kinase receptors, thereby suggesting that the surface immunophenotype of human $CD34^{-}$ SRCs is $Lin^{-}CD34^{-}c\text{-}kit^{-}flt3^{-}$ [11,13]. However, the $18Lin^{-}CD34^{-}$ cells weekly expressed the mRNAs of c-kit and flt3 by RT-PCR (Supplementary Figure E1; online only, available at www.exphem.org). Except for the expression of c-kit, these findings are consistent with the earlier report of Adolfsson and colleagues [23] that very primitive murine long-term HSCs ($CD34^{-}$ KSL cells) did not express flt3. We also reported that the CB-derived $CD34^{-}$ SRCs could produce $CD34^{+}flt3^{-}$ and $CD34^{+}flt3^{+}$ SRCs in vitro [11]. Collectively, the $CD34^{-}$ SRCs are a distinct class of the HSC population and appear to be more immature than the $CD34^{+}CD38^{+}$ and the $CD34^{+}CD38^{-}$ SRCs.

In order to more precisely elucidate the hierarchical organization of human primitive HSCs/hematopoietic progenitor cells, including $CD34^{-}$ SRCs, $CD34^{+}CD38^{-}$ SRCs, and $CD34^{+}CD38^{+}$ SRCs, we recently investigated the functional characteristics of these three classes of SRCs [12]. In that study, the $CD34^{-}$ SRCs and $CD34^{+}CD38^{-}$ SRCs could both support human cell repopulation for up to 24 weeks in NOG mice [12]. On the other hand, the $CD34^{+}CD38^{+}$SRCs could only support short-term human

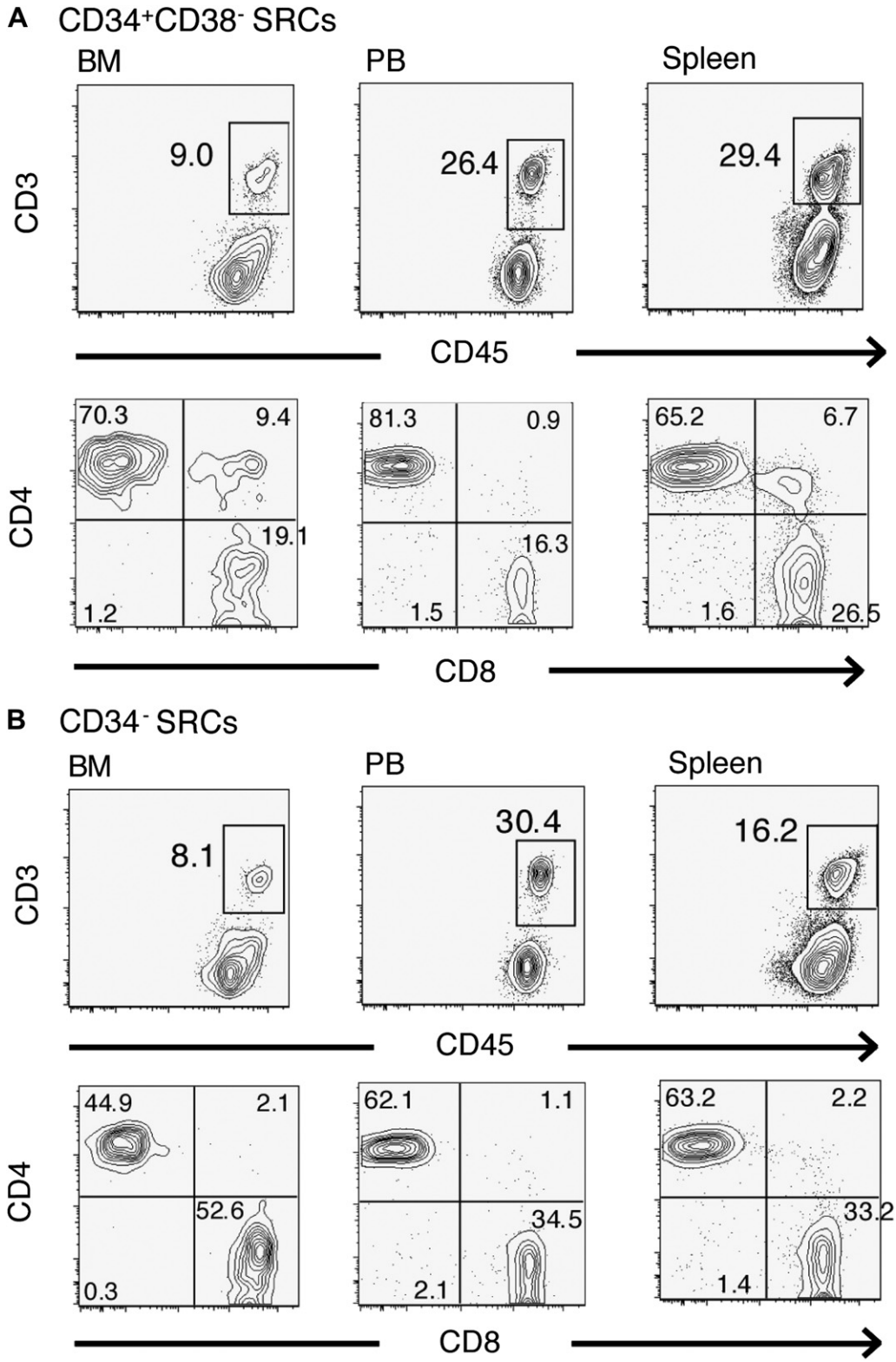

Figure 7. T-lymphoid reconstitution abilities of $CD34^{+}38^{-}$ and $CD34^{-}$ SRCs. The human T cells detected in the BM, PB, and spleen obtained from these two representative NOG mice that received five $CD34^{+}CD38^{-}$ SRCs (**A**, upper column) or two $CD34^{-}$ SRCs (**B**, lower column) 24 weeks after transplantation are presented. First, $CD3^{+}$ cell gates were set on the human $CD45^{+}$ cells, and then the expression of CD4/CD8 on the $CD3^{+}$ cells was analyzed by six-color FCM. The percentages of each fraction are indicated.

cell repopulation in NOG mice as reported previously [12,20]. The frequency of $CD34^+CD38^-$ SRCs in $13Lin^-CD34^+CD38^-$ cells is approximately 1/40 by IBMI [12]. In contrast, the frequency of $CD34^-$ SRCs in $13Lin^-CD34^-$ cells is approximately 1/25,000 by IBMI [12], which is significantly lower than that in $CD34^+CD38^-$ SRCs. Therefore, it is very important to highly purify the CB-derived $CD34^-$ SRCs in order to more precisely elucidate their HSC characteristics.

In this study, we added five more Lin-specific mAbs (anti-CD11b, anti-CD33, anti-CD45RA, anti-CD66c, and anti-CD127) to remove the retained myeloid and lymphoid cells in the $13Lin^-CD34^-$ cell fraction. We then succeeded in highly purifying the $CD34^-$ SRCs (Fig. 1). An in vivo limiting dilution analysis demonstrated the frequency of $CD34^-$ SRCs in the $18Lin^-CD34^-$ cells to be approximately 1/1,000 (Fig. 4). The 2×10^3 $18Lin^-CD34^-$ cells (containing only two $CD34^-$ SRCs) could support long-term (up to 24 weeks) as well as high-level human hematopoiesis in NOG mice (Fig. 5) and showed extensive multilineage human cell repopulation into different cell lineages including $CD34^+$ stem/progenitor, $CD19^+$ B-lymphoid, $CD4^+/CD8^+$ T-lymphoid, $CD14^+$ monocytic, $CD33^+$ myeloid, $CD41^+$ megakaryocytic, and $CD235a^+$ erythroid lineages (Figs. 6 and 7). These results unveiled that the $CD34^-$ SRCs had potent human cell repopulation potentials in NOG mice at least for 24 weeks.

In our model, the $CD34^-$ SRCs are more immature than the $CD34^+CD38^-$ SRCs, because the $CD34^-$ SRCs could generate $CD34^+$ SRCs in vitro as well as in vivo [9,11]. These results may suggest that the hierarchical position of $CD34^+CD38^-$ SRCs is in the intermediate stage of the HSC compartment between $CD34^-$ SRCs and $CD34^+CD38^+$ SRCs [13]. However, further studies will be required to fully elucidate the proposed model of the human HSC hierarchy [13]. For this purpose, our goal was to purify the $CD34^-$ SRCs at the single-cell level. In this study, we developed an efficient purification method using a negative selection technique to deplete lineage-positive cells. It would be more efficient to use specific positive markers of the $CD34^-$ SRCs/HSCs. Thus, it is inevitably essential to detect/identify novel and/or known specific positive markers that are expressed on the $CD34^-$ SRCs/HSCs. Nakamura et al. recently reported that tie 2, a receptor tyrosine kinase, is a candidate positive marker of human BM-derived $CD34^-$ SRCs [24]. However, CB-derived $18Lin^-CD34^-$ cells did not express the tie 2 receptor (Sasaki and Sonoda, unpublished data). Using this newly developed high-resolution purification method, studies to identify specific positive markers of $CD34^-$ SRCs are now under investigation in our laboratory.

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Conflict of interest disclosures

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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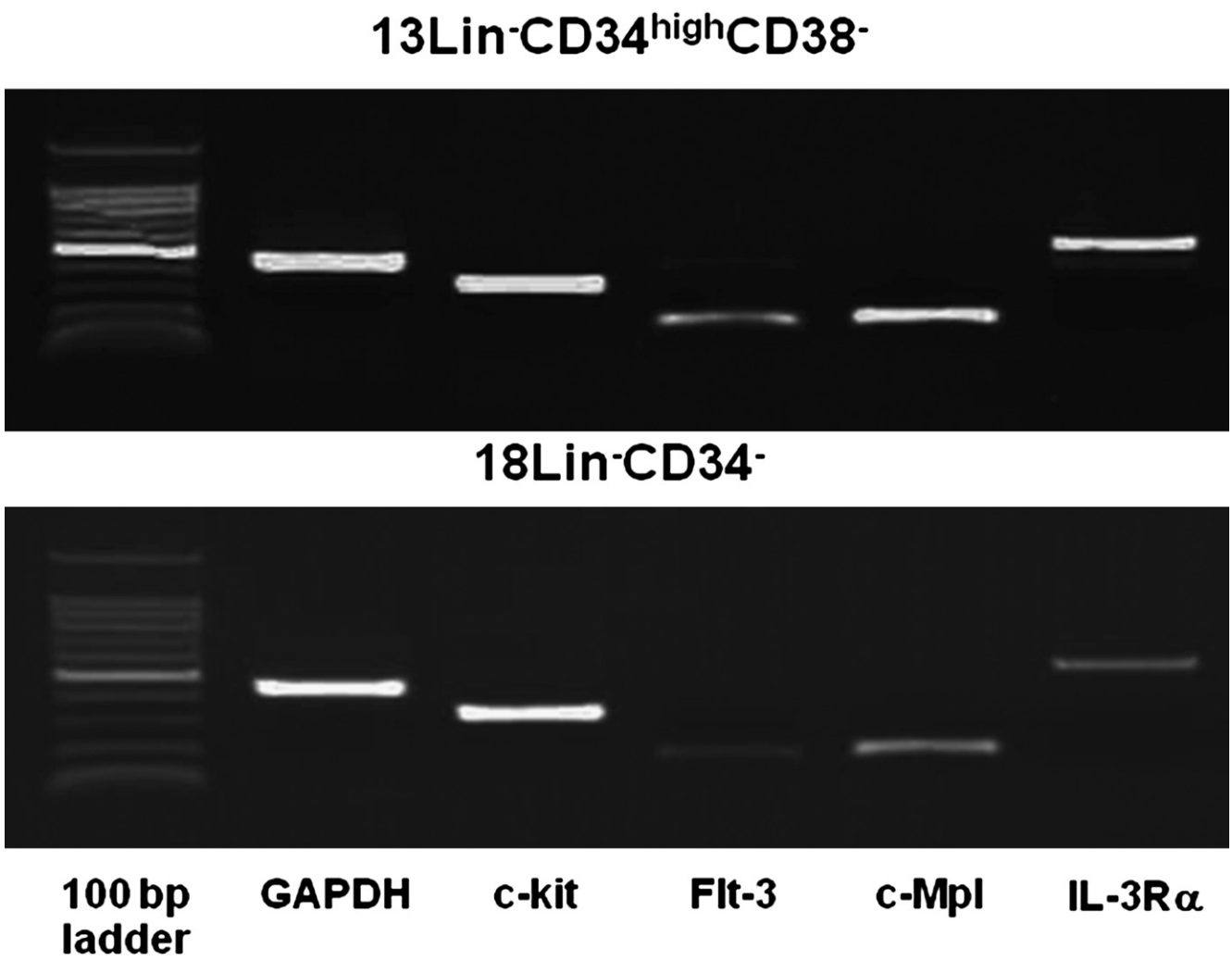

Supplementary Figure E1. RT-PCR analyses for mRNAs of c-kit, Flt3, c-Mpl, and IL-3Rα.

Supplementary Table E1. Primer pairs used for RT-PCR

Gene name	Strand	Primer sequences	Product length (bp)
c-kit	F	5′-AAGGACTTGAGGTTTATTCCT-3′	345
	R	5′-CTGACGTTCATAATTGAAGTC-3′	
Flt-3	F	5′-CCATCTTGGACCTGGAAGAA-3′	193
	R	5′-AAGATGTGCCAAGGGAATTG-3′	
c-Mpl	F	5′-TGGAGATGCAGTGGCACTTG-3′	206
	R	5′-AGAACTGTGGGGTCTGTAG-3′	
IL-3Rα	F	5′-ACAGGTCAGAGACAGAACCTCC-3′	555
	R	5′- CTGTTCTTCTTCCTGGCAGC-3′	
GAPDH	F	5′-ACCACAGTCCATGCCATCAC-3′	450
	R	5′-TCCACCACCCTGTTGCTGTA-3′	