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Rapid ex vivo expansion of human umbilical cord hematopoietic progenitors using a novel culture system

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Cell numbers limit the widespread clinical use of cord blood (CB) for gene therapy and marrow replacement in adults; a simple and effective method for ex vivo expansion of CB primitive progenitor cells (PPC) is required. Recently, the combination of thrombopoietin (TPO) and Flk-2/Flt-3 ligand (FL-2) was reported to support slow proliferation of CB-PPC in stroma-free liquid culture. We established a novel culture system in which the murine stromal cell line HESS-5 dramatically supports the rapid expansion of cryopreserved CB-PPC in synergy with TPO/FL-2. Furthermore, while HESS-5 cells directly adhered to human progenitors during culture, the cultured human cells could easily be harvested without contamination by HESS-5 cells. Within 7 days of culture, a 100-fold increase in CD34bright/CD38dim cells was obtained in serum-containing culture. When HESS-5 cells were physically separated from human progenitor cells in the presence of TPO/FL-2, synergy was blocked, suggesting that HESS-5 cells support proliferation of PPC by direct cell-to-cell interaction. The hematopoietic-supportive effects of this xenogeneic coculture system were then assessed in a very short-term (5 days) serum-free culture. Expansion was further enhanced by addition of stem cell factor (SCF) or interleukin-3 (IL-3). As a result, a 50- to 100-fold increase in CD34bright/CD38dim cells was noted. Colony-forming units in culture (CFU-C) and mixed colonies (CFU-GEMM) were enhanced by 10- to 30-fold and 10- to 20-fold, respectively. Moreover, generation of long-term-culture–initiating cells (LTC-IC) from CD34bright/CD38dim cells was amplified by 25-fold. The severe-combined immunodeficient (SCID) mouse-repopulating cell (SRC) assay confirmed extensive ability of the expanded cells to reconstitute long-term hematopoiesis. These results indicate that this xenogeneic coculture system, in combination with human cytokines, can rapidly generate PPC from cryopreserved CB.

Keywords: Ex vivo expansion—Progenitor cells—Stromal cell line—Thrombopoietin—Flk-2/Flt-3 ligand

Introduction

Human umbilical cord blood (CB) is an attractive alternative to bone marrow or growth factor mobilized peripheral blood as a source of hematopoietic progenitors because CB contains a high number of primitive progenitor cells (PPC) [1,2]. To date, over 500 transplants have been performed worldwide using unfractionated CB from related and unrelated donors, and the results over the past 9 years of CB transplantation for children with malignant and nonmalignant diseases have been promising [3]. However, there are potential limitations to the widespread use of CB; there may be enough hematopoietic stem cells to reconstitute children, but the ability to engraft an adult from CB may require ex vivo manipulations.

Many studies to identify culture conditions able to support expansion of PPC have been attempted [4–7]. Recently it was reported that the combination of thrombopoietin (TPO) and Flk-2/Flt-3 ligand (FL-2) maintained production of CB-PPC for more than 6 months in stroma-free liquid culture [8]. Both early-acting cytokines were reported to sustain cell viability and promote proliferation preferentially of a minor subpopulation of CD34bright cells, i.e., CD34bright/CD38dim cells [9–12]; PPC are reported to be included in this subset [13].

On the other hand, contact with murine stromal cells was reported to preserve human PPC quality during ex vivo manipulation [14,15]. We have established several murine stromal cell lines from bone marrow and spleen [16]. Among

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these, we discovered a novel hematopoietic-supportive cell line, HESS-5, which effectively supports not only formation of murine granulocyte and macrophage colonies but also proliferation of human CB CD34bright/CD38dim cells in the presence of human cytokines [17,18]. Progenitors expanded by this xenogeneic coculture system generate a number of high proliferative potential colony-forming cells and mixed colony-forming units and differentiate to CD10+/CD19+ B-lymphoid cells in the presence of FL-2.

Although it requires a relatively long process to obtain a sufficient number of PPC by ex vivo manipulation [19–21], prolonged cultures often result in the exhaustion of the proliferative and lineage potential of most PPC [22,23]. An effective culture system in which PPC can be rapidly expanded is more suitable for clinical settings because this prevents infections or transformation. Rapid expansion also is economical and timely for transplantation. Here, we demonstrate marked supportive effects of HESS-5 cells on proliferation of PPC isolated from cryopreserved CB in synergy with TPO/FL-2 using a novel culture system by which contamination with murine stromal cells can be avoided. The number of CD34bright/CD38dim cells was dramatically increased in a very short time. The long-term–culture–initiating cell (LTC-IC) assay and the severe-combined immunodeficient (SCID) mouse-repopulating cell (SRC) assay indicated extensive abilities of the expanded cells to sustain and reconstitute long-term hematopoiesis. This novel culture system will be a good ex vivo model that provides valuable information for regulatory mechanisms of rapid ex vivo expansion of PPC.

Materials and methods

CD34bright cell purification
CB, collected according to institutional guidelines, was obtained from normal full-term deliveries. Mononuclear cells (MNC) were isolated from CB using Ficoll-Hypaque (Lymphoprep, 1.077 ± 0.001 g/mL; Nycomed, Oslo, Norway) density gradient centrifugation. CD34bright cell purification utilized positive selection using the MACS immunomagnetic separation system (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer’s instructions. Briefly, MNC were suspended in buffer containing phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2 mM EDTA (BSA-EDTA-PBS), and incubated for 15 minutes with monoclonal hapten-conjugated anti-CD34 antibody (clone: QBEND/10) and human Ig to prevent nonspecific binding. Washed cells were resuspended in BSA-EDTA-PBS and incubated for 15 minutes with colloidal super-paramagnetic microbeads conjugated to an anti-hapten antibody. After labeling, the cell suspension was passed through a column (VS+ separation column) held within a magnetic field causing CD34bright cells to be retained in the column. CD34bright cells were collected by removal of the column from the magnet and washing with BSA-EDTA-PBS. The cells were then applied to a second column (RS+ separation column) and the purification step repeated. Ninety-five percent or more of the enriched cells were CD34bright by flow cytometric analysis.

CB cryopreservation and thawing
CD34bright cells purified from CB were frozen in a medium supplemented with dimethylsulfoxide and FCS (Cell-Banker; Nihon Zenyaku Kohgyo, Fukushima, Japan) using a step-down freezing procedure and placed in liquid nitrogen. Aliquots of frozen samples were thawed before use. The thawed cells were washed twice and trypan blue viability determination was performed. When cell viability was more than 98%, the samples were subjected to further studies.

Murine stromal cell line
The murine hematopoietic-supportive stromal cell line HESS-5 was previously established from murine bone marrow [16]. HESS-5 cells were maintained in minimal essential medium α (MEM-α; Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% (v/v) horse serum (GibcoBRL, Grand Island, NY) at 37°C under 5% CO2 in humidified air.

Human cytokines
Recombinant human TPO, interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin (EPO) were generously provided by Kirin Brewery (Tokyo, Japan). Recombinant human stem cell factor (SCF) was a gift from Amgen Biologicals (Thousand Oaks, CA). Recombinant human FL-2 was purchased from R&D Systems (Minneapolis, MN). The final concentrations of cytokines were as follows: TPO, 50 ng/mL; FL-2, 50 ng/mL; IL-3, 20 ng/mL; SCF, 50 ng/mL; GM-CSF, 10 ng/mL; and EPO, 3 U/mL.

Culture systems
Diagrams of several culture systems used in this study are displayed in Fig. 1. Stromata-free culture and classical coculture with stromal cells were performed in culture media in 24-well microplates (Iwaki Glass, Chiba, Japan). In the classical coculture, when contamination of stromal cells in the harvested cells was negligible (<2%) by microscopic visualization, cells were subjected to further examination. In some experiments, human hematopoietic cells were physically separated from the stromal layer by a polyethylene terephthalate track-etched membrane in cell culture inserts (Becton Dickinson Labware, Franklin Lakes, NJ) (noncontact culture), as described by Verfaillie et al. [24,25]. In this study, a novel culture system was devised by modifying the cell culture insert system. First, HESS-5 cells were cultured on the reverse (back) side of the track-etched membrane of the insert in MEM-α supplemented with 10% horse serum. After obtaining a confluent feeder layer, stromal cells were washed five times and the medium was changed for coculture. CD34bright cells isolated from CB were seeded on the upper side of the membrane of the insert where the cytoplasmic villi of HESS-5 cells passed through the etched 0.45 μm pores. Therefore, while HESS-5 cells directly adhered to human hematopoietic cells during culture, expanded cells could easily be harvested without contamination with HESS-5 cells. Low pore density (LPD, 1.6 μm pores) and high pore density membranes (HPD, 1.0 × 106/cm2) and high pore density membranes (HPD, 1.0 × 106/cm2) were used in the present study.

Short-term ex vivo expansion of hematopoietic progenitors
Serum-containing liquid culture was carried out using a medium containing 12.5% horse serum, 12.5% fetal bovine serum, 10−4 M 2-mercaptoethanol, 2 mM L-glutamine, 0.2 mM i-inositol, 20 μM 2-mercaptoethanol, 2 mM L-glutamine, 0.2 mM i-inositol, 20 μM
folic acid, and MEM-α (MyeloCult; StemCell Technologies, Vancouver, Canada) supplemented with 10⁻⁶ M hydrocortisone (Sigma, St. Louis, MO) and penicillin/streptomycin (GibcoBRL) with or without designated cytokines. Three thousand and 100,000 CD34bright cells were subjected to cytokine-containing and cytokine-free culture, respectively. Culture plates were incubated at 37°C in a humidified atmosphere consisting of air enriched with 5% CO₂. On Day 7 of culture, the medium in each well was removed and replaced with fresh medium. On Days 7 and/or 14 of culture, aliquots of cultured cells were harvested and subjected to cell count, clonal cell culture, and flow cytometric analysis. Serum-free liquid culture was carried out using StemPro™-34SFM (GibcoBRL) supplemented with StemPro™-34 Nutrient Supplement (GibcoBRL), 2 mM L-glutamine (GibcoBRL), and penicillin/streptomycin with or without designated cytokines. Five thousand to 100,000 CD34bright cells were cultured under the same conditions as serum-containing culture. On Day 5 of culture, cells were harvested and counted. Aliquots were applied to studies including the LTC-IC assay.

**Immunophenotyping by flow cytometry**

Aliquots of cells were suspended in EDTA-BSA-PBS and incubated with mouse IgG (Inter-Cell Technologies, Hopewell, NJ) to block nonspecific binding. Cells were then reacted for 15 minutes with FITC- and PE-conjugated several monoclonal antibodies at 4°C. Unbound antibodies were removed by two washes, and cells were resuspended in EDTA-BSA-PBS. Stained cells were then passed through a nylon mesh filter and subjected to two-color flow cytometric analysis. Cells labeled with FITC- and PE-conjugated mouse isotype-matched antibodies were used as negative controls. The analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) with LYSIS II software (Becton Dickinson). At least 10,000 events were acquired for each analysis. Antibodies used were as follows; FITC-conjugated CD2, CD3, CD14, CD15, CD19, CD33, CD41, glycophorin A, and HLA-DR antibodies; PE-conjugated CD38 and CD45 antibodies. CD41 antibody was purchased from Nichirei (Tokyo, Japan). CD3 and glycophorin A antibodies were from Immunotech (Marseille, France). CD14, CD33, and CD45 antibodies were from Pharmingen (San Diego, CA) and all others were from Becton Dickinson. Furthermore, in some experiments, aliquots of cultured cells were subjected to three-color flow cytometric analysis to assess the lineage commitment of progenitors. Samples were incubated for 15 minutes with biotin-conjugated anti-CD34 (Immunotech, Marseille, France). Cells labelled with a biotin-conjugated mouse isotype-matched antibody were used as a negative control. After washing, cells were labeled with streptavidin PerCP (Becton Dickinson), PE-conjugated


in methylcellulose media at concentrations of 1–2

Aliquots from initial CB samples or cultured cells were incubated

Clonal cell culture

Three-color flow cytometry was performed using a FACSCalibur

anti-CD38, and various FITC-conjugated monoclonal antibodies. Three-color flow cytometry was performed using a FACSCalibur (Becton Dickinson) with CellQuest software (Becton Dickinson).

Table 1. Evaluation of synergistic effects between HESS-5 cells and TPO/FL-2 on ex vivo expansion of hematopoietic progenitors

<table>
<thead>
<tr>
<th>Cell population or colony</th>
<th>None</th>
<th>HESS-5</th>
<th>TPO/FL-2</th>
<th>HESS-5 &amp; TPO/FL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of cells</td>
<td>0.7 ± 0.1</td>
<td>2.9 ± 0.5</td>
<td>98.7 ± 20.7</td>
<td>414.7 ± 10.1</td>
</tr>
<tr>
<td>CD34&lt;sup&gt;bright&lt;/sup&gt; cells</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td>4.3 ± 0.4</td>
<td>16.0 ± 2.5</td>
</tr>
<tr>
<td>CD34&lt;sup&gt;bright&lt;/sup&gt;/CD38&lt;sup&gt;bright&lt;/sup&gt; cells</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td>4.3 ± 0.4</td>
<td>14.7 ± 2.1</td>
</tr>
<tr>
<td>CD34&lt;sup&gt;bright&lt;/sup&gt;/CD38&lt;sup&gt;dim&lt;/sup&gt; cells</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.3</td>
<td>4.2 ± 2.0</td>
<td>150.3 ± 84.3*</td>
</tr>
<tr>
<td>CFU-C</td>
<td>ND</td>
<td>1.7 ± 0.0*</td>
<td>13.3 ± 2.3</td>
<td>74.3 ± 11.2**</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>ND</td>
<td>2.4 ± 0.3*</td>
<td>7.3 ± 5.3</td>
<td>35.7 ± 7.6*</td>
</tr>
</tbody>
</table>

Cord blood (CB) CD34<sup>bright</sup> cells were cultured in the presence or absence of TPO/FL-2 with or without HESS-5 cells. In coculture, HESS-5 cells directly adhered to the CB cells through 0.45-μm pores of low pore-density membranes in the cell culture insert system. Each number for the above-mentioned cell populations indicates the mean fold increase ± SEM of three different experiments on Day 14 of culture. Suitable aliquots of cultured cells were assayed for CFU-C and CFU-GEMM. After 2 weeks of clonal cell culture, colonies were scored and the results represent the fold increase (mean ± SEM of three different experiments). *Only one experiment was performed in triplicate; **p < 0.01 vs those without HESS-5; ND = not determined.

Clonal cell culture

Aliquots from initial CB samples or cultured cells were incubated in methylcellulose media at concentrations of 1–2 × 10<sup>5</sup> cells/mL for purified CD34<sup>bright</sup> cells and 5–10 × 10<sup>4</sup> cells/mL for cultured cells in 35-mm tissue culture dishes (Iwaki Glass). One milliliter of culture mixture contained 1.2% 1500 cp methylcellulose (Sigma), MEM-α, 1% deionized fraction v BSA (Sigma), 10<sup>−4</sup> M/L 2-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan), 30% fetal calf serum (JRH Biosciences, Lenexa, KS), EPO, IL-3, SCF, GM-CSF, and cells. Dishes were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air. All cultures were done in triplicate. Total colony-forming units in culture (CFU-C) and mixed colonies containing erythroid and myeloid cells and megakaryocytes (CFU-GEMM) consisting of 50 or more cells were scored on an inverted microscope at 14 days of culture. To assess the accuracy of in situ identification, individual colonies were lifted with an Eppendorf micropipette under direct microscopic visualization, spread on glass slides using a cytocentrifuge and studied with May-Grunwald-Giemsa staining.

LTC-IC assay

LTC-IC assay was performed as described by Sutherland et al. [26] with slight modifications. Briefly, bone marrow stromal cells derived from hematologically normal donors were seeded at 10<sup>3</sup> cells per well in 96-well flat-bottomed plates with MEM-α supplemented with 10% FCS. After obtaining semiconfluent feeder layers, stromal cells were irradiated with 15-Gy using a 137Cs γ-iradiator. CD34<sup>bright</sup> cell subpopulations (i.e., CD34<sup>bright</sup>/CD38<sup>bright</sup> and CD34<sup>bright</sup>/CD38<sup>dim</sup> cells) purified from CB or those isolated from cultured cells by sorting with a FACSVantage (Becton Dickinson) were seeded at limiting dilution on the feeder layer in serum-containing media. For each evaluation, at least three cell concentrations were used with 24 replicates per concentration. Culture plates were incubated at 37°C with 5% CO<sub>2</sub> for 3 to 5 days and then incubated at 34°C with weekly changes of medium. After 5 weeks of culture, cells were assayed for CFU-C in methylcellulose medium. Colonies were scored 2 weeks later. The frequency of wells in which there were no clonogenic progenitors was determined ac-

Figure 2. Electron microscopic view of the novel culture system. HESS-5 cells were cultured at the reverse (back) side of the polyethylene terephthalate track-etched membrane of the cell culture insert. After obtaining a confluent feeder layer, the novel coculture was initiated by seeding cord blood (CB) CD34<sup>bright</sup> cells on the upper side of the membrane of the cell culture insert. On Day 5 of coculture, the membrane to which both cultured cells were adherent was removed from the cell culture insert and subjected to electron microscopy. Photos represent views of the upper side of the membrane, demonstrating direct contact between cultured human hematopoietic cells and cytoplasmic villi of HESS-5 cells, which passed through the etched 0.45-μm pores of membrane.
According to the number of the initial input population, and the frequency of LTC-IC was calculated.

**SRC assay**

SRC assay was performed as previously described [13], with slight modifications. Briefly, 8-week-old male NOD/Shi-scid (NOD/SCID) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All animals were handled under sterile conditions and maintained under microisolators in the animal facility located at the Tokai University School of Medicine. Human hematopoietic cells at the indicated doses were transplanted by tail-vein injection into sublethally irradiated mice (350 cGy using a 4 × 10^6 V linear accelerator). Cells were co-transplanted with irradiated (15 Gy using a 137Cs γ-irradiator) nonrepopulating CD34^dim_ cells as accessory cells. Mice were killed 7 weeks after transplantation, and the bone marrow (from the femurs and tibiae), spleen, and peripheral blood cells (from the retro-orbital venous plexus using heparin-coated micropipettes) were harvested. The presence of human hematopoietic cells was determined by detection of cells positively stained with FITC-conjugated antihuman CD45 using flow cytometry. Southern blot analysis using a human chromosome 17-specific α-satellite probe was also performed to confirm flow cytometric results, as described previously [27,28].

**Scanning electron microscopy**

CB CD34^bright_ cells were cocultured with HESS-5 cells in the LPD culture system. On Day 5 of coculture, the membrane to which both cultured cells were adherent was removed from the cell cul-

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**Figure 3.** Effects of several culture systems on ex vivo expansion of hematopoietic progenitors. CD34^bright_ cells were cocultured with HESS-5 cells in serum-containing medium supplemented with TPO and FL-2 using several culture systems. The results represent the mean fold increase ± SEM of three different experiments on Days 7 and 14 of culture. *No significant differences between different culture systems (p > 0.05); **p < 0.05 as compared with low pore density (LPD) culture or noncontact culture. ■ = high pore density (HPD) culture; □ = LPD culture; ▲ = noncontact culture; ● = classical culture.
ture insert; the membrane was fixed in 2.5% glutaraldehyde at 4°C for 1 hour, stained with 1% OsO₄/2% tannic acid, then freeze-dried with t-butyl alcohol. The specimen was coated with gold by ion sputtering and examined with a scanning electron microscope (JSM 840A, JEOL, Tokyo, Japan).

Statistical analysis
Data were compared using analysis of variance. Where significant differences were inferred, sample means were compared using the Student’s t-test.

Results
Evaluation of supportive effects of HESS-5 cells on ex vivo expansion of CB-PPC in synergy with TPO/FL-2. We first assessed the effects of TPO and FL-2 on ex vivo expansion of CB-PPC in the stroma-free serum-containing culture. As single agents, neither TPO nor FL-2 could effectively support proliferation of CD34<sup>bright</sup> cells (data not shown). However, the combination of factors (TPO/FL-2) enhanced production of progenitors; the number of CD34<sup>bright</sup> cells was approximately four times the input number after 14 days of culture (Table 1). The supportive effects of HESS-5 cells on proliferation of CB-PPC were then studied in LPD culture. Electron microscopic examination confirmed direct contact between cultured human hematopoietic cells and cytoplasmic villi of HESS-5 cells, which had passed through the etched 0.45-μm pores of the membrane in the cell culture insert (Fig. 2). Under cytokine-free conditions, although HESS-5 cells alone weakly supported production of nucleated cells, the number of CD34<sup>bright</sup> cells was decreased. However, in the presence of TPO/FL-2, HESS-5 cells could effectively support proliferation of progenitor cells, especially in the CD34<sup>bright</sup>/CD38<sup>dim</sup> cell subpopulation; the mean number of CD34<sup>bright</sup>/CD38<sup>dim</sup> cells was approximately 150 times the input number after 14 days of culture. The output of CFU-C and CFU-GEMM also was enhanced.

![Figure 4](attachment:image.png)

**Figure 4.** Comparison of CD34 and CD38 expression among hematopoietic cells in different culture systems. CD34<sup>bright</sup> cells derived from a single delivery were cocultured with HESS-5 cells in serum-containing medium supplemented with TPO and FL-2 in different culture systems. On Day 7 of culture, aliquots of harvested cells were subjected to flow cytometric analysis. While there were no significant differences in the number of total nucleated cells (p > 0.05), differences in CD34<sup>bright</sup>/CD38<sup>dim</sup> cells were noted between different culture systems. Data represent three different experiments. Results of this trial are displayed quantitatively in Fig. 3. LPD = low pore density culture; HPD = high pore density culture.
In the presence or absence of the above-mentioned cytokines, cord blood (CB) CD34 bright cells were cultured with HESS-5 cells under serum-free conditions. CD34 bright cells 1.1 CFU-C 1.5 CD34 bright/CD38 dim cells 1.1 CD34 bright/CD38 dim cells was approximately 50 to 100 times increased. Moreover, by addition of SCF or IL-3, those effects were amplified despite the short duration of culture (Table 4). Efficacy of CD34 bright/CD38 bright cells was markedly ameliorated from cells cultured by this system was negative for most lineage-committed markers but was positive for HLA-DR (Table 3).

### Table 2. Evaluation of synergistic effects between HESS-5 cells and TPO/FL-2 containing cytokines on ex vivo expansion of hematopoietic progenitors in very short-term serum-free culture

<table>
<thead>
<tr>
<th>Cell population or colony</th>
<th>Cytokine-free</th>
<th>TPO + FL-2</th>
<th>TPO + FL-2 + SCF</th>
<th>TPO + FL-2 + IL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of cells</td>
<td>1.1 ± 0.0</td>
<td>5.3 ± 0.6</td>
<td>14.3 ± 1.2*</td>
<td>17.2 ± 3.9**</td>
</tr>
<tr>
<td>CD34 bright cells</td>
<td>1.1 ± 0.0</td>
<td>2.3 ± 0.4</td>
<td>4.8 ± 0.1*</td>
<td>4.9 ± 1.3*</td>
</tr>
<tr>
<td>CD34 bright/CD38 bright cells</td>
<td>1.1 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>3.0 ± 0.4*</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>CD34 bright/CD38 dim cells</td>
<td>1.1 ± 0.4</td>
<td>48.4 ± 7.6</td>
<td>103.1 ± 10.3*</td>
<td>113.9 ± 21.7**</td>
</tr>
<tr>
<td>CFU-C</td>
<td>1.5 ± 0.1</td>
<td>12.4 ± 2.3</td>
<td>34.2 ± 6.7**</td>
<td>33.2 ± 5.3*</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>0.5 ± 0.1</td>
<td>9.4 ± 2.5</td>
<td>18.3 ± 5.3</td>
<td>11.5 ± 1.2</td>
</tr>
</tbody>
</table>

In the presence or absence of the above-mentioned cytokines, cord blood (CB) CD34 bright cells were cultured with HESS-5 cells under serum-free conditions. HESS-5 cells directly adhered to the CB cells through 0.45-μm pores of high pore-density membranes in the cell culture insert system. Each number for the cell populations indicates the mean fold increase ± SEM of three different experiments on Day 5 of culture. Suitable aliquots of cultured cells were assayed for CFU-C and CFU-GEMM. After 2 weeks of clonal cell culture, colonies were scored and the results represent the fold increase (mean ± SEM of three different experiments). *p < 0.05; **p < 0.01 as compared with TPO + FL-2; SCF = stem cell factor.

### Assessment of the supportive effects of HESS-5 cells on proliferation of CB-PPC in the presence of TPO/FL-2 using several culture systems

Next, we determined suitable culture conditions in which HESS-5 cells could most effectively support proliferation of CB-PPC in synergy with TPO/FL-2. CD34 bright cells were cocultured with HESS-5 cells in several culture systems (Fig. 1) in serum-containing medium supplemented with TPO and FL-2. During culture, there were no significant differences in the number of total nucleated cells between the different culture systems (Fig. 3). However, CD34 bright cells, especially CD34 bright/CD38 dim cells, were generated more by the HPD culture and the classical coculture than by the LpD culture or the noncontact culture; the mean number of CD34 bright/CD38 dim cells was over 100 times the initial input number in HPD culture within 7 days (Figs. 3 and 4). By contrast, synergistic effects of HESS-5 cells with TPO/FL-2 were not seen in the noncontact culture (Fig. 3). Effects of TPO/FL-2 alone or HESS-5 cells alone were identical to those described in Table 1 (data not shown).

### Ex vivo expansion of CB-PPC in a very short-term (5 days) serum-free culture

The synergistic effects of HESS-5 cells and TPO/FL-2 were also studied in serum-free HPD culture for a very short duration. After 5 days of culture, HESS-5 cells could most significantly stimulate production of nucleated cells or progenitors in cytokine-free conditions (Table 2). However, in the presence of TPO/FL-2, the number of progenitor cells, especially CD34 bright/CD38 dim cells, was remarkably increased. Moreover, by addition of SCF or IL-3, those effects were further enhanced. As a result, the mean number of CD34 bright/CD38 dim cells was approximately 50 to 100 times the initial input number. The outputs of CFU-C and CFU-GEMM were increased 10- to 30-fold and 10- to 20-fold, respectively. The combination of TPO/FL-2 and IL-3 was the most effective cytokine combination to obtain expansion of CD34 bright/CD38 dim cells in this system. Three-color flow cytometry revealed that most of the CD34 bright/CD38 dim population amplified by this system was negative for most lineage-committed markers but was positive for HLA-DR (Table 3).

### Table 3. Immunophenotypes of hematopoietic progenitor cells expanded by the very-short-term serum-free culture

<table>
<thead>
<tr>
<th>Marker</th>
<th>CD34 bright/CD38 bright cells</th>
<th>CD34 bright/CD38 dim cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15</td>
<td>29.6 ± 8.2</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>CD19</td>
<td>3.9 ± 2.6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>CD2</td>
<td>4.8 ± 2.3</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>CD41</td>
<td>4.7 ± 3.3</td>
<td>6.4 ± 2.4</td>
</tr>
<tr>
<td>Gly A</td>
<td>4.5 ± 3.8</td>
<td>2.5 ± 1.6</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>88.7 ± 3.8</td>
<td>90.9 ± 3.7</td>
</tr>
</tbody>
</table>

In the presence of TPO, FL-2, and IL-3, cord blood CD34 bright cells were cocultured with HESS-5 cells under serum-free conditions. After 5 days of culture, cells were harvested and immunophenotypes of expanded CD34 bright cells were assessed using three-color flow cytometry. Each number indicates the mean percentage of positive cells ± SEM of three different experiments.
Table 4. Results of LTC-IC assay using CB CD34<sup>bright</sup> cells or those generated by very-short-term serum-free culture

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Pre-expansion</th>
<th>Post-expansion</th>
<th>Fold LTC-IC amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34&lt;sup&gt;bright&lt;/sup&gt;/CD38&lt;sup&gt;bright&lt;/sup&gt; cells</td>
<td>1/215.0 ± 85.0</td>
<td>1/2833.3 ± 1593.3</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>CD34&lt;sup&gt;bright&lt;/sup&gt;/CD38&lt;sup&gt;dim&lt;/sup&gt; cells</td>
<td>1/87.5 ± 32.5</td>
<td>1/134.0 ± 42.1</td>
<td>25.3 ± 16.5</td>
</tr>
</tbody>
</table>

Long-term culture-initiating cells (LTC-IC) frequency was compared between cryopreserved cord blood (CB) CD34<sup>bright</sup> cells (pre-expansion) and those generated by very short-term (5 days) serum-free coculture with HESS-5 in the presence of TPO, FL-2, and IL-3 (post-expansion). The fold increase in LTC-IC was determined by the LTC-IC frequency and the fold increase in CD34<sup>bright</sup>/CD38<sup>bright</sup> or CD34<sup>bright</sup>/CD38<sup>dim</sup> cells during culture. Results represent mean ± SEM of three different experiments.

Discussion

In this study we demonstrated that the murine stromal cell line HESS-5 could effectively support rapid expansion of cryopreserved CB-PPC in synergy with human cytokines. We first assessed the supportive effects of two early-acting cytokines, TPO and FL-2, on proliferation of PPC; synergistic effects of these factors were confirmed [8]. We then assessed the supportive effects of HESS-5 cells on proliferation of human PPC. While HESS-5 cells alone did not effectively support proliferation, HESS-5 cells could dramatically enhance generation of progenitors, especially CD34<sup>bright</sup>/CD38<sup>dim</sup> cells, in synergy with TPO/FL-2 over a short period. The addition of SCF or IL-3 further enhanced production.

In ex vivo expansion of human PPC, there are several benefits to using a xenogenic stromal cell line as the feeder: (1) HESS-5 cells can be maintained easily; (2) consistent hematopoietic-supportive effects are repeatedly obtained; and (3) cell differentiation due to exposure to various factors secreted by stromal cells can be avoided. Although HESS-5 cells produced high GM-CSF activity, murine GM-CSF does not exhibit activity on cells of human origin [16,29]. We previously studied the supportive effects of normal human bone marrow fibroblasts on proliferation of CB-PPC, but variable results were noted because of their marked differentiation-supportive potential (data not shown).

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Effects of the 5-day serum-free stroma-contact culture system on human reconstituting hematopoietic progenitors

Accordingly, we studied the SRC assay to determine whether cells cultured in the very short-term xenogenic coculture system are capable of long-term multilineage reconstitution in vivo. Purified 1 × 10<sup>5</sup> CB CD34<sup>bright</sup> cells were initially cocultured with HESS-5 cells in the presence of TPO, FL-2, and IL-3 in serum-free media for 5 days; harvested cells were then transplanted into NOD/SCID mice. As controls, uncultured 1 × 10<sup>5</sup> CB CD34<sup>bright</sup> cells obtained from the same sources were also transplanted into other mice. Seven weeks after transplantation, human CD45<sup>+</sup> cells were found in the bone marrow, spleen, and peripheral blood cells of mice transplanted with the cultured cells (Fig. 5A, and data not shown). There were marked differences in the percentage of chimeraism between bone marrow cells in mice transplanted with cultured cells and those transplanted with control samples. The proportion of human hematopoietic cells in the murine bone marrow was also quantitatively confirmed by Southern blot analysis using a human chromosome 17-specific α-satellite probe (data not shown). Human CD45<sup>+</sup> cells in the murine bone marrow were further subjected to flow cytometric analysis to determine multilineage reconstitution. As a result, human CD45<sup>+</sup> cells were positive for CD33, CD14, CD41, glycophorin A, or CD19 (Fig. 5B). CD34-positive cells were also determined. However, CD3 was considered to be negative (data not shown).

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Most of the expanded CD34\textsuperscript{bright}/CD38\textsuperscript{dim} cell population did not express various lineage-committed markers, but were positive for HLA-DR. In contrast to previous findings in bone marrow, CB LTC-IC were shown to be present among the CD34\textsuperscript{bright}/HLA-DR\textsuperscript{bright} cell fraction [30,31]. Furthermore, Bhatia et al. [13] recently identified the SRC that were capable of multilineage reconstitution of human hematopoiesis in the bone marrow of NOD/SCID mice; the
CD34-positive SRC were found exclusively in the CD34^{bright}/CD38^{dim} cell fraction [13]. Therefore, the expanded hematopoietic progenitors were expected to sustain long-term hematopoiesis. As a result, 25-fold LTC-IC amplification was observed in CD34^{bright}/CD38^{dim} cells expanded by the very short-term xenogeneic coculture, although the number of LTC-IC in the more mature population of CD34^{bright} cells, i.e., CD34^{bright}/CD38^{bright} cells, was decreased during culture. The SRC assay indicated the reconstituting ability of these cultured human PPC. Although we could not perform

Figure 5B. Determination of human hematopoietic reconstitution in NOD/SCID mice 7 weeks after transplantation. Specific subsets of human CD45^{+} cells in bone marrow cells of the NOD/SCID mouse transplanted with the human cord blood cells (Sample 1, Fig. 5(A)) after the xenogeneic coculture. Harvested cells were stained with PE-conjugated CD45 and various FITC-conjugated monoclonal antibodies.
a quantitative SRC assay, the difference in the percentage of chimerism of human CD45$^+$ cells between bone marrow cells of mice transplanted with cultured cells and those transplanted with control samples strongly suggests the extensive ability of these ex vivo-generated PPC to sustain and reconstitute long-term human hematopoiesis in vivo.

The mechanism of hematopoietic-supportive effects of HESS-5 cells, especially in the CD34$^{bright}$/CD38$^{dim}$ cell fraction, remains unknown. Recently, a cell surface molecule identified as delta-like/preadipocyte factor-1 (dlk) on a murine stromal cell line (AFT024) was reported to show activity for murine stem cells by promoting the formation of cobblestone areas that contain both high-proliferative potential progenitors and in vivo repopulating cells [32]. Although expression of dlk in HESS-5 cells could not be detected by Northern blot analysis (in preparation), a novel molecular pathway should play an important role in stem cell regulation by HESS-5 cells.

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