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ORIGINAL ARTICLE

In vivo dynamics of human cord blood-derived CD34$^-$ SCID-repopulating cells using intra-bone marrow injection

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The identification of human CD34-negative (CD34$^-$) hematopoietic stem cells (HSCs) provides a new concept for the hierarchy in the human HSC compartment. This study investigated the long-term repopulating capacity and redistribution kinetics of human cord blood-derived CD34$^-$ severe combined immunodeficiency (SCID)-repopulating cells (SRCs) and compared them with those of CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ SRCs using the intra-bone marrow injection (IBMI) to clarify the characteristics of CD34$^-$ SRCs. On the basis of the limiting dilution analyses data, estimated numbers of CD34$^+$CD38$^+$, CD34$^+$CD38$^-$, and CD34$^-$ SRCs were transplanted to NOD/SCID mice by IBMI. The human cell repopulation at the site of injection and the other bones were serially investigated. Interestingly, CD34$^+$CD38$^+$, CD34$^+$CD38$^-$, and CD34$^-$ SRCs began to migrate to other bones 2 and 5 weeks after the transplantation, respectively. Accordingly, the initiation of migration seemed to differ between the CD34$^+$ and CD34$^-$ SRCs. In addition, CD34$^+$CD38$^+$ SRCs only sustained a short-term repopulation. However, both CD34$^+$CD38$^-$ and CD34$^-$ SRCs had longer-term repopulation capacity. Taken together, these findings showed that CD34$^-$ SRCs show different *in vivo* kinetics, thus suggesting that the identified CD34$^-$ SRCs are a distinct class of primitive HSCs in comparison to the CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ SRCs.
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Introduction

The biology of hematopoietic stem cell (HSC) is a current topic of interest, which has important implications for clinical HSC transplantation as well as for the basic research of HSCs. A number of studies have revealed that the severe combined immunodeficiency (SCID)-repopulating cell (SRC) activity reside in CD34-positive (CD34$^+$) cell factions derived from bone marrow (BM), cord blood (CB), and peripheral blood.[1–6] Recently, we successfully identified human CB-derived CD34-negative (CD34$^-$) SRCs with extensive lymphoid and myeloid repopulating ability using the intra-bone marrow injection (IBMI) method.[7] Functional studies revealed that these CD34$^-$ SRCs have different HSC characteristics from earlier-reported CD34$^+$ SRCs.[7–9] These data suggested that the CD34$^-$ SRCs are a novel class of primitive repopulating HSCs that can only be detected by the sensitive IBMI method.[7–10]

On the other hand, Hogan *et al.*[11] clearly showed that CB-derived SRCs present in the CD34$^+$ cell fraction could be segregated into two subpopulations with distinct repopulation characteristics. The CD34$^+$CD38$^+$ SRCs could rapidly repopulate the recipient NOD/SCID mice, but they could only maintain a human cell repopulation for 12 weeks, while showing no secondary repopulating potential. In contrast, the more primitive CD34$^+$CD38$^-$ SRCs could repopulate recipients more gradually and maintain human hematopoiesis for at least 20 weeks. Importantly, they had a secondary repopulating potential throughout the engraftment period. These results showed that human CB-derived CD34$^+$CD38$^-$ SRCs are more primitive SRCs than CD34$^+$CD38$^+$ SRCs.

To further clarify the HSC characteristics of CD34$^-$ SRCs, this study investigated the *in vivo* redistribution kinetics and the long-term (LT) repopulating potential of human CB-derived CD34$^-$ SRCs and compared them with those of CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ SRCs using the IBMI method.

Materials and methods

Collection of CB samples and processing of lineage-negative cells

CB samples were obtained from normal full-term deliveries with the signed informed consent. This study was approved by the Institutional Review Boards of Kansai Medical University. The CB-derived lineage-negative (Lin$^-$) mononuclear cells were separated using a StemSep device (StemCell Technologies, Vancouver, BC, Canada), as reported earlier.[7–9]

Purification of Lin$^-$CD34$^+$CD38$^+$, Lin$^-$CD34$^+$CD38$^-$, and Lin$^-$CD34$^-$ cells

The pooled Lin$^-$ cells from multiple donors were stained with fluorescein isothiocyanate-conjugated anti-CD45 monoclonal antibody (mAb) (Beckman Coulter, Inc., Fullerton, CA, USA), PC5-conjugated anti-CD34 mAb (Coulter), and phycoerythrin-conjugated anti-CD38 mAb (Becton Dickinson Immunocytometry, San Jose, CA, USA), as reported earlier.[7–9] These stained cells were then sorted into three fractions, including

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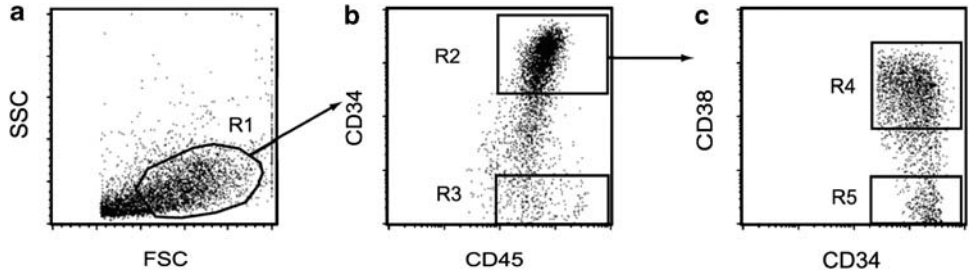

Figure 1 Representative FACS profile of sorted CB-derived Lin$^-$CD45$^+$CD34$^-$ and Lin$^-$CD45$^+$CD34$^+$CD38$^+$ or Lin$^-$CD45$^+$CD34$^+$CD38$^-$ cells. (**a**) The forward scatter/side scatter (FSC/SSC) profile of immunomagnetically isolated Lin$^-$ cells. The R1 gate was set on the lymphocyte/blast window. (**b**) The R1-gated cells were subdivided into two fractions: Lin$^-$CD45$^+$CD34$^+$ (R2) and CD45$^+$CD34$^-$ (R3) cells, respectively. The definitions of CD34$^+$ and CD34$^-$ cells are as follows: the CD34$^+$ fraction contains cells expressing maximum PC-5 fluorescent intensity (FI) to 5% level of FI and the CD34$^-$ fraction contains cells expressing $<0.2\%$ level of FI, respectively. (**c**) CD34$^+$ (R2) cells were further subdivided into two populations according to their expression levels of the CD38 antigen. The cells in the R4 and R5 gates were classified as CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ cells, respectively. The definitions of CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ cells are as follows: the CD34$^+$CD38$^+$ fraction contains cells expressing maximum phycoerythrin FI to 5% level of FI and the CD34$^+$CD38$^-$ fraction contains cells expressing $<0.5\%$ level of FI, respectively.

Lin$^-$CD34$^+$CD38$^+$, Lin$^-$CD34$^+$CD38$^-$, and Lin$^-$CD34$^-$ cells as shown in Figure 1 using a FACSVantage (BD Biosciences, San Jose, CA, USA).

Semiquantitative reverse transcriptase polymerase chain reaction analysis for CD34 mRNA

The detailed methods are presented in the legend to Supplementary Figure 1.

NOD/SCID and NOG mice

Female 5-week-old 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[12] were also 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.

SRC assay and serial analysis of human cell engraftment in NOD/SCID or NOG mice by flow cytometry

An SRC assay was performed using an earlier reported method[1–5] with slight modifications.[7–9,13] In this study, purified 2.5×10^3 to 2×10^4 CB-derived Lin$^-$CD34$^+$CD38$^+$, 20–4×10^2 CB-derived Lin$^-$CD34$^-$CD38$^-$ cells, or 2–7×10^4 CB-derived Lin$^-$CD34$^-$ cells were transplanted by IBMI[7–9] into sublethally irradiated (250 cGy using a ^{137}Cs-γ irradiator) 8- to 10-week-old NOD/SCID or NOG mice. The repopulation of human hematopoietic cells in murine BMs was serially analyzed by the aspiration method. Two to 18 weeks after transplantation, the BMs were separately aspirated from the injected left tibia and the contra-lateral site (right tibia) of each mouse using a heparinized tuberculin syringe and were flushed into α-medium. In LT human hematopoietic cell reconstitution experiments using NOG mice, BMs were serially aspirated from the contra-lateral sites (right tibia) of IBMI of each mouse. In some of these experiments, the mice were killed 5, 8, 12, and 24 weeks after transplantation and the BMs from the pairs of femurs and tibiae of each mouse were flushed into α-medium. The repopulation of human hematopoietic cells in murine BMs was determined by detecting the number of cells positively stained with PC5-conjugated anti-human CD45 mAb (Coulter) by flow cytometry (FACS Calibur, Becton Dickinson). The mice were scored as positive if over 0.1% of the total murine BM cells were human CD45$^+$. The cells were also stained with phycoerythrin-conjugated anti-human CD34 mAb (Becton Dickinson) and fluorescein isothiocyanate-conjugated mAbs for human lineage-specific Ags, including CD19 (eBioscience, San Diego, CA, USA) and CD33 (Coulter) for the detection of human stem/progenitor, lymphoid, and myeloid hematopoietic cells, respectively.

Limiting dilution analysis

To assess the frequency of SRCs in the CB-derived Lin$^-$CD34$^+$CD38$^+$, Lin$^-$CD34$^+$CD38$^-$, and Lin$^-$CD34$^-$ cells, various numbers of Lin$^-$CD34$^+$CD38$^+$ cells (2500, 5000, 10 000, and 15 000; $n=32$), Lin$^-$CD34$^+$CD38$^-$ cells (20, 50 and 100; $n=39$), and Lin$^-$CD34$^-$ cells (5000, 20 000, and 40 000; $n=21$) were transplanted to NOD/SCID mice by IBMI as reported earlier.[7,8]

Statistical analysis

In the limiting dilution analysis, the frequencies of SRCs were calculated using the Poisson statistics as reported earlier.[3,5] For each cell population, the data from several limiting dilution experiments were pooled and analyzed by applying the Poisson statistics to the single-hit model.[14] The frequency of SRC in each cell population was calculated using the weighted mean (WM) estimator, as reported.[15]

In the analysis of the *in vivo* migration pattern, the difference between the ratios of the human cell repopulation at the site of injection and the contra-lateral site of injection was checked by Fisher's exact test.

Results

Isolation of Lin$^-$CD34$^+$CD38$^+$, Lin$^-$CD34$^+$CD38$^-$, and Lin$^-$CD34$^-$ cells by FACS

First, the R1 gate was set on the blast–lymphocyte window as shown in Figure 1a. Next, Lin$^-$CD45$^+$CD34$^+$ and Lin$^-$CD45$^+$CD34$^-$ cells were gated as R2 and R3 as shown in Figure 1b. These Lin$^-$CD45$^+$CD34$^+$ cells were further

subdivided into two distinct populations gated as R4 and R5 based on their surface CD38 expression (Figure 1c). These three fractions were then sorted for the subsequent SRC assays.

The contamination of the Lin$^-$CD34$^-$ cell fraction with Lin$^-$CD34$^+$ cells was ruled out by a semiquantitative reverse transcriptase polymerase chain reaction analysis as shown in the Supplementary Figure 1.

Limiting dilution analysis of Lin$^-$CD34$^+$CD38$^+$, Lin$^-$CD34$^+$CD38$^-$, and Lin$^-$CD34$^-$ cells

Next, a limiting dilution analysis was performed to clarify the frequency of SRCs in CB-derived Lin$^-$CD34$^+$CD38$^+$ and Lin$^-$CD34$^+$CD38$^-$ cells. As shown in Figure 2a and b, limiting dilution analyses clearly showed that the frequencies of SRCs in CB-derived Lin$^-$CD34$^+$CD38$^+$ and Lin$^-$CD34$^+$CD38$^-$ cells using IBMI were estimated to be 1/6640 and 1/44, respectively. The frequency of SRCs in Lin$^-$CD34$^+$CD38$^-$ cells by tail vein injection is reported to be approximately 1/600.[3,16] On the other hand, the frequency of SRCs in the same cell population by IBMI is also reported to be 1/44 or 1/121.[16,17] Therefore, these data are comparable with these earlier reports. According to these results, the IBMI method seemed to be very sensitive and could detect the CD34$^+$ SRCs with >6–15-fold higher frequency than that by the conventional tail vein injection method. In contrast, the frequency of CD34$^-$ SRCs in Lin$^-$CD34$^-$ cells was estimated to be 1 out of 25 523 (Figure 3c), which was consistent with the findings of earlier reports.[7,8]

Characteristics of in vivo migration patterns of CD34$^+$CD38$^+$, CD34$^+$CD38$^-$, or CD34$^-$ SRCs

On the basis of the data obtained from these limiting dilution analyses (Figure 2), 100 Lin$^-$CD34$^+$CD38$^-$ cells, 15 000–20 000 Lin$^-$CD34$^+$CD38$^+$ cells, or 60 000–70 000 Lin$^-$CD34$^-$ cells (each containing approximately 2–3 SRCs) were transplanted into primary recipient mice. Thereafter, the human CD45$^+$ cell repopulation in the site of injection and the other bones were serially investigated, separately for 2–8 weeks (Figure 3). Interestingly, CD34$^+$CD38$^+$ SRCs completed their migration from the site of injection to the other bones 3 weeks after the transplantation (Figure 3a). On the other hand, CD34$^+$CD38$^-$ SRCs began to migrate 3 weeks after the transplantation, as shown in Figure 3b and the Supplementary Table. At this time point, the human CD45$^+$ cells were detected in the other bones of two out of four mice that received CD34$^+$CD38$^-$ SRCs. In addition, most of them completed their migration from the site of injection to the other bones 5 weeks after the transplantation (Figure 3b). The median rates of human CD45$^+$ cells at 5 weeks at both sites were comparable (3.5 vs 2.8%) (Supplementary Table).

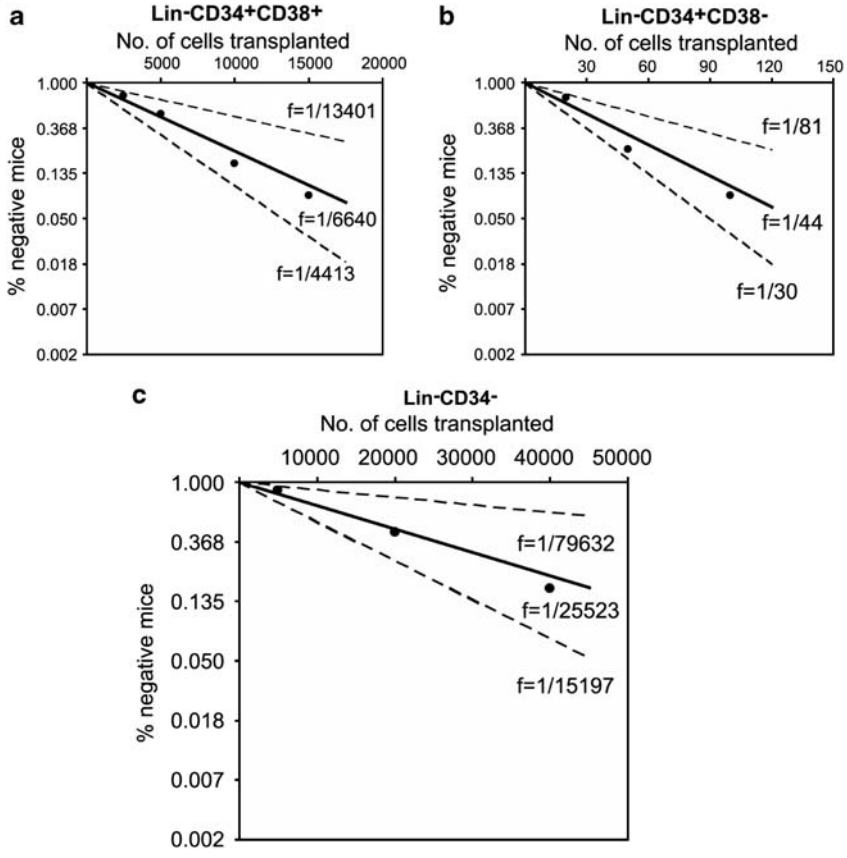

Figure 2 Comparison of the frequency of SRC in Lin$^-$CD34$^+$CD38$^+$, Lin$^-$CD34$^+$ CD38$^-$, and Lin$^-$CD34$^-$ cells. Various numbers of Lin$^-$CD34$^+$CD38$^+$ cells ($n=32$; **a**), Lin$^-$CD34$^+$CD38$^-$ cells ($n=39$; **b**), and Lin$^-$CD34$^-$ cells ($n=21$; **c**) were transplanted to NOD/SCID mice. The human CD45$^+$ cell repopulation in the mouse BMs was analyzed by flow cytometry 8 weeks after transplantation. The frequencies of SRCs in these three populations were as follows: 1 per 6640 Lin$^-$CD34$^+$CD38$^+$ cells (**a**), 1 per 44 Lin$^-$CD34$^+$CD38$^-$ cells (**b**), and 1 per 25 523 Lin$^-$CD34$^-$ cells (**c**). For each frequency determination, the middle solid line represents the estimate f_{WM}. The lower and the upper dotted lines represent the 95% confidence limit of f_{WM}.

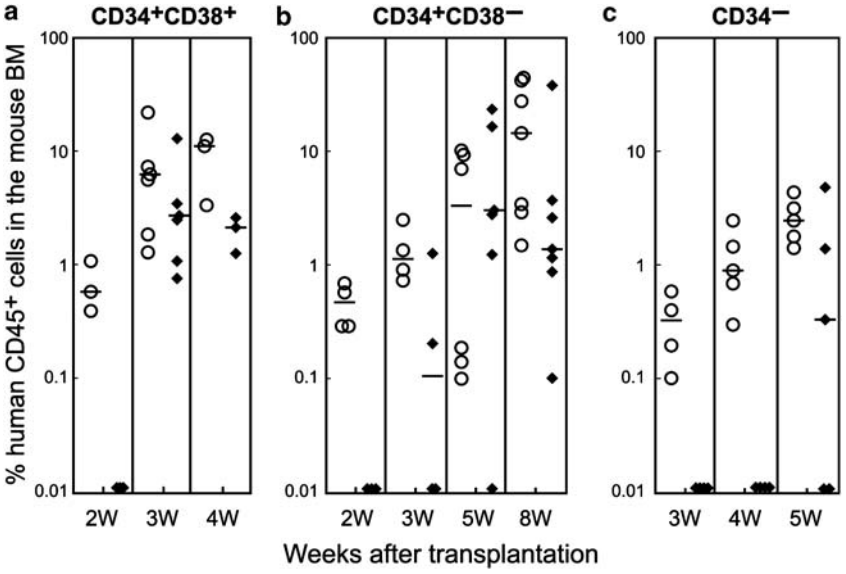

Figure 3 *In vivo* migration patterns of $CD34^+CD38^+$, $CD34^+CD38^-$, and $CD34^-$ SRCs. On the basis of the limiting dilution analyses (Figure 2), 100 $Lin^-CD34^+CD38^-$ cells, 15 000–20 000 $Lin^-CD34^+CD38^+$ cells, or 60 000–70 000 Lin^-CD34^- cells (each containing approximately 2–3 SRCs) were injected into the left tibia of each mouse. The human $CD45^+$ cell rates in the left tibia (open circle) as well as in the other bones (closed diamond) were separately and serially analyzed. Three to seven mice were analyzed at each time point. Interestingly, $CD34^+CD38^+$ and $CD34^+CD38^-$ SRCs completed or began to migrate 3 weeks after transplantation (**a**, **b**). In contrast, $CD34^-$ SRCs first began to migrate 5 weeks after transplantation (**c**). The horizontal bars show the median values. Precise data are presented in the Supplementary Table.

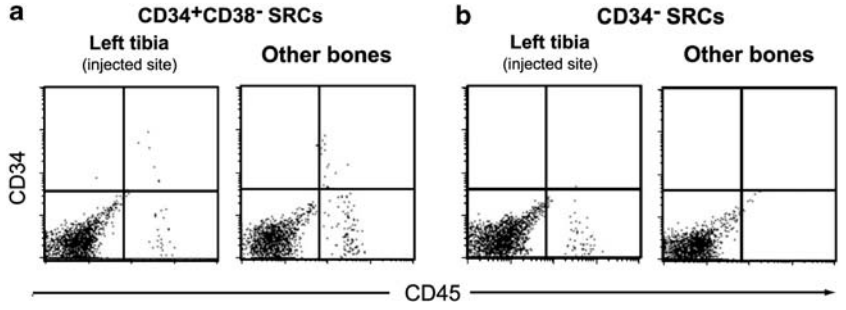

Figure 4 Delayed *in vivo* migration pattern of $CD34^-$ SRCs in comparison to that of $CD34^+CD38^-$ SRCs. In these representative mice, 200 (five SRCs) of $Lin^-CD34^+CD38^-$ and 7×10^4 (three SRCs) of Lin^-CD34^- cells were injected into the left tibia of each mouse, respectively. The number of SRCs transplanted were estimated based on the limiting dilution analyses data presented in Figure 2. The human $CD45^+$ cell repopulation of the injected left tibia and other bones of these mice were analyzed 5 weeks after transplantation. (**a**) The mice receiving transplants of five $CD34^+CD38^-$ SRCs showed human cell repopulation in the injected left tibia as well as in the other bones. Both the human $CD45^+CD34^+$ and $CD45^+CD34^-$ cells are detected. (**b**) The mice receiving transplants of three $CD34^-$ SRCs showed human cell repopulation in the injected left tibia; however, only $CD45^+CD34^-$ cells were detected. In contrast, the human $CD45^+$ cells were not detected in the other bones.

In contrast, $CD34^-$ SRCs stayed at the site of injection until 4 weeks after the transplantation and first began to migrate to other bones 5 weeks after the transplantation. As shown in Figure 3c and Supplementary Table, two out of five mice showed the human cell repopulation only in the site of injection 5 weeks after transplantation. The median rates of human $CD45^+$ cells at the contra-lateral site (0.3%) seemed to be lower than that (2.4%) at the site of injection (Supplementary Table). However, the differences between the ratios of human cell repopulation at the contra-lateral site of injection in the mice receiving $CD34^+CD38^-$ SRCs and $CD34^-$ SRCs were 2/4 vs 0/4 at 3 weeks and 5/6 vs 3/5 at 5 weeks, respectively. These differences were not statistically significant ($P > 0.05$). Therefore, further studies will be needed to elucidate the *in vivo* migration patterns of these two classes of SRCs more precisely.

Next, a precise comparative analysis of the *in vivo* migration patterns of $CD34^+CD38^-$ and $CD34^-$ SRCs was performed. In these experiments, 200 $Lin^-CD34^+CD38^-$ cells (containing approximately five SRCs) and 7×10^4 Lin^-CD34^- cells (containing approximately three SRCs) were transplanted into eight mice. All of the three mice that received five $CD34^+CD38^-$ SRCs showed the human $CD45^+$ cell repopulation in both sites. On the other hand, three out of five mice that received three $CD34^-$ SRCs showed the human $CD45^+$ cell repopulation only in the site of injection. As shown in Figure 4a, a representative mouse that received transplants of five $CD34^+CD38^-$ SRCs showed the human $CD45^+$ cell repopulation in the injected left tibiae as well as in the other bones 5 weeks after the transplantation. In addition, human $CD45^+CD34^+$ and $CD45^+CD34^-$ cells were detected at both sites. In contrast, a

representative mouse that received three CD34$^-$ SRCs showed the human CD45$^+$ cell repopulation only in the injected left tibiae 5 weeks after the transplantation (Figure 4b, left panel). In addition, human CD45$^+$CD34$^-$ cells were only detected in the injected left tibia. Figure 4b (right panel) clearly showed that no human CD45$^+$ cells were detected in the other bones. These results suggest that CD34$^-$ SRCs may, therefore, remain or slowly proliferate as a CD34$^-$ phenotype at the site of injection for at least 5 weeks.

All these findings indicated that CD34$^-$ SRC as well as CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ SRCs could actively migrate from the site of injection to the other bones. However, the timing of the initiation of migration may differ between the CD34$^+$ and CD34$^-$ SRCs. These results suggested, for the first time, that CD34$^-$ SRCs show different redistribution kinetics and also suggest that the identified CD34$^-$ SRCs[7–10] are a distinct class of primitive HSCs in comparison to CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ SRCs.[3,11,16]

LT repopulation patterns of CD34$^+$CD38$^+$, CD34$^+$CD38$^-$, and CD34$^-$ SRCs in NOG mice

The next approach to characterize the CD34$^-$ SRCs with respect to self-renewal potential as well as LT repopulating potential was to serially analyze the kinetics of engraftment for 24 weeks in the contra-lateral sites of IBMI in NOG mice that received transplants of only one CD34$^-$ SRC. This was compared with the repopulating pattern in mice that received 2–3 CD34$^+$CD38$^+$ SRCs and 10 CD34$^+$CD38$^-$ SRCs using NOG mice as shown in Figure 5. The numbers of SRCs transplanted were estimated based on the data obtained from the limiting dilution analyses presented in Figure 2.

In these experiments, both the mice that received transplants of CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ SRCs showed sign of human cell repopulation at 5 weeks after the transplantation (Figure 5). Moreover, the mice that received transplants of CD34$^+$CD38$^+$ SRCs showed the peak level of repopulation at 5 weeks, as their repopulation rates were continuously decreased to under the 1% level at 24 weeks after transplantation. However, the repopulation rates of the mice that received transplants of CD34$^+$CD38$^-$ SRCs increased during 5–12 weeks and showed the peak levels at 12 weeks after transplantation. These repopulation levels were maintained thereafter.

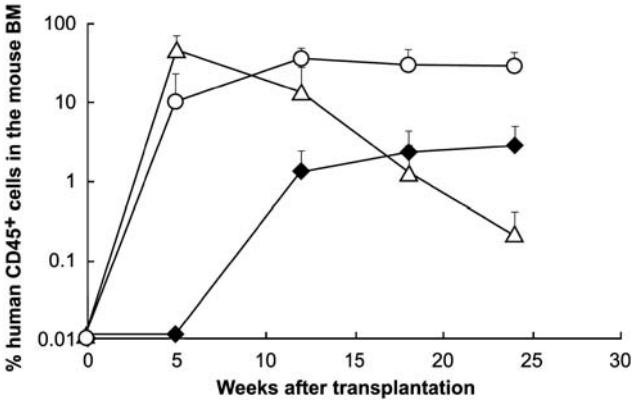

Figure 5 LT human hematopoietic cell reconstitution in NOG mice. A total of 2 to 3 CD34$^+$CD38$^+$ SRCs (open triangle), 10 CD34$^+$CD38$^-$ SRCs (open circle), and only 1 CD34$^-$ SRCs (closed diamond) were injected into the left tibia of NOG mice, respectively. The number of SRCs transplanted was estimated based on the limiting dilution analyses data presented in Figure 2. The human CD45$^+$ cell rates in the contra-lateral sites of IBMI of recipient NOG mice were serially analyzed by the aspiration method 5, 12, 18, and 24 weeks after transplantation by flow cytometry. Data represent the mean ± s.d. of the results from five mice at each time point.

Interestingly, the mice that received transplants of CD34$^-$ SRCs did not show any sign of human cell repopulation at 5 weeks after transplantation in the contra-lateral site of IBMI as shown in Figure 4b. The difference between the ratios of human cell repopulation at the contra-lateral site of injection in the NOG mice receiving CD34$^+$CD38$^-$ SRCs vs CD34$^-$ SRCs were 5/5 vs 0/5 at 5 weeks after the transplantation, as shown in Figure 5, and this difference was statistically significant ($P = 0.00794$). These findings also suggest that the timing of the initiation of migration may differ between the CD34$^+$ and CD34$^-$ SRCs. However, the repopulation rates of CD34$^-$ SRCs gradually increased at 12 weeks after the transplantation. They showed a peak level of repopulation at 24 weeks after transplantation. Next, the lympho-myeloid repopulating patterns were analyzed in the mice that received these three classes of SRCs. As shown in Supplementary Figure 2, both CD34$^-$ SRCs and CD34$^+$CD38$^-$ SRCs showed lympho-myeloid repopulation; however, the majority of CD45$^+$ human cells were CD19$^+$ B-lymphoid cells. In contrast, CD34$^+$CD38$^+$ SRCs showed only CD33$^+$ myeloid cell repopulation.

These results indicated that CD34$^-$ SRC, as well as CD34$^+$CD38$^-$ SRCs, could, therefore, sustain LT human cell repopulation in NOG mice. However, the CD34$^+$CD38$^+$ SRCs could only support short-term (ST) human cell repopulation as reported earlier.[11]

Discussion

The hierarchical organization of human primitive HSC has been extensively investigated by means of the xenotransplantation system using NOD/SCID mice, which is a commonly used surrogate assay and allows for the evaluation of primitive human lympho-myeloid reconstituting HSC *in vivo*.[1–11,16] A number of clonal tracking studies using retro- or lenti-viral vector have shown that CB-derived CD34$^+$ cells are a heterogeneous population and contain ST- and LT-repopulating SRCs.[16,18–21] Guenechea *et al*.[18] revealed that the type of repopulation in NOD/SCID mice that receive transplants of retrovirally transduced CB-derived HSCs is generally oligoclonal with an extensive variability in the life span and proliferative capacity of individual SRCs. In addition, McKenzie *et al*.[21] clearly showed that the functionally equivalent SRC daughter cell pairs can have distinct and unpredictable repopulation kinetics. Very recently, Yahata *et al*.[22] reported that human CB-derived LT-SRCs exist in a rare population of CD34$^+$CD38$^-$ cells that localized to the stem cell niche and maintained their stem cell activities while being in a quiescent state. Moreover, clonally distinct LT-SRCs control hematopoietic homeostasis and create a stem cell pool hierarchy by asymmetric self-renewal division that produced lineage-restricted ST-SRCs and long-lasting LT-SRCs.[22] They also showed that the CD34$^+$CD38$^+$ ST-SRCs are subdivided to myeloid-restricted ST-SRCs and lympho-myeloid ST-SRCs.[22] All these studies clearly show the existence of different classes of HSCs with variable proliferation and self-renewal potentials.[16,18–22]

This study investigated the characteristics of CB-derived primitive HSCs/HPCs, including CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ SRCs and the identified CD34$^-$ SRCs,[7–10] using the sensitive IBMI method[7–9,16,17,21] to more precisely elucidate

the hierarchical organization of these three classes of human primitive HSCs/HPCs. The IBMI method can improve the homing efficiency;[7–9,16,17] therefore, the frequency of these SRCs in target populations increased in comparison with those analyzed by the tail vein injection method.[7,8,16] As reported earlier,[7,8] limiting dilution analyses showed that the frequency of CD34$^+$ and CD34$^-$ SRCs in CB-derived Lin$^-$CD34^{high} and Lin$^-$CD34$^-$ cells were estimated to be 1/1010 and 1/24 100, respectively.[7,8] The frequencies of SRCs in CB-derived Lin$^-$CD34$^+$CD38$^+$, Lin$^-$CD34$^+$CD38$^-$, and Lin$^-$CD34$^-$ cells were approximately 1/6600, 1/40, and 1/25 500 cells, respectively, using the IBMI (Figure 2). On the basis of these data, the estimated but comparable numbers of these three classes of SRCs were transplanted, and their HSC characteristics were compared using a serial analysis of human cell repopulation in BMs of recipient mice at the site of injection as well as at the contra-lateral sites of IBMI separately. The data clearly showed that CD34$^+$CD38$^+$ SRCs completed the migration from the site of injection to the other bones 3 weeks after the transplantation, which is at an earlier time point than that of CD34$^+$CD38$^-$ SRCs (Figure 3; Supplementary Table). Interestingly, a substantial number of CD34$^-$ SRCs were retained at the site of injection at least for 5 weeks (Figures 3 and 4) and began to migrate from the site of injection to the other bones thereafter. The specific homing of circulating HSCs into the BM niche has been reported to depend on their responsiveness to stromal-derived factor-1.[23] In addition, the stromal-derived factor-1 and its receptor CXCR4 were found to have a critical function in murine BM engraftment by human SRCs.[24] An earlier study[7] showed that CB-derived CD34$^-$ SRCs showed poor stromal-derived factor-1/CXCR4-mediated homing abilities, possibly because of their low levels of homing receptor expression, including CXCR4, CD62L, and CD106. Therefore, the different expression patterns of these homing receptors on CD34$^+$ and CD34$^-$ SRCs may affect the *in vivo* redistribution and homing kinetics of these three classes of SRCs. Moreover, homing receptor expression of CD34$^-$ SRCs may be changed/modified after their homing into the BM niche using the IBMI method. However, the molecular mechanisms involved in this migratory (redistribution and homing) process have yet to be clarified.

More importantly, this study clearly showed that only one (estimated number) human CB-derived CD34$^-$ SRCs could sustain human lympho-myeloid cell repopulation for at least 24 weeks after the transplantation (Figure 5). It is well documented that more primitive HSCs, which show a higher self-renewal potential, can sustain the human cell repopulation in NOD/SCID or NOG mice much longer than differentiated HSCs/HPCs.[11,18,19,22] On the basis of this concept, CD34$^+$CD38$^-$ as well as CD34$^-$ SRCs could sustain up to 24 weeks of human hematopoiesis, thus suggesting that both SRCs are primitive HSCs. In addition, CD34$^-$ SRCs showed different redistribution and homing kinetics in comparison to CD34$^+$CD38$^-$ SRCs. All these findings again suggest that the identified human CB-derived CD34$^-$ SRCs represent a novel class of primitive human HSC.[7–10]

Primitive LT-HSCs in mice have been reported to lack CD34 expression.[25] However, Ogawa proposed the concept that the expression of CD34 by murine HSCs is reversible.[26] On the other hand, human BM-derived CD34$^+$ HSCs have been reported to convert to CD34$^-$ HSCs *in vivo*.[27,28] Both these studies showed that the conversion of the CD34 expression occurs at least 8–15 months after the transplantation of purified Lin$^-$CD34$^+$ or Lin$^-$CD34$^+$CD38$^-$ cells in either preimmune fetal sheep or homozygous bg.bg/nu.nu/xid.xid (bnx) mice. These studies indicate the possibility that the human BM-derived CD34$^+$ cell population can act as a reservoir for the generation of CD34$^-$ cells. Conversely, the human BM-derived CD34$^-$ cell population generates CD34$^+$-repopulating cells *in vivo*, thus indicating that the expression of CD34 antigen can be reversible on human BM-derived HSCs.[27,28] As we reported earlier,[7] human CB-derived CD34$^-$ SRCs generate CD34$^+$ SRCs *in vitro* as well as *in vivo*. In contrast to BM-derived HSCs,[27,28] human CB-derived CD34$^+$ SRCs do not convert to CD34$^-$ SRCs for at least 16 weeks after transplantation.[7] However, it remains to be determined whether human CB-derived CD34$^+$ SRCs can convert to CD34$^-$ SRCs. Therefore, much longer observation periods ($>$1 year) will thus be needed, as suggested in earlier reports.[27,28]

Interestingly, human BM-derived Lin$^-$CD34$^-$ cells did not show any SRC activity when they were directly transplanted into NOD/SCID mice by IBMI (Sonoda *et al*. unpublished data). These data are consistent with the findings of a recent report,[29] which describes that mouse BM stromal cell line, HESS-5, engineered to produce angiopoietin-1, supports the induction of hematopoietic activity in human BM-derived CD34$^-$ cells, thus suggesting that the characteristics of CD34$^-$ LT-HSC found in human CB and BM are different classes of primitive HSC.

In summary, this study showed, for the first time, that the CD34$^-$ SRCs show different *in vivo* redistribution kinetics in comparison to CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ SRCs. In addition, the earlier study[7] did not elucidate whether the CD34$^-$ cell population obtained from primary mice transplanted with CB-derived Lin$^-$CD34$^-$ cells still contain CD34$^-$ HSCs, which cannot home into the BM niche in secondary recipients even by IBMI. Further studies are now underway to examine this important issue. To more precisely elucidate CB-derived CD34$^-$ SRCs, it is very important to identify a specific positive marker for the higher degree purification of this rare class of primitive HSCs. Additional studies are also currently being conducted to clarify the candidate positive markers for these human primitive HSCs.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)