



## CD34<sup>high</sup>+CD38<sup>low</sup>/- cells generated in a xenogenic coculture system are capable of both long-term hematopoiesis and multiple differentiation

T Tsuji<sup>1</sup>, K Itoh<sup>2</sup>, Y Nishimura-Morita<sup>1</sup>, Y Watanabe<sup>1</sup>, D Hirano<sup>1</sup>, KJ Mori<sup>3</sup> and K Yatsunami<sup>1</sup>

<sup>1</sup>Division of Hematology, Pharmaceutical Frontier Research Laboratories, JT Inc., Kanagawa; <sup>2</sup>Department of Clinical Molecular Biology, Faculty of Medicine, Kyoto University, Kyoto; and <sup>3</sup>Department of Biology, Faculty of Science, Niigata University, Niigata, Japan

CD34<sup>+</sup> cells isolated from human umbilical cord blood (HUCB) are thought to have potential in clinical applications such as transplantation and gene therapy. Recently, we developed a xenogenic coculture system involving HUCB-CD34<sup>+</sup> cells and murine bone marrow stromal cells, HESS-5 cells, in combination with human interleukin-3 and stem cell factor. Under these xenogenic coculture conditions, the numbers of CD34<sup>high</sup>+ cells and primitive progenitor cells, such as CD34<sup>high</sup>+CD38<sup>low</sup>/- cells and high proliferative potential colony-forming cells (HPP-CFCs), increased dramatically by a factor of 102.1, 66.5 and 104.9, respectively. In the present study, we used a secondary culture of B progenitor cells and long-term culture (LTC)-initiating cells to characterize and compare the progenitor capability of re-isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells, which have been identified as one of the most primitive progenitor cells, with that of freshly isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells. Compared with freshly isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells, the re-isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells were equally as capable of proliferating and differentiating into myeloid and B progenitor cells. No significant differences were observed in the frequency of LTC-initiating cells in the re-isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells compared with that in freshly isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells. Furthermore, the re-isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells were capable of long-term reconstitution and multiple differentiation in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice). The results demonstrate that this xenogenic coculture system can be used for successful *in vitro* expansion of HUCB-progenitor cells that possess the capability for both long-term hematopoiesis as well as multipotent differentiation into myeloid and lymphoid cells both *in vivo* and *in vitro*.

**Keywords:** *in vitro* expansion; transplantation; hematopoietic primitive progenitor cells; long-term culture-initiating cells; hematopoiesis

### Introduction

Human umbilical cord blood (HUCB) offers potential in a number of clinical applications including transplantation<sup>1–3</sup> due to the abundance of hematopoietic stem/progenitor cells in the CD34<sup>+</sup> cell population.<sup>4,5</sup> The *in vitro* expansion of CD34<sup>+</sup> cells contained in HUCB is an attractive and useful method for hematopoietic reconstitution in recipients who fail to exhibit engrafting after transplantation of HUCB from typical HUCB collections.<sup>1</sup> Therefore, successful *in vitro* expansion of HUCB-hematopoietic stem/progenitor cells, which can restore a recipient's hematopoiesis, is expected to result in wide applicability of HUCB for clinical purposes including the treatment of adults.

Many researchers have tried to expand hematopoietic stem/progenitor cells contained in HUCB, peripheral blood, bone marrow and fetal liver.<sup>1,6–8</sup> Most of these studies involved the amplification of CD34<sup>+</sup> cells by means of a com-

bination of cytokines. Although the numbers of total hematopoietic cells and colony-forming units increased dramatically with the cytokine combination protocol, the number of CD34<sup>+</sup> cells, which are capable of multiple differentiation ability, decreased.<sup>7</sup>

The proliferation and differentiation of hematopoietic stem/progenitor cells is thought to be regulated by cellular interactions between hematopoietic cells and stromal cells comprising the hematopoietic microenvironment in hematopoietic organs.<sup>9</sup> These cellular interactions are mediated by direct cell-to-cell interactions via cell contact, and also by humoral factors produced by stromal cells.<sup>10–12</sup> Long-term cultures *in vitro* have demonstrated the important roles of the maintenance, self-renewal, proliferation and differentiation of stem/progenitor cells. However, in *in vitro* cultures in the absence of stromal cells, the proliferation and differentiation of stem/progenitor cells is dependent only on the presence of cytokines.<sup>13,14</sup>

Recently, we developed a xenogenic coculture system involving HUCB-CD34<sup>+</sup> cells and a murine stromal cell line, HESS-5 cells, in the presence of human cytokines such as recombinant human interleukin-3 (rh-IL-3), stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO).<sup>15</sup> The most potent combination of cytokines in this coculture system for increasing the number of CD34<sup>high</sup>+ cells and CFUs was recombinant human interleukin-3 (rh-IL-3) and stem cell factor (SCF). The proliferation of CD34<sup>high</sup>+ and primitive progenitor cells was calculated by FACS analysis and was expressed in terms of the number of CD34<sup>high</sup>+ and CD34<sup>high</sup>+CD38<sup>low</sup>/- cells, which have been identified as among the most primitive progenitor cells, and high-proliferative potential colony-forming cells (HPP-CFCs). HPP-CFCs in human and murine bone marrow were reported to be earlier in the stem/progenitor cell hierarchy than CFU-GEMM, and a counterpart of murine HPP-CFCs has been detected in human bone marrow, umbilical cord blood and fetal liver.<sup>16–18</sup>

In the present study, we characterized the re-isolated CD34<sup>+</sup> and CD34<sup>high</sup>+CD38<sup>low</sup>/- cells after *in vitro* expansion in a large-scale xenogenic coculture system. The CD34<sup>+</sup>CD38<sup>-</sup> immunophenotype defines a primitive subpopulation of hematopoietic stem/progenitor cells in hematopoietic organs.<sup>19–22</sup> The cell surface markers, colony-forming ability and cell-cycle status of the re-isolated CD34<sup>+</sup> cells after *in vitro* expansion in this xenogenic coculture system are described. We compared the capability for secondary expansion and differentiation of re-isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells into B progenitor cells after *in vitro* expansion with that of freshly isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells. We also performed quantitation analysis on long-term culture (LTC)-initiating cells and clonogenic B progenitor cells in freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low</sup>/- cells using limiting dilution analysis. Furthermore, long-term reconstitution ability of the

Correspondence: T Tsuji, Pharmaceutical Frontier Research Laboratories, JT Inc., 1-13-2 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan; Fax: +81-45-786-7692  
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re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) was analyzed.

## Materials and methods

### *Cytokines and monoclonal antibodies*

Purified recombinant human stem cell factor (rh-SCF) was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human interleukin-3 (rh-IL-3) was obtained from Genzyme (Cambridge, MA, USA). Monoclonal antibodies (mAbs) were used for cell-surface marker analysis by flow cytometry: fluorescein isothiocyanate (FITC)-conjugated anti-CD34 mAb ( $\alpha$ CD34; clone HPCA-2) was obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA), and R-phycoerythrin (PE)-conjugated  $\alpha$ CD13 (clone WM-15),  $\alpha$ CD33 (clone WM-53),  $\alpha$ CD38 (clone HIT2),  $\alpha$ CD45RA (clone HI100),  $\alpha$ CD10 (clone HI10a),  $\alpha$ CD19 (clone HIB19),  $\alpha$ CD5 (clone UCHT2), and  $\alpha$ CD2 (clone RPA-2.10) mAbs were purchased from Pharmingen (San Diego, CA, USA).

### *Murine stromal cells and their culture*

The murine stromal cell line was established from bone marrow of C3H/HeN mice, and its hematopoietic supportive ability was analyzed by means of *in vitro* long-term cultures as described previously.<sup>23</sup> HESS-5 cells were maintained in alpha-minimal essential medium ( $\alpha$ -MEM; Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% (v/v) horse serum (HS; Nichimen America, Los Angeles, CA, USA).

### *Collection and preparation of CD34<sup>+</sup> cells from HUCB and in vitro expanded cells*

HUCB was obtained on full-term deliveries, and under the guidelines established by the Review Boards of Tokyo Senbai Hospital and the Pharmaceutical Frontier Research Laboratories, JT Inc. Blood was collected in sterile tubes containing 20 U/ml heparin from the umbilical cord vein at the placental end by needle aspiration while the placenta remained *in situ*.

Low-density cells (<1.077 g/ml), CD34<sup>+</sup> and CD34<sup>-</sup> cells were isolated according to the instruction manuals and as described previously.<sup>15,24</sup> Cells after *in vitro* expansion were also subjected to the same separation procedure as for CD34<sup>+</sup> cells.

### *Xenogenic coculture of murine stromal cells and HUCB CD34<sup>+</sup> cells*

HUCB-CD34<sup>+</sup> cells ( $1 \times 10^5$  cells/ml) were cocultured with or without a confluent layer of the murine stromal cell line, HESS-5 cells, in 30 ml of Myelocult H5100 ( $\alpha$ -MEM supplemented with 12.5% HS, 12.5% fetal calf serum (FCS), and  $10^{-4}$  m 2-mercaptoethanol; Stem Cell Technologies, Vancouver, British Columbia, Canada) in a 75 cm<sup>2</sup> culture flask (Falcon, Lincoln Park, NJ, USA) with rh-IL-3 and rh-SCF.<sup>15,25</sup> After 10 days culture, the cells were harvested by pipetting, filtration through a nylon mesh and then centrifugation. These cells were subjected to immunofluorescence staining, and cell surface markers were analyzed by flow cytometry, and also

colony formation ability was analyzed by semi-solid methylcellulose assaying.

### *Flow cytometric analysis and cell sorting of CD34<sup>high</sup>+CD38<sup>low/-</sup> cell populations*

Cells were collected, and then stained with FITC- or PE-conjugated mAbs. The stained cells were analyzed as described previously.<sup>15</sup>

Cell sorting was also performed with a FACSsort flow cytometer (Becton Dickinson). HUCB-CD34<sup>+</sup> cells and CD34<sup>+</sup> cells after *in vitro* expansion were isolated as described above. The cells were stained with FITC- $\alpha$ CD34 and PE- $\alpha$ CD38 mAbs. The CD34<sup>high</sup>+CD38<sup>low/-</sup> cell population was defined as to high CD34 antigen expression and PE-fluorescence less than the maximum PE-fluorescence of the isotype control. The content of the CD34<sup>high</sup>+CD38<sup>low/-</sup> cell population in the freshly isolated HUCB-CD34<sup>+</sup> cells was usually 3.0–4.5%.

### *Semisolid methylcellulose assay*

The assay for the detection of hematopoietic progenitor cells was performed in a complete pretested mixture, Methocult GF H4434V (Stem Cell Technologies), comprising 0.9% Iscove's methylcellulose, 30% FCS, 1% BSA,  $10^{-4}$  m 2-ME, 2 mm l-glutamine, 3 U/ml erythropoietin, 50 ng/ml rh-SCF, 10 ng/ml rh-GM-CSF, 10 ng/ml rh-G-CSF and 20 ng/ml rh-IL-3, as described previously.<sup>15,16,26</sup> The cells were incubated at 37°C under a humidified atmosphere of 5% CO<sub>2</sub> for 28 days, and then assayed for colony formation as to CFU-GM, HPP-CFCs and erythroid colonies including BFU-E and CFU-GEMM.<sup>15,16</sup>

### *Cell cycle analysis*

Mononuclear cells and CD34<sup>+</sup> cells in HUCB, and cultured cells after expansion in the xenogenic culture system were subjected to cell cycle analysis. The repopulated CD34<sup>+</sup> cells were re-isolated from total hematopoietic cells after expansion as described above. These cells were stained using a Cycle TEST PLUS DNA Reagent Kit (Becton Dickinson) according to the instruction manual. Cell samples were analyzed with a FACSsort flow cytometer (Becton Dickinson) calibrated with a DNA Quality Control Kit (Becton Dickinson) using the nuclei of chicken erythrocytes and thymocytes. The percentages of cells in the G<sub>0</sub>/G<sub>1</sub>-, S- and G<sub>2</sub>/M-phases of the cell cycle were determined using CellFIT software (Becton Dickinson).

### *Comparison of CD34<sup>high</sup>+CD38<sup>low/-</sup> cell populations isolated from HUCB-CD34<sup>+</sup> cells and CD34<sup>+</sup> cells after in vitro expansion on coculture with HESS-5 cells and the B progenitor culture*

Sorted CD34<sup>high</sup>+CD38<sup>low/-</sup> cells from HUCB-CD34<sup>+</sup> cells and re-isolated CD34<sup>+</sup> cells after *in vitro* expansion were cultured in this xenogenic coculture system in combination with rh-IL-3 and rh-SCF as described above.

On the other hand, the B progenitor culture reported by Rawlings *et al*<sup>27</sup> was modified and performed using HESS-5 cells. Briefly,  $3 \times 10^3$  cells/well of a 12-well culture plate of the sorted CD34<sup>high</sup>+CD38<sup>low/-</sup> cells were plated on a confluent layer of HESS-5 cells in 2 ml of RPMI-1640 medium

(Nikken Bio Medical Laboratory) supplemented with 3% defined FCS (GIBCO-BRL, Rockville, MD, USA),  $5 \times 10^{-5}$  M 2-mercaptoethanol and 50 ng/ml of rh-flk-2/flt-3 ligand (PeproTech EC, London, UK). At weekly intervals, half of the medium was changed. For analysis after 3 weeks in culture, the cultured cells were harvested, and then stained with FITC- $\alpha$ CD19 and PE- $\alpha$ CD10 mAbs. Quantitation of B progenitor cells was performed by limiting dilution analysis. For limiting dilution experiments, the CD34<sup>high+</sup>CD38<sup>low/-</sup> cells sorted HUCB or the expanded cells were seeded on to the confluent layer of HESS-5 cells at the concentration of 2–200 cells per well with 24 replicates in 150  $\mu$ l of the same medium as described above. After 3 weeks in culture, the frequency of the B-progenitor cells in these populations was calculated by Poisson statistics and weighted mean method.<sup>28,29</sup>

#### Quantitation of LTC-initiating cells in CD34<sup>high+</sup>CD38<sup>low/-</sup> cells sorted from HUCB and the *in vitro* expanded cells by limiting dilution analysis

To determine the frequency of LTC-initiating cells by limiting dilution analysis, we developed the long-term culture method on HESS-5 cells for 5 weeks. Confluent layers of HESS-5 cells were established on 96-well type plate (Falcon), and then the CD34<sup>high+</sup>CD38<sup>low/-</sup> cells sorted from HUCB and the *in vitro* expanded cells were seeded on the layer at the range of 2–200 cells per well with 24 replicates in Myelocult H5100 (Stem Cell Technologies). At weekly intervals, half of the culture medium was changed for fresh medium. After 5 weeks in culture, wells were assayed for CFU-C in methylcellulose culture as described above and colonies were counted after 2 weeks. Individual wells were scored as a positive if at least one colony was detected. The frequency of LTC-initiating cells in the seeded population was calculated by Poisson statistics and the weighted mean method.<sup>28,29</sup>

#### Transplantation of CD34<sup>high+</sup>CD38<sup>low/-</sup> cells into immune-deficient mice

Sublethally irradiated (350 cGy) 8-week-old NOD/LtSz-scid/scid (NOD/SCID) mice, which were obtained from Clea Japan (Tokyo, Japan), received transplantation by tail-vein injection as described in previous reports.<sup>30–32</sup> CD34<sup>high+</sup>CD38<sup>low/-</sup> cells ( $2 \times 10^5$  cells/mouse) were transplanted into five mice together with irradiated (15 Gy) nonrepopulating lineage-positive CD34<sup>-</sup> cells ( $2 \times 10^6$  cells/mouse) as accessory cells. After 12 weeks, bone marrow cells of the transplanted mice were analyzed by flowcytometry.

## Results

#### Expansion of HUCB-CD34<sup>+</sup> cells in a xenogenic coculture system

Recently, we reported a xenogenic coculture system involving HUCB-CD34<sup>+</sup> cells and murine hematopoietic-supportive stromal cells, HESS-5 cells.<sup>15</sup> The optimal cytokine combination for the proliferation of CD34<sup>+</sup> cells and HPP-CFCs was rh-IL-3 and rh-SCF in the coculture system. To characterize the re-isolated CD34<sup>+</sup> cells and CD34<sup>high+</sup>CD38<sup>low/-</sup> cells after the expansion, we performed on a practical large scale

of 75 cm<sup>2</sup>. The FACS profiles on staining with  $\alpha$ CD34-FITC and  $\alpha$ CD38-PE are shown in Figure 1.

When HUCB-CD34<sup>+</sup> cells were cultured on stimulation with rh-IL-3 and rh-SCF in the absence of HESS-5 cells, the total number of hematopoietic cells greatly increased to  $169.7 \pm 4.0$ -fold that of starting cells. However, the content of CD34<sup>high+</sup> cells among total hematopoietic cells stimulated with rh-IL-3 and rh-SCF dramatically decreased (2.0%), and the calculated number of CD34<sup>high+</sup> cells slightly increased ( $5.5 \pm 1.6$ -fold). In contrast, the number of total hematopoietic cells and CD34<sup>high+</sup> cells dramatically increased  $396.4 \pm 82.5$ - and  $102.1 \pm 23.1$ -fold, respectively, in the xenogenic coculture system in combination with rh-IL-3 and rh-SCF. Under these culture conditions, surprisingly, a very primitive stem/progenitor population, CD34<sup>high+</sup>CD38<sup>low/-</sup> cells, was clearly detected (1.0%).

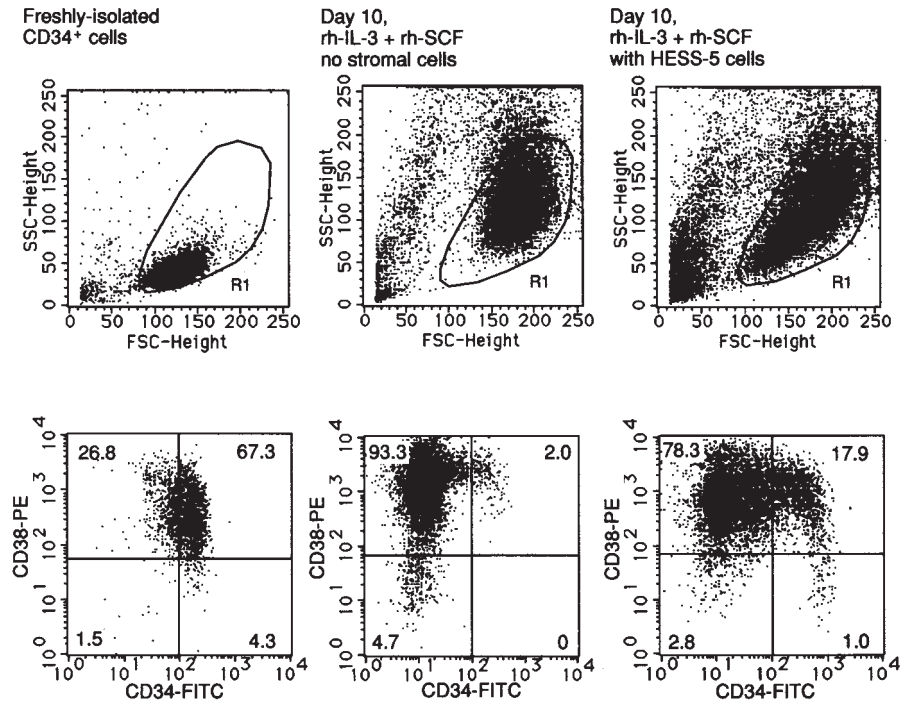
We also examined colony formation assay after the *in vitro* expansion as described above. In the presence of rh-IL-3 and rh-SCF without HESS-5 cells, only myeloid progenitor cells (CFU-GM) dramatically increased ( $180 \pm 47$ -fold), small HPP-CFC and erythroid progenitor cells only slightly increasing (4.9-fold and 5.7-fold). Large HPP-CFC were not detected under these assay conditions. Under the xenogenic coculture condition in combination with rh-IL-3 and rh-SCF, on the other hand, the number of progenitor cells dramatically increased. The large and small HPP-CFC proliferated 65.6-fold and 161.4-fold, respectively. The number of CFU-GM increased to 734.2-fold, ie more than under the culture conditions without the stromal cells (244.9-fold). An increase in the number of erythroid colonies, including BFU-E and CFU-GEMM, was observed under these culture conditions (12.4-fold).

#### Characterization of the expanded CD34<sup>+</sup> cells in the xenogenic coculture system in combination with rh-IL-3 and rh-SCF

To characterize the expanded CD34<sup>+</sup> cells, CD34<sup>+</sup> cells were re-isolated from the total expanded cells, which were cultured with stimulation with rh-IL-3 and rh-SCF in the xenogenic coculture system as described above, by magnetic cell sorting. After *in vitro* expansion in this system, the re-isolated CD34<sup>+</sup> cells of  $63.4 \pm 20.5 \times 10^5$  cells could be collected, when the culture was started with  $10^5$  HUCB-CD34<sup>+</sup> cells per 75 cm<sup>2</sup> flask.

The efficiency of colony formation of the freshly isolated and re-isolated CD34<sup>+</sup> cells was the same (35.4% and 34.2%, respectively: Table 1). Although the number of small HPP-CFC among the re-isolated CD34<sup>+</sup> cells per  $10^5$  cells was the same as that among the freshly isolated HUCB-CD34<sup>+</sup> cells, the content of large HPP-CFC and erythroid colonies in the re-isolated CD34<sup>+</sup> cells after *in vitro* expansion was decreased to 58.9% and 7.7%, respectively, that of HUCB-CD34<sup>+</sup> cells. In contrast, the number of CFU-GM of the re-isolated CD34<sup>+</sup> cells increased to 2.9-fold that of the starting HUCB-CD34<sup>+</sup> cells.

The FACS profiles of the freshly isolated HUCB-CD34<sup>+</sup> cells and re-isolated CD34<sup>+</sup> cells are shown in Figure 2. The size and density of the re-isolated CD34<sup>+</sup> cells were greater than those of freshly isolated HUCB-CD34<sup>+</sup> cells. The percentages of CD34<sup>high+</sup> and CD34<sup>low+</sup> cells among HUCB-CD34<sup>+</sup> cells did not change after the expansion. The percentages of CD34<sup>high+</sup>CD33<sup>+</sup> and CD34<sup>high+</sup>CD45RA<sup>+</sup> cells among the re-isolated CD34<sup>+</sup> cells significantly increased to more than



**Figure 1** Flow cytometric analysis of HUCB-CD34<sup>+</sup> cells and cells amplified from HUCB-CD34<sup>+</sup> cells under various culture conditions, with staining with  $\alpha$ CD34-FITC and  $\alpha$ CD38-PE. The culture conditions are shown above each panel. Forward (size) and side scatter (density) are shown, showing region R1 in which the CD34<sup>+</sup> cells are located. The HESS-5 cells are located outside this region. The percentages of CD34<sup>high</sup>+ and CD34<sup>low/-</sup> cells at each analysis point are given in the upper and lower quadrants of each histogram. These results are expressed as the mean values for triplicate wells and are representative of three to five independent experiments.

**Table 1** Comparison of colony-forming cells among CD34<sup>+</sup> cells freshly isolated from human umbilical cord blood and re-isolated CD34<sup>+</sup> cells after *in vitro* expansion in the xenogenic coculture system in combination with rh-IL-3 and rh-SCF

Conditions	Number of colonies (colonies/10 <sup>2</sup> CD34 <sup>+</sup> cells)			
	Large HPP-CFC	Small HPP-CFC	CFU-GM	Erythroid colonies
Day 0, freshly isolated CD34 <sup>+</sup> cells	5.6 ± 1.6	3.9 ± 1.2	9.0 ± 2.3	16.9 ± 2.5
Day 10, re-isolated CD34 <sup>+</sup> cells after expansion	3.3 ± 0.6	3.6 ± 0.6	26.0 ± 2.6	1.3 ± 0.6

The results are expressed as mean numbers of colonies per 10<sup>2</sup> CD34<sup>+</sup> cells ± s.e., isolated from human umbilical cord blood and *in vitro* expanded CD34<sup>+</sup> cells in the xenogenic coculture system in combination with rh-IL-3 (20 ng/ml) and rh-SCF (50 ng/ml) as described under Materials and methods. The colony formation assay was performed at the density of 100–300 cells/dish, 35 mm in diameter, and in triplicate. These results are representative of four independent experiments.

that of the freshly isolated CD34<sup>+</sup> cells (each  $P < 0.01$ ; Student's *t*-test). The percentage of the CD34<sup>high</sup>+CD38<sup>low/-</sup> cell population among the re-isolated CD34<sup>+</sup> cells decreased to 65.1% that of HUCB-CD34<sup>+</sup> cells ( $P < 0.05$ ; Student's *t*-test). In contrast, the CD34<sup>+</sup>CD13<sup>low/-</sup> cell population among HUCB-CD34<sup>+</sup> cells increased on expansion. The expression of CD10 and CD19 did not change before or after expansion. On examination of the expression of CD2 and CD5, surprisingly, CD34<sup>+</sup>CD2<sup>+</sup> and CD34<sup>+</sup>CD5<sup>+</sup> cell populations were clearly detected among the re-isolated CD34<sup>+</sup> cells (8.7% and 0.6%, respectively).

#### Cell cycle analysis of HUCB-CD34<sup>+</sup> cells and expanded CD34<sup>+</sup> cells

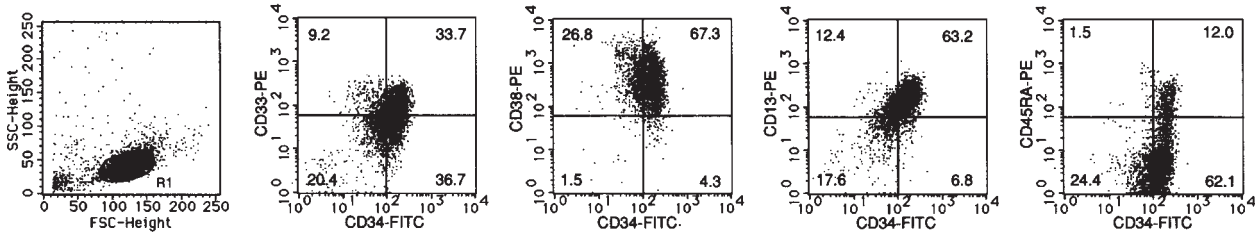
To confirm the proliferation of re-isolated CD34<sup>+</sup> cells stimulated with rh-IL-3 and rh-SCF in the xenogenic coculture sys-

tem, we next performed cell cycle analysis of the CD34<sup>+</sup> cells (Figure 3). Although freshly isolated HUCB-mononuclear cells and CD34<sup>+</sup> cells, 96.8% and 97.5%, respectively, were in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle, CD34<sup>+</sup> cells re-isolated from the total hematopoietic cells of an *in vitro* expansion culture with stimulation with rh-IL-3 and rh-SCF, and coculturing with HESS-5 cells, which were the culture conditions for the self-renewal of CD34<sup>+</sup> cells, were found to pass to the cycling phase (41.6%) from the G<sub>0</sub>/G<sub>1</sub> phase (58.4%), like the total hematopoietic cells.

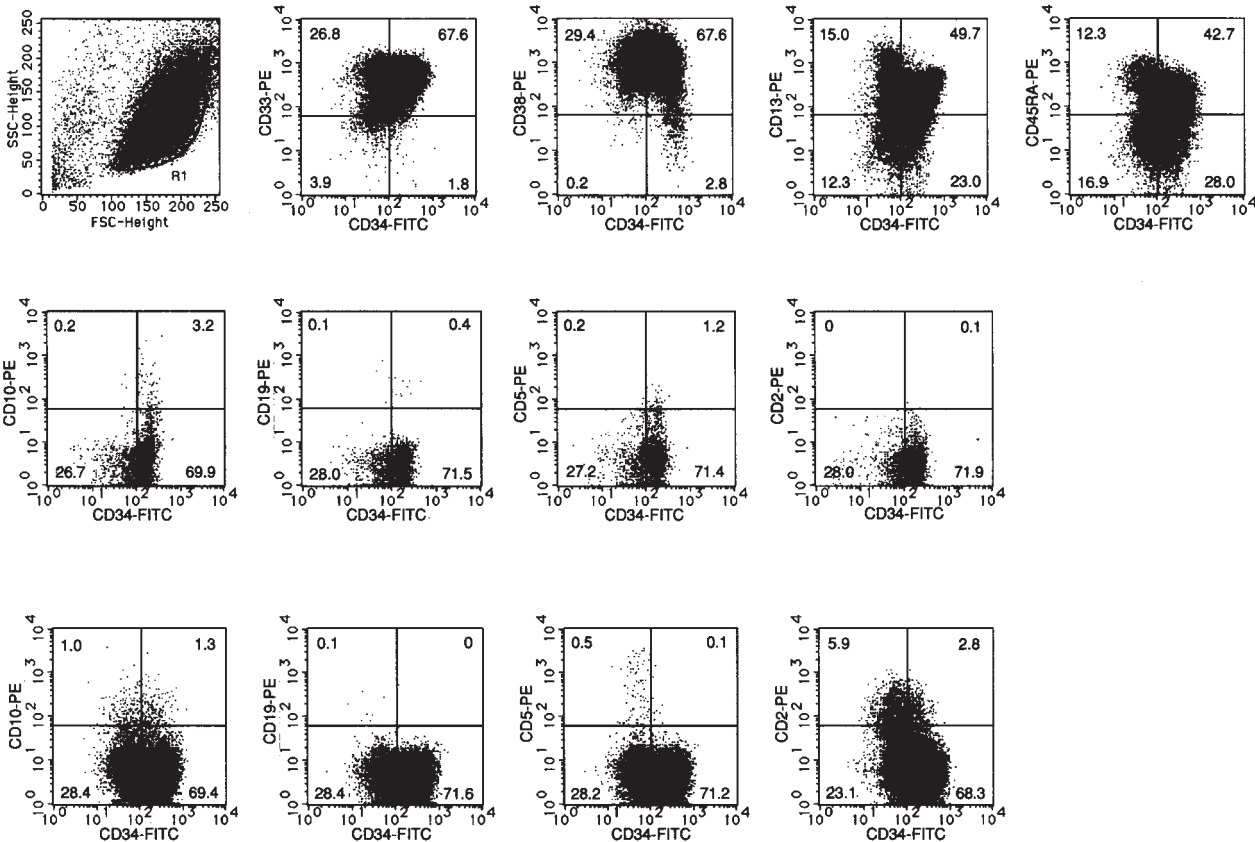
#### Comparison of the characteristics of the CD34<sup>high</sup>+CD38<sup>low/-</sup> cell population between before and after *in vitro* expansion

The primitive progenitor cells are known to be enriched in the CD34<sup>+</sup>CD38<sup>-</sup> subpopulation.<sup>24–27</sup> After expansion in the

Freshly-isolated CD34<sup>+</sup> cells



Re-isolated CD34<sup>+</sup> cells from *in vitro* expanded cells with rh-IL-3, rh-SCF, and HESS-5 cells



**Figure 2** Phenotypic characterization of freshly isolated HUCB-CD34<sup>+</sup> cells and re-isolated CD34<sup>+</sup> cells after *in vitro* expansion, with double-staining with  $\alpha$ CD34-FITC and  $\alpha$ CD33-PE,  $\alpha$ CD38-PE,  $\alpha$ CD13-PE,  $\alpha$ CD45RA-PE,  $\alpha$ CD10-PE,  $\alpha$ CD19-PE,  $\alpha$ CD5-PE or  $\alpha$ CD2-PE. Region R1 was used for analysis of cell surface marker expression on the cells. The percentages of cells expressing high and low/undetectable levels of the CD34, CD33, CD38, CD13, CD45RA, CD10, CD19, CD5 and CD2 antigens are given in the upper and lower quadrants of each histogram. These results are expressed as the mean values for triplicate wells and representative of four independent experiments.

xenogenic coculture system, a minor primitive CD34<sup>high</sup>+CD38<sup>low/-</sup> cell population was clearly detected. To determine whether or not this subpopulation after expansion possesses the characteristics of primitive stem/progenitor cells, we performed a secondary culture on HESS-5 cells and a B progenitor culture of this subpopulation *in vitro*, and then the abilities of CD34<sup>high</sup>+CD38<sup>low/-</sup> cells after the *in vitro* expansion were compared with those of the subpopulation in HUCB-CD34<sup>+</sup> cells.

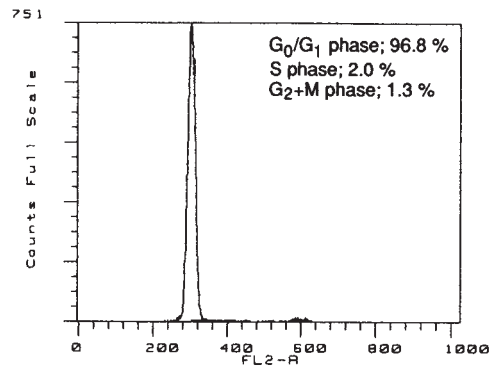
The freshly and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cell populations derived from HUCB-CD34<sup>+</sup> cells and CD34<sup>+</sup> cells after *in vitro* expansion, respectively, are shown in Figure 4a. The re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells were subjected to the secondary xenogenic coculture on a confluent layer of HESS-5 cells in combination with rh-IL-3 and rh-SCF. After 10

days, the contents of CD34<sup>high</sup>+ cells in the total hematopoietic cells among the freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells were 16.6 and 12.4%, respectively, and no essential differences were observed between these two FACS profiles (Figure 4b). When these cells were cultured in the absence of HESS-5 cells with rh-IL-3 and rh-SCF, the CD34<sup>high</sup>+ cells dramatically decreased, like the culture of the freshly isolated HUCB-CD34<sup>+</sup> cells, as described above. The numbers of total hematopoietic cells and CD34<sup>high</sup>+ cells among the freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells are shown in Table 2.

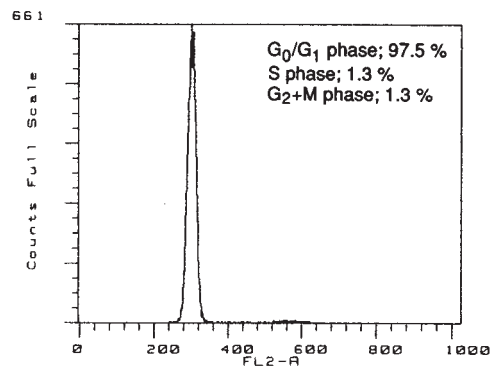
Next, we examined the production of B lymphoid progenitor cells from these subpopulations *in vitro* reported by Rawlings *et al*<sup>27</sup> except for the cocultured stromal cells. We cultured fresh and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells in the

**Day 0,  
umbilical cord blood**

mononuclear cells

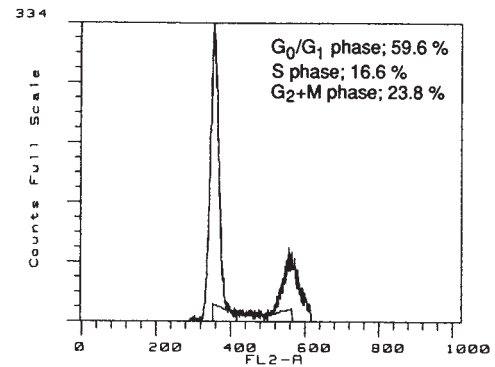


CD34<sup>+</sup> cells

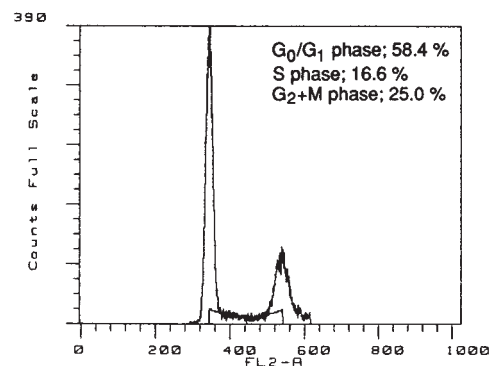


**Day 10,  
rh-IL-3 and rh-SCF,  
cocultured with HESS-5 cells**

total hematopoietic cells



CD34<sup>+</sup> cells



**Figure 3** Cell cycle analysis of mononuclear cells and CD34<sup>+</sup> cells isolated from HUCB, and cultured cells after expansion in the xenogenic culture system. HUCB-CD34<sup>+</sup> cells were stimulated with rh-IL-3 and rh-SCF in the xenogenic coculture system, and then CD34<sup>+</sup> cells were re-isolated from the total hematopoietic cells as described under Materials and methods. The cells were stained with propidium iodide and then analyzed by FACS. The contents of cells in the G<sub>0</sub>/G<sub>1</sub>-, S- and G<sub>2</sub>M-phases of the cell cycle were determined using CellFIT software on a FACSsort (Becton Dickinson). The percentage in each phase is expressed as the mean value for three independent experiments, and is given in each histogram.

presence of HESS-5 cells because HESS-5 cells also stimulate the production of B progenitor cells in the condition. The harvested cells were small and showed low-density and they were typically lymphoid (Figure 5). Furthermore, almost all the cells were CD19CD10-double positive B progenitor ones (Figure 5), and these cells exhibited no staining with myeloid lineage-specific markers such as CD13 and CD33 (data not shown). The numbers of the cells increased to 114.7- and 101.7-fold those of starting cells, respectively (Table 3). The frequency of clonogenic B progenitor cells in the freshly isolated and the re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells was calculated to be 1 per 41 and 19.5 cells, respectively (Figure 6). Taken together, these results indicate that the re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells after *in vitro* expansion have the same abilities as to proliferation and differentiation into myeloid and B lymphoid cells as the freshly isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells, and possess the characteristics of primitive progenitor cells.

*Quantitation of LTC-initiating cells in CD34<sup>high</sup>+CD38<sup>low/-</sup> cells isolated from HUCB and the *in vitro* expanded cells by limiting dilution analysis*

To determine whether the sorted CD34<sup>high</sup>+CD38<sup>low/-</sup> cells have the ability to sustain long-term hematopoiesis, the fre-

quencies of LTC-initiating cells in CD34<sup>high</sup>+CD38<sup>low/-</sup> cells sorted from HUCB and the *in vitro* expanded cells were quantified by the modified mini-LTC in 96-well culture plate with the confluent layers of irradiated murine stromal cell line, HESS-5 cells. The testing cells were seeded on to the layer with at least five concentrations with 24 replicates and cultured for 5 weeks. The frequency of LTC-initiating cells in the freshly isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells from HUCB could be calculated to be 1 cell per 19.5 cells of the freshly isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells (Figure 7). After *in vitro* expansion, CD34<sup>high</sup>+CD38<sup>low/-</sup> cells were sorted from the total expanded cells and the frequency of LTC-initiating cells in the population was found to be 1 per 18.8 cells. There was no significant difference in the frequency of LTC-initiating cells between the freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells ( $P > 0.7$ ; Student's *t*-test).

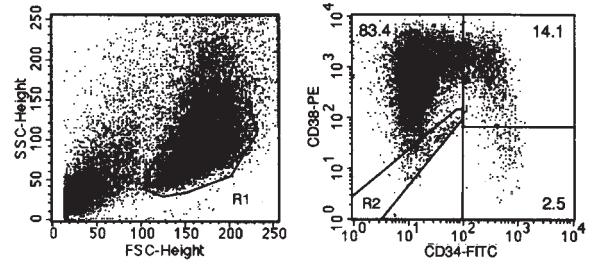
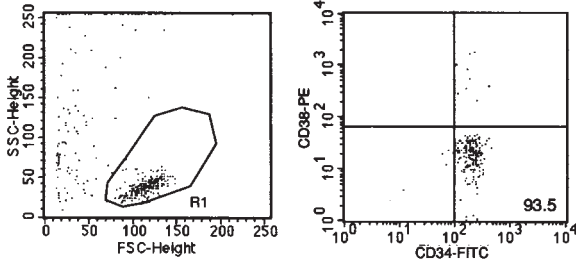
*Reconstitution of immuno-deficient mice by injection of re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells after xenogenic coculture*

Re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells after xenogenic coculture possess long-term reconstitution ability in immuno-

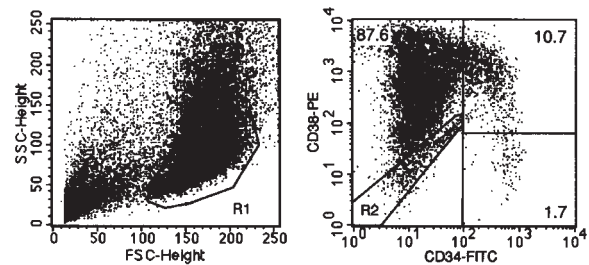
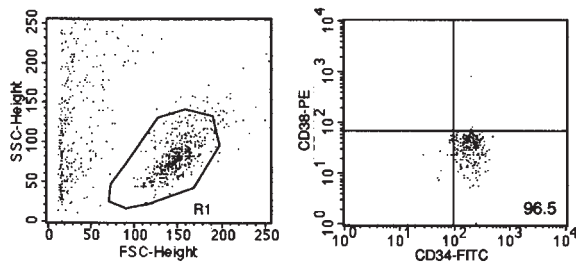
**a** Sorted CD34<sup>high</sup>+ CD38<sup>low/-</sup> cells

**b** Total hematopoietic cells after secondary culture

Freshly-isolated CD34<sup>high</sup>+ CD38<sup>low/-</sup> cells.



Re-isolated CD34<sup>high</sup>+ CD38<sup>low/-</sup> cells.



**Figure 4** Flow cytometric analysis of the freshly isolated and the re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells, with double-staining with FITC- $\alpha$ CD34 and PE- $\alpha$ CD38 mAbs. (a) Sorted CD34<sup>high</sup>+CD38<sup>low/-</sup> cells derived from CD34<sup>+</sup> cells of HUCB and after *in vitro* expansion. Region R1 was used for analysis of the expression of cell surface markers on the cells. CD34<sup>high</sup>+CD38<sup>low/-</sup> cells were detected in the lower right of quadrants and their purities are given in the lower quadrants of each histogram. (b) Phenotypic characterization of total hematopoietic cells after *in vitro* expansion of freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells. Regions R1 and R2 were used as described above. The percentages of cells expressing high and low/undetectable levels of the CD34 antigen, and high/intermediate and undetectable levels of the CD38 antigens are given in the upper and lower quadrants of each histogram. These results are representative of three independent experiments.

**Table 2** Expansion of freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells in the xenogenic coculture system in combination with rh-IL-3 and rh-SCF *in vitro*

Input cells	Number of total hematopoietic cells (cells/well $\times 10^{-5}$ )	Fold-increase in total hematopoietic cells	Calculated number of CD34 <sup>high</sup> + cells (cells/well $\times 10^{-5}$ )	Fold-increase in CD34 <sup>high</sup> + cells
Freshly isolated CD34 <sup>high</sup> +CD38 <sup>low/-</sup> cells	15.0 $\pm$ 2.0	500 $\pm$ 67	2.5 $\pm$ 0.2	83.1 $\pm$ 6.7
Re-isolated CD34 <sup>high</sup> +CD38 <sup>low/-</sup> cells	24.6 $\pm$ 3.1	821 $\pm$ 103	3.1 $\pm$ 0.4	102.0 $\pm$ 12.0

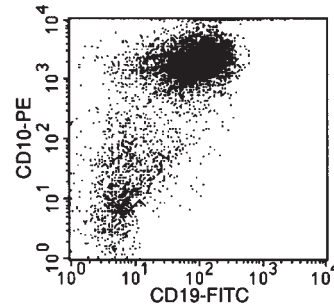
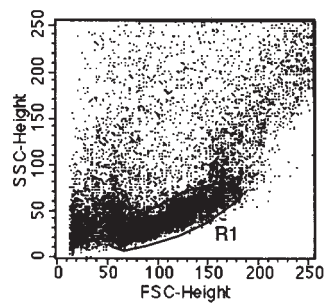
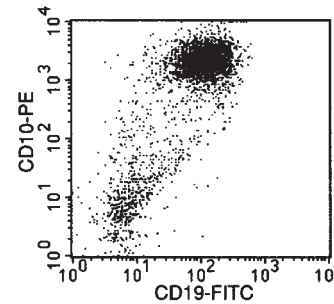
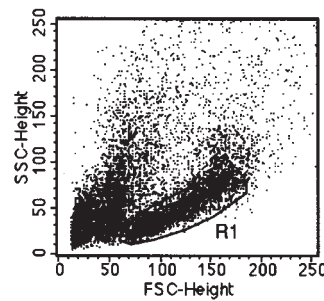
Sorted CD34<sup>high</sup>+CD38<sup>low/-</sup> cells derived from the HUCB-CD34<sup>+</sup> cells and re-isolated CD34<sup>+</sup> cells after expansion were cultured on HESS-5 cells in the presence of rh-IL-3 (20 ng/ml) and rh-SCF (50 ng/ml) for 10 days as described under Materials and methods. The cells was harvested and the numbers of total hematopoietic cells determined. The number of CD34<sup>high</sup>+ cells were calculated from the total hematopoietic cell number and the percentage of CD34<sup>high</sup>+ cells by FACS analysis. The results are expressed as the mean number per well of 12-well culture plate  $\pm$  s.e., and are representative of three independent experiments.

deficient NOD/SCID mice. Purified  $2 \times 10^5$  cells CD34<sup>high</sup>+CD38<sup>low/-</sup> cells isolated from HUCB and total expanded cells after xenogenic coculture were transplanted into five NOD/SCID mice. Twelve weeks after transplantation, human CD45<sup>+</sup> cells were detected in all transplanted mice and in the bone marrow of both NOD/SCID mice transplanted with freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells (Table 4). No significant differences were observed in the percentages of chimerism for CD45<sup>+</sup> cell in bone marrow between the freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells. A typical histogram of the expression of human CD45<sup>+</sup> cells is shown in Figure 8a. Human CD45<sup>+</sup> cells were contained at above 70% of total bone marrow cells in NOD/SCID mice transplanted with the re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells. Human CD34<sup>+</sup> cells were clearly detected in bone marrow cells in NOD/SCID

mice (Figure 8b). CD38<sup>+</sup> cells, which were expressed on the cell surface of mature myeloid and lymphoid cells, were also detected in the bone marrow cells (Figure 8b), and the transplanted cells were capable of multilineage differentiation into myeloid (CD13<sup>+</sup>) and B lymphoid cells (CD19<sup>+</sup>) in NOD/SCID mice (data not shown).

**Discussion**

Some researchers have reported analysis of the proliferation of human hematopoietic cells using a xenogenic coculture system.<sup>19,20</sup> The number of clonogenic progenitor cells increased 3- to 10-fold on 5–10 weeks coculture, and the clonogenic output on long-term culture of CD34<sup>+</sup>/CD38<sup>-</sup> cells on murine stromal cells was greater than that on human

**a** Freshly-isolated CD34<sup>high</sup>+CD38<sup>-</sup> cells.

**b** Re-isolated CD34<sup>high</sup>+CD38<sup>-</sup> cells after *in vitro* expansion.


**Figure 5** Phenotypic characterization of total hematopoietic cells after B progenitor culture of (a) freshly-isolated and (b) re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells, with double staining with FITC- $\alpha$ CD19 and PE- $\alpha$ CD10 mAbs. The specified lymphoid gate was set on the forward and side scatter histogram (Region R1), and used for analysis of the expression of cell surface markers on the cells.

**Table 3** Production of CD19<sup>+</sup>CD10<sup>+</sup> B progenitor cells on HESS-5 cells from freshly isolated or re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells

Experiment	Freshly isolated CD34 <sup>high</sup> +CD38 <sup>low/-</sup> cells		Re-isolated CD34 <sup>high</sup> +CD38 <sup>low/-</sup> cells	
	Fold-increase in cell number	B progenitors (%)	Fold-increase in cell number	B progenitors (%)
1	107	86.0	84	79.0
2	124	78.8	105	82.2
3	113	90.4	116	85.6
Mean	114.7	85.1	101.7	82.3

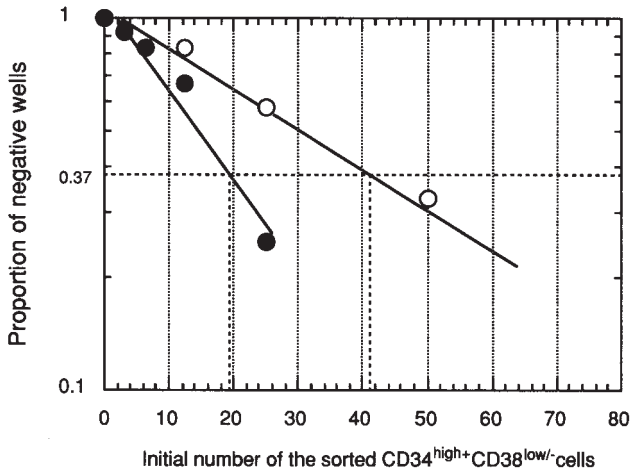
The cells ( $3 \times 10^3$  cells/well) were cultured on HESS-5 cells in 2 ml of RPMI-1640 medium supplemented with 3% FCS,  $5 \times 10^{-5}$  M 2-mercaptoethanol and 50 ng/ml flk-2/flt-3 ligand as described under Materials and methods. After 3 weeks culture, the cells were counted and analyzed with a flow cytometer. The small and low-density CD19<sup>+</sup>CD10<sup>+</sup> cell population was calculated as B progenitor cells. The reproducibility of these results was confirmed by four independent experiments.

stromal cells.<sup>19</sup> However, this expansion needs a long culture period and it seems to be difficult to put this method into practical use for clinical applications. Recently, we developed a short-term xenogenic coculture system involving HUCB-CD34<sup>+</sup> cells and HESS-5 cells.<sup>15</sup> With this xenogenic coculture system, the combination of rh-IL-3 and rh-SCF was the most effective for the proliferation of CD34<sup>high</sup>+ and primitive progenitor cells among a wide variety of combinations of human cytokines, such as rh-IL-3, rh-SCF, rh-G-CSF, rh-GM-CSF, and h-EPO.<sup>15</sup> HESS-5 cells strongly promoted the proliferation of HUCB-CD34<sup>+</sup> cells in combination with rh-IL-3 and rh-SCF and is necessary to expand CD34<sup>+</sup> cells *in vitro*.

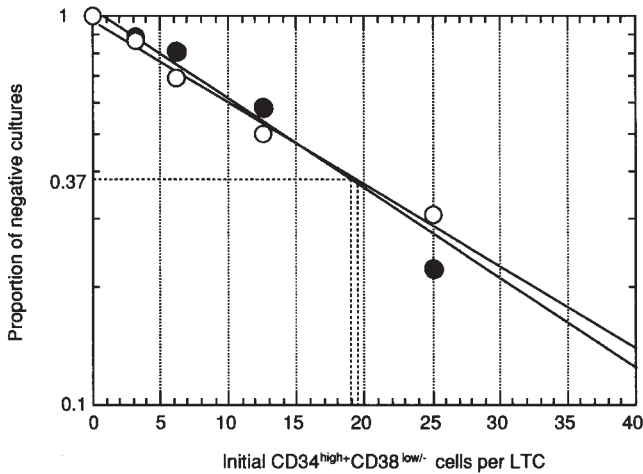
In the present study, we characterized and compared in detail the potencies as hematopoietic progenitor cells of the re-isolated CD34<sup>+</sup> and CD34<sup>high</sup>+CD38<sup>low/-</sup> cells with freshly isolated ones. Previously, the cell cycle of total hematopoietic cells amplified from CD34<sup>+</sup> cells with a combination of cyto-

kines was reported by several groups.<sup>33,34</sup> However, these groups did not examine the re-isolated CD34<sup>+</sup> cells after expansion. We performed cell cycle analysis of both total hematopoietic cells and re-isolated CD34<sup>+</sup> cells. Although almost all the freshly isolated HUCB-CD34<sup>+</sup> cells existed in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle (97.6%), the re-isolated CD34<sup>+</sup> cells after expansion had exited from the quiescent phase, and many cells had entered the S- and G<sub>2</sub>/M phases (41.6%), as judged on cell cycle analysis. Moreover, how to get CD34<sup>+</sup> cells to enter the growth phase for successful genetic modification of hematopoietic cells with a retroviral vector is a difficult problem.<sup>35</sup> This system may be an effective means of genetic modification of progenitor cells with a retroviral vector.

To determine whether or not the CD34<sup>high</sup>+CD38<sup>low/-</sup> cell population after *in vitro* expansion has the characteristics of functionally immature cells, like the freshly isolated



**Figure 6** Limiting dilution analysis of clonogenic B progenitor cells in CD34<sup>high</sup>+CD38<sup>low/-</sup> cells sorted from HUCB and the *in vitro* expanded cells. The freshly isolated (open circle) and the re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells (closed circle) were seeded on to irradiated HESS-5 cell feeders in RPMI-1640 medium supplemented with 3% defined medium, 5 × 10<sup>-5</sup> M 2-ME and 50 ng/ml of rh-flk-2/flt-3 ligand, and then the number of clonogenic cells detectable after 3 weeks was determined. The frequency of the clonogenic B progenitor cells in the input cells was calculated by Poisson statistics and weighted mean method. The results are representative of four independent experiments.



**Figure 7** Quantitative analysis of LTC-initiating cells in CD34<sup>high</sup>+CD38<sup>low/-</sup> cells sorted from HUCB and the *in vivo* expanded cells using the xenogenic coculture by limiting dilution analysis. The freshly isolated (open circle) and the re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells were plated on to the irradiated HESS-5 cells and cultured for 5 weeks. Then, the number of clonogenic cells was determined. The results are representative of five independent experiments.

CD34<sup>high</sup>+CD38<sup>low/-</sup> cells, we focused on CD34<sup>high</sup>+CD38<sup>low/-</sup> cell populations, and compared the characteristics of the re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells with the freshly isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells. In a secondary culture of re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells in this expansion system, the cells proliferated and differentiated like the freshly isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells. The re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells could also differentiate into CD19<sup>+</sup>CD10<sup>+</sup>CD13<sup>-</sup>CD33<sup>-</sup> B progenitor cells, like the freshly isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells. The frequency of clonogenic B progenitor cells in the re-isolated

CD34<sup>high</sup>+CD38<sup>low/-</sup> cells was found to be 1 per 19.5 cells and increased compared to that in the freshly isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells (1 per 41.0 cells). Taken together, these results indicate that CD34<sup>high</sup>+CD38<sup>low/-</sup> cells could proliferate in this xenogenic coculture system and possess the ability to differentiate into myeloid and B lymphoid cells.

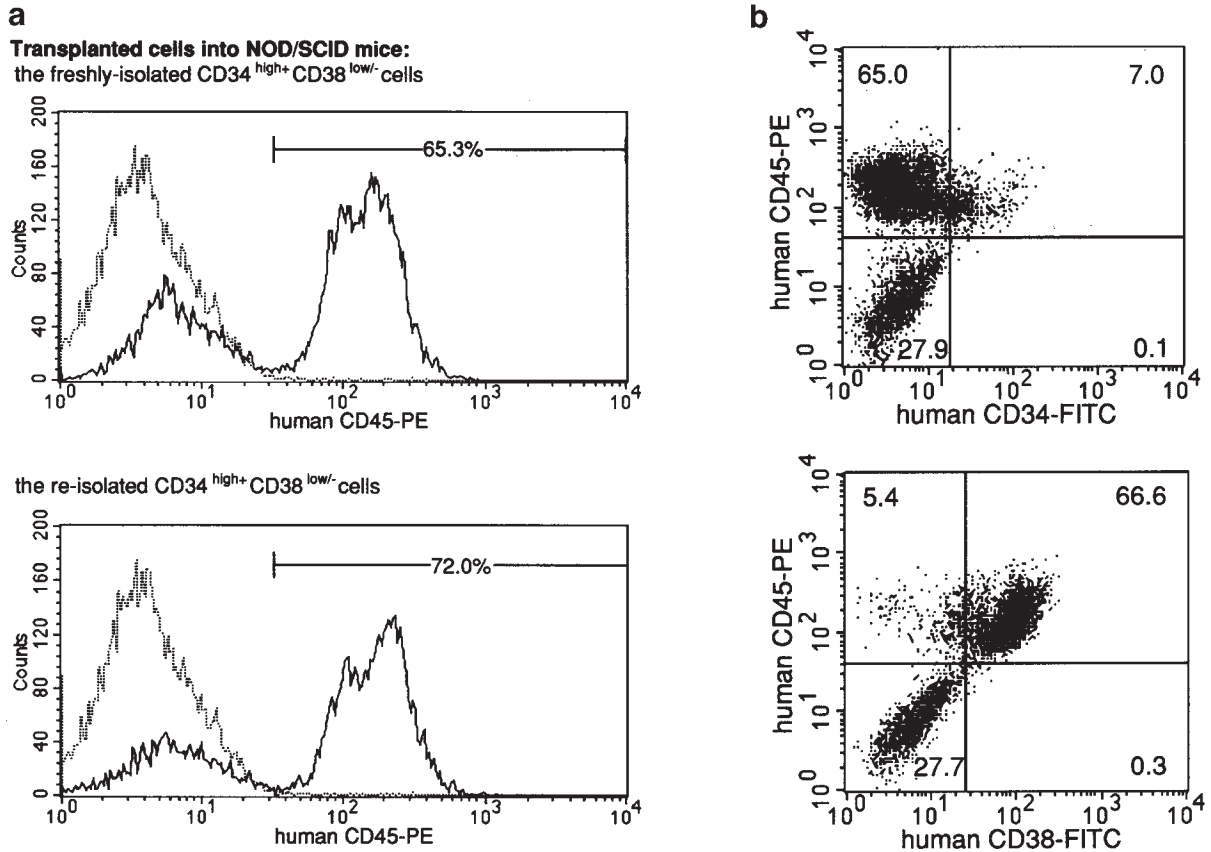
LTC-initiating cells had been detected in cells that could generate myeloid clonogenic cell progeny in long-term cultures for 5 weeks.<sup>25</sup> This assay appears to be useful since many of the LTC-initiating cells identified in human marrow share unique and rare phenotype characteristics of transplantable murine repopulating cells.<sup>36,37</sup> As judged from the results by limiting dilution assay, the frequencies of LTC-initiating cells in the freshly isolated and the re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells were found to be 1 per 18.8 and 19.5 cells, respectively, and did not change before and after the *in vitro* expansion. The expansion-fold of LTC-initiating cells in CD34<sup>high</sup>+CD38<sup>low/-</sup> cells was calculated to be 68.9. These results indicate that this expansion system can lead the successful expansion of LTC-initiating cells which are able to generate clonogenic cell progeny in LTC for 5 weeks.

Furthermore, SCID repopulating cells (SRCs) were identified and found to be capable of long-term and multilineage reconstitution of human hematopoiesis in NOD/SCID mice.<sup>30-32</sup> As judged from gene marking analysis by retroviral infection, SRCs were primitive and distinct from most CFCs and LTC-ICs.<sup>30</sup> SRCs were highly enriched in the CD34<sup>+</sup>CD38<sup>-</sup> cell fractions.<sup>31</sup> The re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells possessed multilineage repopulating ability in NOD/SCID mice and no significant differences were seen in the numbers of human CD45<sup>+</sup> cells between bone marrow cells after transplantation with the freshly and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells. These results strongly suggest that the CD34<sup>high</sup>+CD38<sup>low/-</sup> cells generated in this xenogenic coculture system possess the characteristics of hematopoietic primitive progenitor cells.

This xenogenic coculture system should be a useful basic method to provide valuable information on the regulation mechanisms of stem/progenitor cells and the evaluation of cis-activities of various kinds of retroviral vectors for human hematopoietic cells. This coculture would have the potential clinical application for expansion of hematopoietic cells and gene therapy. For the clinical purpose, this coculture system is suboptimal from the viewpoint of biosafety. We are making efforts to establish an indirect coculture system between human cells and murine stromal cells using microporous membrane<sup>15</sup> and a serum-free defined medium. After these efforts, the xenogenic coculture system would be worthy of consideration for the clinical application of transplantation, gene therapy and the development of hematopoietic stem/progenitor banking. Further studies on the regulatory molecule for the proliferation of primitive progenitor cells on HESS-5 cells would also provide valuable information on the role of hematopoietic-supportive stromal cells in the hematopoietic microenvironment.

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**Figure 8** Flow cytometric analysis of human hematopoietic cells in NOD/SCID mice transplanted with freshly or re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells.  $2 \times 10^5$  cells of freshly or re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells were injected into five NOD/SCID mice with  $2 \times 10^6$  cells of human lineage<sup>+</sup>CD34<sup>-</sup> cells as carrier cells. Twelve weeks after transplantation, bone marrow cells were harvested and analyzed by flow cytometry. (a) Cells stained with  $\alpha$ -human CD45-PE antibody (thick line) compared with cells in NOD/SCID mice transplanted only with carrier cells (dotted line). Percentage of human CD34<sup>+</sup> cells in total hematopoietic cells including mouse cells is given in each quadrant. (b) Detection of human CD34<sup>+</sup> cells in bone marrow cells of NOD/SCID mice transplanted with re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells. Cells are stained with  $\alpha$ -human CD45-PE,  $\alpha$ -human CD34-FITC and/or  $\alpha$ -human CD38-FITC antibodies. Percentage of cells in total hematopoietic cells including mouse cells is given in each quadrant.

**Table 4** Reconstitution of human hematopoietic cells in NOD/SCID mice transplanted with the freshly isolated and re-isolated CD34<sup>high</sup>+CD38<sup>low/-</sup> cells in the xenogenic coculture system in combination with rh-IL-3 and rh-SCF *in vitro*

Input cells	Input number (cells/mouse)	% of human cell engraftment (number of mice)	% of CD45 <sup>+</sup> cells in bone marrow cells
Freshly isolated CD34 <sup>high</sup> +CD38 <sup>low/-</sup> cells	$2 \times 10^5$	100 (5)	$60.1 \pm 9.3$
Re-isolated CD34 <sup>high</sup> +CD38 <sup>low/-</sup> cells	$2 \times 10^5$	100 (5)	$65.7 \pm 6.4$

Sorted CD34<sup>high</sup>+CD38<sup>low/-</sup> cells derived from the HUCB-CD34<sup>+</sup> cells and re-isolated CD34<sup>+</sup> cells after expansion were injected into 8-week-old NOD/SCID mice as described under Materials and methods. After 12-weeks, bone marrow cells were collected and stained with  $\alpha$ -human CD45 antibody. The percentages of CD45<sup>+</sup> cells are by FACS analysis. The results are expressed as the mean  $\pm$  s.e. of five mice.

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