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Research Article

Marrow mesenchymal stem cells transduced with TPO/FL genes as support for ex vivo expansion of hematopoietic stem/progenitor cells

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Abstract. A new marrow-derived mesenchymal stem cell (hMSC) line that could support expansion of hematopoietic stem/progenitor cells (HSPCs) was developed. Primary hMSCs were infected with retrovirus containing Flt-3 ligand and thrombopoietin genes. CD34+ cells from cord blood were expanded with primary hMSCs or transduced hMSCs. The expansion of total nucleated cells, CD34+ cells and mixed colonies containing erythroid and myeloid cells and megakaryocytes for 2 weeks coculture with transduced hMSCs was remarkably increased. The outputs of long-term culture-initiating cells for 2 and 4 weeks coculture with transduced hMSCs were also largely increased. The expansion rates of HSPCs with transduced hMSCs were unchanged for 6 weeks. In contrast, the expansion rates of HSPCs with primary hMSCs declined drastically through 6 weeks. SCID-repopulating cell expansion with transduced hMSCs for 4 weeks was significantly higher than that of uncultured CD34+ cells and HSPCs expanded with primary hMSCs.

Key words. Mesenchymal stem cell; transduction; thrombopoietin; Flt-3 ligand; hematopoietic stem/progenitor cell; expansion.

Hematopoietic stem cells (HSCs) are generally defined as cells having self-renewing potential and the capacity to give rise to differentiated cells of all hematopoietic lineages [1]. Therefore, HSC transplantation is performed for complete healing of hematologic disorders and as a supportive therapy after high-dose chemotherapy against malignant diseases. HSCs can be collected from peripheral blood (PB), bone marrow (BM), and umbilical cord blood (UCB). Human UCB is thought to be an attractive alternative to BM or growth-factor-mobilized PB as a source of hematopoietic progenitors because UCB contains a high number of primitive progenitor cells (PPCs) [2–4], and the frequency of graft-versus-host disease, which is a severe side effect of HSC transplantation in patients, is reduced among patients receiving transplants from UCB [5]. However, the total number of UCB HSCs harvested from one donor's UCB may be enough to reconstitute children, but it is not sufficient for HSC transplantation in an adult patient. To overcome this problem, attention has been increasingly focused on ex vivo expansion of HSCs. Many approaches have been reported during the last decade, and they can be divided into two categories. The first category is the treatment of HSCs with various combinations of cytokines. The treatment with the following combinations of cytokines

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increased the stem/progenitor cell population by 2- to 30-fold in the relatively short period of 10–14 days: the combination of thrombopoietin (TPO), stem cell factor (SCF) and Flt-3 ligand (FL); the combination of SCF, megakaryocyte growth and development factor (MGDF) and granulocyte-colony stimulating factor (G-CSF); the combination of G-CSF, SCF, FL, interleukin-3 (IL-3) and interleukin-6 (IL-6); and the combination of FL, SCF and IL-6 [6–9]. However, hematopoietic stem/progenitor cell maintaining (HSPC) activity in long-term cultures, even if the total number of hematopoietic cells can be expanded, is difficult to maintain. Hence, these methods should be improved for use in clinical settings. The second category involves using stromal cells, including mesenchymal stem cells (MSCs). The SCID-repopulating activity (SRA) of human HSPCs could be maintained by coculture with murine stromal cells for 7 weeks [10], and the SRA could be maintained by coculture with the AGM-S3 stromal cell line for 4 weeks [11]. MS-5 expanded SCID-repopulating cells (SRCs) for 2 weeks [12], FBMD-1 expanded cobblestone-area-forming cells by 90-fold [13]. HESS-5 expanded SRCs for only 5 days [14, 15]. Contact between HSPCs and stromal cells is important for maintaining the function of HSPCs [16, 17]. However, when human HSPCs are cocultured with nonhuman stromal cells, the expanded human HSCs might have a risk of being exposed to unknown viral contamination in animal stromal cells.

Several methods of ex vivo expansion using human primary stromal cells have been reported [18, 19]. When HSPCs were cocultured with human primary stromal cells, the HSPCs were expanded for 2–4 weeks. However, in general, when human primary somatic cells divide in an in vitro culture, the replication of human primary cells slows (aging occurs), and the cells finally cease to divide (crisis phase) [20, 21]. To solve this problem, trials in establishing human stromal cell lines using transduction of viral antigens or the telomerase catalytic subunit (hTERT) gene have been reported [22–26]. However, the actual hematopoietic support activity of these transduced stromal cells was similar to that of primary stromal cells.

Recently, our laboratory and others have shown that mesenchymal stem/progenitor cells in human UCB, BM and placenta could support ex vivo expansion of UCB HSPCs [27–29] and that MSCs from BM did not enter crisis phase. The results strongly suggest that BM-derived MSCs may be a good feeder layer for ex vivo expansion of HSPCs from UCB. Previous research has shown that TPO and FL, two early acting cytokines, could lead to significant expansion of UCB PPCs for a short time, including long-term culture-initiating cells (LTC-ICs) [6, 7, 9], and investigations have indicated that maximum proliferation in vitro of the hematopoietic cell types depends on stimulation by the highest concentrations of cytokines [30]. In the present study, we attempted to introduce the TPO and FL gene into human BM-derived mesenchymal stem cells (hMSCs) and to assess the supportive effects of the transduced MSCs on ex vivo expansion of HSPCs from UCB. The result showed that TPO/FL-transduced hMSCs are capable of expanding HSPCs from UCB in synergy with extra cytokines in vitro.

Materials and methods

Isolation and culture expansion of hMSCs. Human BM samples were collected from healthy human donors (28–46 years old) at the First Affliated Hospital, Zhejiang University, under an Institutional-Review-Board-approved protocol. Mononuclear cells (MNCs) were plated in hMSC medium at a density of 2 x 10^7 cells per 75-cm^2 plastic flask (Nunc, Naperville, Ill.). Human MSC medium consisted of minimal essential medium α (MEM-α; HyClone, Logan, Utah) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, N. Y.) and 1% antibiotic-antimycotic solution (Life Technologies, Gaithersburg, MD.). Attached, well-spread hMSCs were collected and expanded by the removal of nonadherent and loosely attached cells during the medium changes. Confluent cultures were detached by trypsin-EDTA (Life Technologies) and replated at 8 x 10^5 cells per 75-cm^2 flask and denoted passage 1 cells.

Generation of recombinant retrovirus and transduction of hMSCs. The retroviral vectors pfl30, pOT20 and pN2neo were employed in these experiments (fig. 1). Vectors were made using the retroviral pLXIN backbone (Clontech, Palo Alto, Calif.). The retroviral vector pfl30, expressing human FL and TPO, was constructed by amplifying (using RT-PCR) the TPO and FL cDNA from human fetal hepatocyte RNA, followed by cloning the FL fragment into the ECORV site of pBluescript (Stratagene, La Jolla, Calif.). The TPO cDNA was ligated behind the internal ribosomal entry site (IRES) sequence and the IRES-TPO fragment was cloning into the pBluescript (behind the FL fragment). The FL-IRES-TPO fragment was transferred from the pBluescript vector to the retroviral vector pLXIN using the XhoI and BamHI sites, resulting in pfl30. The retroviral vector pOT20 was constructed by subcloning the enhanced green fluorescent protein (GFP) gene from pEGFP-N3 (Clontech) into pLXIN (between XHoI and BAMHI sites). The plasmid pN2neo is a modification of the parent plasmid pLXIN, in which the IRES was removed and the protein initiation codon in the neomycin phosphotransferase gene was changed to GAAAGATGT.

Retroviral infection was performed as described elsewhere [31], with several modifications. Briefly, each viral supernatant was produced from the packaging cell line PA317 by transfection of 8 μg plasmid DNA

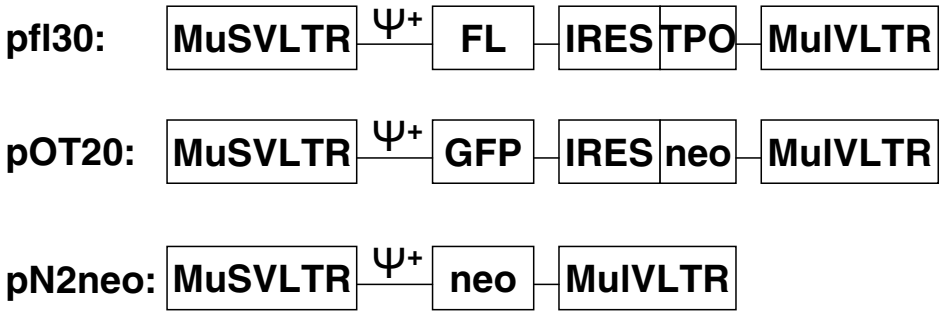

Figure1. Schematic representation of the retrovirus vector constructions. The construction of the vectors used in this study, pfl30, pOT20, pN2neo, is described in detail in Materials and methods. MuSVLTR, murine sarcoma virus long terminal repeat; MuLVLTR, murine leukemia virus long terminal repeat; Ψ^+, extended viral packaging signal; IRES, internal ribosomal entry site; neo, neomycin phosphotransferase gene.

in LipofectAMINE transfection reagent (Life Technologies). Transduced cells was selected with 1 mg/ml G418 for 4 days. The colony-forming units (CFUs) were analyzed using NIH 3T3 target cells, with varied dilutions of retroviral supernatants. The titers of pfl30-, pOT20- and pN2neo-derived retroviruses were 6.4 x 10^5, 2.0 x 10^5 and 4.2 x 10^5 CFUs/ml, respectively. For 8 h, in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO.), 2.0 x 10^5 primary hMSCs (passage 3 hMSCs) in a 10-cm dish were exposed to viral supernatant containing each retrovirus at an approximate multiplicity of infection (MOI) of 1 to ensure single-copy integration. After washing with phosphate-buffered saline (PBS), the transduced cells were incubated for 48 h and selected with 1 mg/ml G418. The surviving cells were noted as TPO/FL-transduced hMSCs for passage 1. Transduction efficiency was assessed by two methods: neomycin-resistant colony formation and GFP fluorescence by flow cytometry analysis. For colony formation, hMSCs were transduced with the retroviral vector pN2neo. For GFP fluorescence, hMSCs were transduced with the pOT20 retroviral vector (fig. 1).

Analysis of expression of the TPO or FL gene by immunoblotting and RT-PCR. For immunoblot analysis, TPO/FL-transduced hMSCs for passage 1 were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P40 (NP40), 150 mM NaCl and the protease inhibitor mixture. Then, 50 μg lysate was subjected to electrophoresis on a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to nitrocellulose membranes in a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, Calif.). Anti-TPO antibody (R&D systems, Minneapolis, Minn.) and anti-FL antibody (BIODESIGN International, Saco, ME.) were used as the primary antibody. Horseradish-peroxidase-conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech (Hong Kong, China). Anti-β-actin monoclonal antibody was purchased from Sigma. Proteins were visualized by means of enhanced chemiluminescence (ECL) (Sigma).

For RT-PCR analysis, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, Calif.) according to the manufacturer's protocol. 500 ng of RNA was used for the RT-PCR reaction in a volume of 25 μl using gene-specific primers and primers for an internal control, β-actin. PCRs were done for 30 cycles each consisting of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 90 s. The primers used for β-actin were: forward 5'-GACTCGACACCGTGTCACCTAC-3'; reverse 5'-CAAACATGATCTGGGTCATCTTCTC-3'. The sequences of primers for detecting TPO and FL were as follows: TPO forward: 5'-GGGCGTTAACATGGAGCTGACTGAATTGC-3'; TPO reverse: 5''-TATGGGATCCTTACCCTTCCTGAGACAG-3''; FL forward 5'-GGGCGTTAACATG ACAGTGCTGGCGCCAGCCT-3' and FL reverse 5'-ATTAGGATCCTTACGGGGCTGTCGG GGCT-3'. The PCR products were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Flow cytometry analysis of GFP fluorescence. Analysis of GFP fluorescence from hMSCs was performed by flow cytometry as previously reported [32]. Briefly, cell layers were washed twice with PBS, and cells were detached with 0.25% trypsin-EDTA. Human MSCs were recovered by centrifugation and washed in flow cytometry buffer consisting of 2% BSA (Sigma) and 0.1% sodium azide (Sigma) in PBS. Cells were collected by centrifugation and resuspended in flow cytometry buffer containing 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA.) before being analyzed. Nonspecific fluorescence was determined using primary hMSCs. Samples were analyzed by collecting 10,000 events on a Becton-Dickinson Vantage instrument (San Jose, Calif.) using CellQuest software (Becton-Dickinson).

Analysis of expression of the TPO and FL genes by enzyme-linked immunosorbent assay. Primary and TPO/FL-transduced hMSCs were cultured in MEM-α (HyClone) supplemented with 10% (v/v) FBS (Gibco BRL) at 37 °C under 5% CO_2 in humidified air. For assaying TPO and FL secretion, primary hMSCs and TPO/

FL-transduced hMSCs were passaged when cells reached 90% confluence by transferring 2.5–5.0 x 10^6 cells into a 75-cm^2 flask with 12 ml of hMSCs medium. Twenty-four hours later, 1 ml of culture supernatant was collected and stored at –80 °C. The assay was performed in triplicate using the TPO and FL ELISA kit (BioSource International, Camarillo, Calif.). The level of TPO and FL was normalized to the level of endogenously expressed IL-6 measured with an IL-6 ELISA kit (BioSource International). Plates were read on a microplate reader (Bio-Rad Laboratories) and the data were analyzed using Microsoft Excel 2003.

Purification of UCB CD34+ cells. UCB was collected from normal full-term pregnancies according to the regulations of the Research Ethics Committee of the Women's Hospital School of Medicine Zhejiang University. MNCs were isolated using Ficoll-Hypaque (1.077 ± 0.001g/ml; Sigma), and resuspended in Iscove's modified Dulbecco's medium (IMDM; HyClone) supplemented with 10% FBS (Gibco BRL). CD34+ cell purification utilized positive selection using the miniMACS immunomagnetic separation system (Miltenyi Biotec, Glodbach, Germany). Briefly, MNCs were suspended in buffer containing PBS, 0.5% bovine serum albumin (BSA; Sigma), and 2 mM EDTA (BSA-EDTA-PBS), and incubated for 15 min with monoclonal anti-CD34 antibody (clone QBEND/10) and human immunoglobulin to prevent nonspecific binding. Washed cells were incubated for 15 min with colloidal super-paramagnetic microbeads. After labeling, the cell suspension was passed through a separation column. CD34+ cells were collected by removal of the column from the magnet and washing with BSA-EDTA-PBS. Ninety-six percent or more of the enriched cells were CD34+ by flow cytometric analysis.

Coculture of hematopoietic cells with primary hMSCs or TPO/FL-transduced hMSCs. We plated 2 x 10^5 primary hMSCs or TPO/FL-transduced hMSCs for passage 4 in a 25-cm^2 flask (Nunc) with 5 ml of hMSC medium and irradiated them with 12 Gy when they reached greater than 90% confluence. hMSCs were washed before the addition of UCB CD34+ cells. We seeded 1 x 10^4 UCB CD34+ cells in a 25-cm^2 flask with either primary hMSCs or TPO/FL-transduced hMSCs and 5 ml of complete medium consisting of IMDM supplemented with 50 ng/ml SCF (Amgen Biologicals, Thousand Oaks, Calif.), 10 ng/ml GM-CSF (Peprotech, London, UK), 20 ng/ml IL-3 (RELIATech, Braunschweig, Germany), 12.5% horse serum (HS; HyClone), 12.5% FBS, 10^{-4} M 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Sigma) and 10^{-6} M hydrocortisone (Sigma). One week later, fresh complete medium was added, and coculture was continued for 1 week. At the end of the second week of coculture, nonadherent and adherent hematopoietic cells (HPCs) that were weakly attached to hMSCs were collected by gentle pipetting and subjected to cell count, clonal cell culture, and flow cytometric analysis when contamination of hMSCs in the harvested cells was negligible (<2%) by microscopic visualization. hMSCs and cobblestone-forming HPCs growing below the hMSC layer were left in the culture flasks, and fresh complete medium was added to continue expansion of HPCs. Likewise, the nonadherent and weakly adherent HPCs were harvested at the end of every week.

Colony-forming cell assay. The total number of colony-forming units in culture (CFU-Cs) and mixed colonies containing erythroid and myeloid cells and megakaryocytes (CFU-GEMM) in uncultured CD34+ or cocultured cells were evaluated. Aliquots from uncultured CD34+ cells or cocultured cells were incubated in methylcellulose medium at concentrations of 1–2 x 10^2 cells/ml for uncultured CD34+ cells and 5–10 x 10^2 cells/ml for cocultured cells in 35-mm dishes (Costar, Bethesda, MD.). All cultures were done in triplicate. One milliliter of culture mixture contained 1.2% 1500 cp methylcellulose (Sigma), MEM-α, 1% deionized fraction V BSA (Sigma), 10^{-4} M 2-mercaptoethanol, 30% fetal calf serum (JRH Biosciences, Lenexa, KS.), 3 U/ml erythropoietin (Peprotech), 20 ng/ml IL-3, 50 ng/ml SCF, and 10 ng/ml GM-CSF. After 14 days of culture, colonies consisting of 50 or more cells were scored under a microscope.

LTC-IC assay. The LTC-IC assay was performed as described by Sutherland et al. [33], with slight modifications. Briefly, stromal cells derived from hematologically normal donors were seeded at 1 x 10^4 cells per well in 96-well flat-bottomed plates (Costar) with MEM-α supplemented with 10% FBS. After obtaining semiconfluent feeder layers, stromal cells were irradiated with 15 Gy using a ^{60}Co γ-irradiator. CD34+ cell subpopulations purified from UCB or from harvested HPCs by sorting with a FACSVantage were seeded at limiting dilution on the feeder layer with 100 µl medium containing 12.5% horse serum, 12.5% FBS, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 10^{-6} M hydrocortisone and IMDM. 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_2 with weekly changes of medium. After 5 weeks of culture, cells were assayed for CFU-Cs. Colonies were scored 2 weeks later. The frequency of wells in which there were no clonogenic progenitors was determined according to the number of the initial input population. Poisson statistics were applied to the single-hit model and the frequency of LTC-ICs was calculated with the maximum-likelihood estimator.

Transplantation into NOD/SCID mice (SRC assay). The SRC assay was performed as previously described [34], with slight modifications. Briefly, 8-week-old NOD/

LtSz-scid/scid (NOD/SCID) mice were bred from breeding pairs originally obtained from the Central Institute for Experimental Animals, Shanghai Institutes for Biological Sciences, CAS, and maintained in the defined flora animal facility located at Zhejiang Academy of Medical Sciences. All animals were handled under sterile conditions. In the presence of SCF, GM-CSF and IL-3, 1 x 10^4 UCB CD34+ cells were cocultured for 2 or 4 weeks on a layer of either primary hMSCs or TPO/FL-transduced hMSCs for passage 4. The HPCs expanded for 2 weeks or 4 weeks were harvested and transplanted by tail vein injection into sublethally irradiated mice (350 cGy using a linear accelerator). Cells were cotransplanted with irradiated (15 Gy using a ^{60}Co γ-irradiator) nonrepopulating $CD34^-$-cells as accessory cells. Mice were killed by cervical dislocation 7 weeks after transplantation, and MNCs from BM (from the femurs and tibiae) and PB (from the retro-orbital venous plexus) were harvested. The presence of human HPCs was determined by detection of cells positively stained with fluorescein isothiocyanate (FITC)- and/or phycoerythrin (PE)-conjugated antihuman monoclonal antibodies using flow cytometry.

PCR amplification of the human Alu repetitive sequence gene was employed as a second test for the presence of human cells in the NOD/SCID mice that had received transplants. Genomic DNA was isolated from the BM cells of mice that had received transplants. The sequences of these primers were 5' – GTGGGCGACAGAACGAGA TTCTAT-3' and 5' – CTCACTACTTGGAGACAGGT-TCA-3'. DNA samples were denatured at 94 ^{0}C for 5 min, and then amplified by rounds consisting of 94 °C for 1 min (denaturation), 55 °C for 45 s (annealing), and 72 °C for 1 min (extension) for 30 cycles. The amplification product was visualized as a 300-bp band on 1.5% agarose gel electrophoresis and ethidium bromide staining.

Immunophenotyping of ex-vivo-expanded HPCs and SRCs. Aliquots of cells were suspended in EDTA-BSA-PBS and incubated with mouse IgG (InterCell Technologies, Hopewell, N. J.) to block nonspecific Fc receptors at 4 °C for 10 min prior to staining. Cells were then reacted for 15 min with several FITC- and PE-conjugated monoclonal antibodies at 4 °C. Unbound antibodies were removed by two washes, and cells were resuspended in EDTA-BSA-PBS. Stained cells were 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 FACsort flow cytometer (Becton-Dickinson) with CellQuest software (Becton-Dickinson). At least 10,000 events were acquired for each analysis. Antibodies used were as follows: FITC-conjugated CD14, CD15, CD19, CD33, CD34 and CD41; PE-conjugated CD38 and CD45. Glycophorin A antibodies were from Immunotech (Marseille, France). CD14, CD33 and CD45 antibodies were from Pharmingen (San Diego, Calif.) and all other antibodies 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 min with biotin-conjugated anti-CD34 (Immunotech). Cells labeled 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 anti-CD38, and various FITC-conjugated monoclonal antibodies. Three-color flow cytometry was performed using a FACSCalibur (Becton-Dickinson) with CellQuest software (Becton- Dickinson).

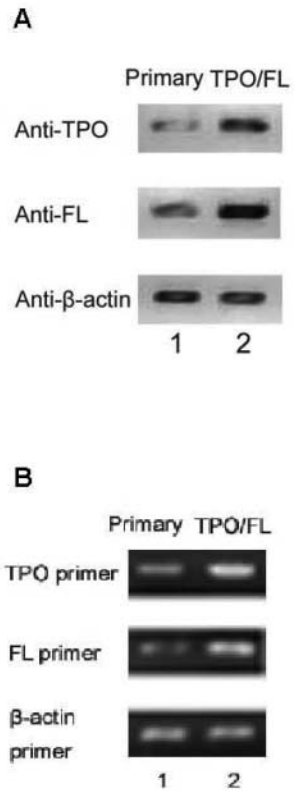

Figure 2. Expression of human TPO or FL in the transduced human BM mesenchymal stem cells (hMSCs) and primary hMSCs. Expression of the transgene was analyzed by immunoblot analysis (A) and by RT-PCR (B). (A) Lane 1, primary hMSCs; lane 2, TPO/FL-transduced hMSCs; anti-TPO, indicates primary antibody for human TPO; anti-FL, antibody for human Flt-3 ligand; anti-β-actin, antibody specific for β-actin protein, as an internal standard. (B) Lane 1, primary hMSCs; lane 2, TPO/FL-transduced hMSCs. RT-PCR was performed using TPO- or FL-specific primers and the β-actin sequence as an internal standard.

Statistical analysis. Results are expressed as the mean ± SD. The significance of differences was assessed by Student's t test or the Mann-Whitney U test. Iterative approximation of Newtons method was performed using Microsoft Visual Basic 6.0 software.

Results

Analysis of expression of TPO and FL by Western blotting and RT-PCR. First, we confirmed the expression of TPO or FL in the transduced BM MSCs by Western blot analysis (fig. 2A). Exogenous FL was strongly expressed

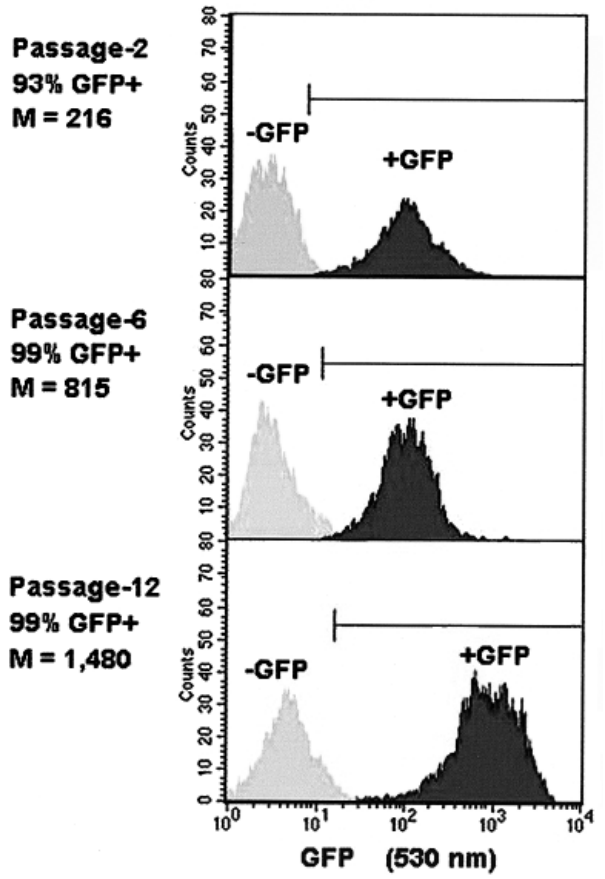

Figure 3. Maintenance of transgene expression in vitro from GFP-transduced hMSCs. Cultures of GFP-transduced hMSCs were maintained for 12 passages and assayed for GFP expression after passages 2, 6 and 12. Flow cytometry histograms for these cultures are shown. Untransduced cultures showed a slight decrease in autofluorescence at higher passage. M represents the average mean fluorescent value observed for GFP fluorescence detection. Untransduced cultures were 2% GFP+ with a mean fluorescence value of 36. The values for the percentage transduction and mean fluorescence for GFP-transduced cultures are shown under the passage numbers. The retroviral supernatants used in these experiments were obtained from the PA317 packaging cell line. The cells were exposed twice to the retroviral supernatant without any drug selection. These data are representative of results obtained from three different donors.

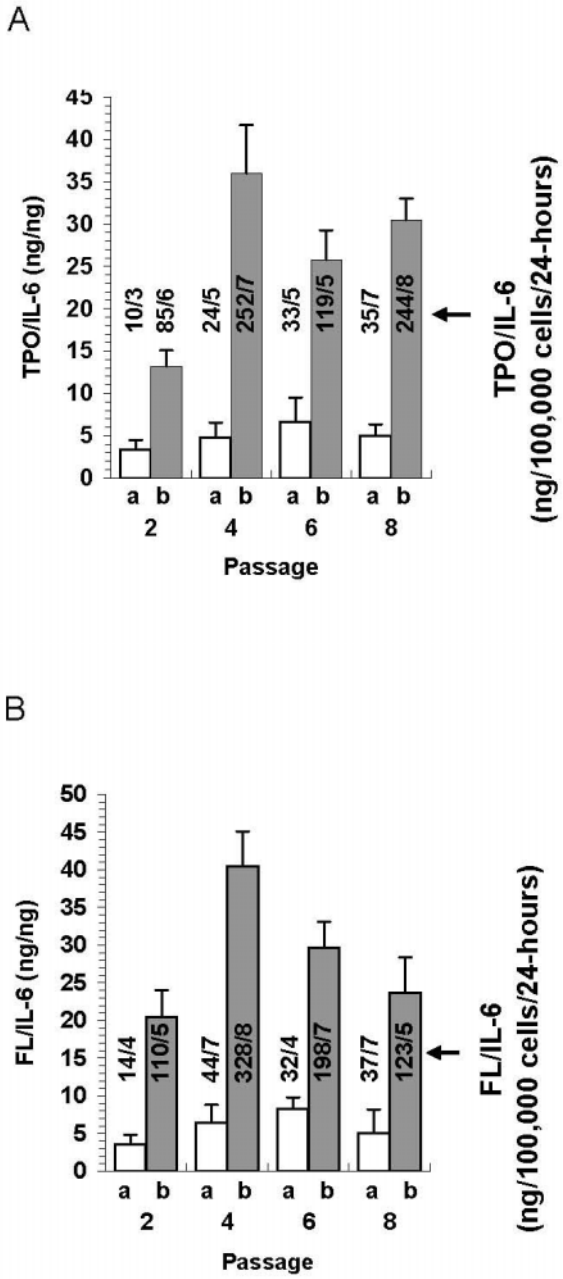

Figure 4. Maintenance of TPO and FL production in vitro. TPO and FL levels secreted from TPO/FL-transduced hMSCs and primary hMSCs and endogenous IL-6 levels were assayed in the medium after 2, 4, 6 and 8 passages in culture. The level of TPO and FL in the supernatant was normalized to the level of endogenous IL-6 and the ratio was plotted for passage number. In addition, the absolute values (ng/100,000 cells per 24 hours) of TPO, FL and IL-6 are shown (arrow). Data were obtained from triplicate ELISA measurements. a, primary hMSCs; b, TPO/FL-transduced hMSCs.

in TPO/FL-transduced hMSCs, and a low level of endogenous FL was detected in primary hMSCs. Immunoblot analysis revealed that exogenous TPO protein was also expressed in TPO/FL-transduced hMSCs, and expression of endogenous TPO was low. Next, we performed RT-PCR analysis to detect TPO and FL mRNA derived from the transgenes. Expression of TPO and FL mRNA was detected in TPO/FL-transduced hMSCs as well as in primary hMSCs (fig. 2B). These results demonstrated that TPO and FL (double genes) had been introduced successfully into primary hMSCs.

Long-term transgene expression in vitro. To illustrate long-term in vitro transgene expression by retrovirally transduced hMSCs, we monitored both GFP and TPO/FL transgene expression in transduced hMSCs. Differ-

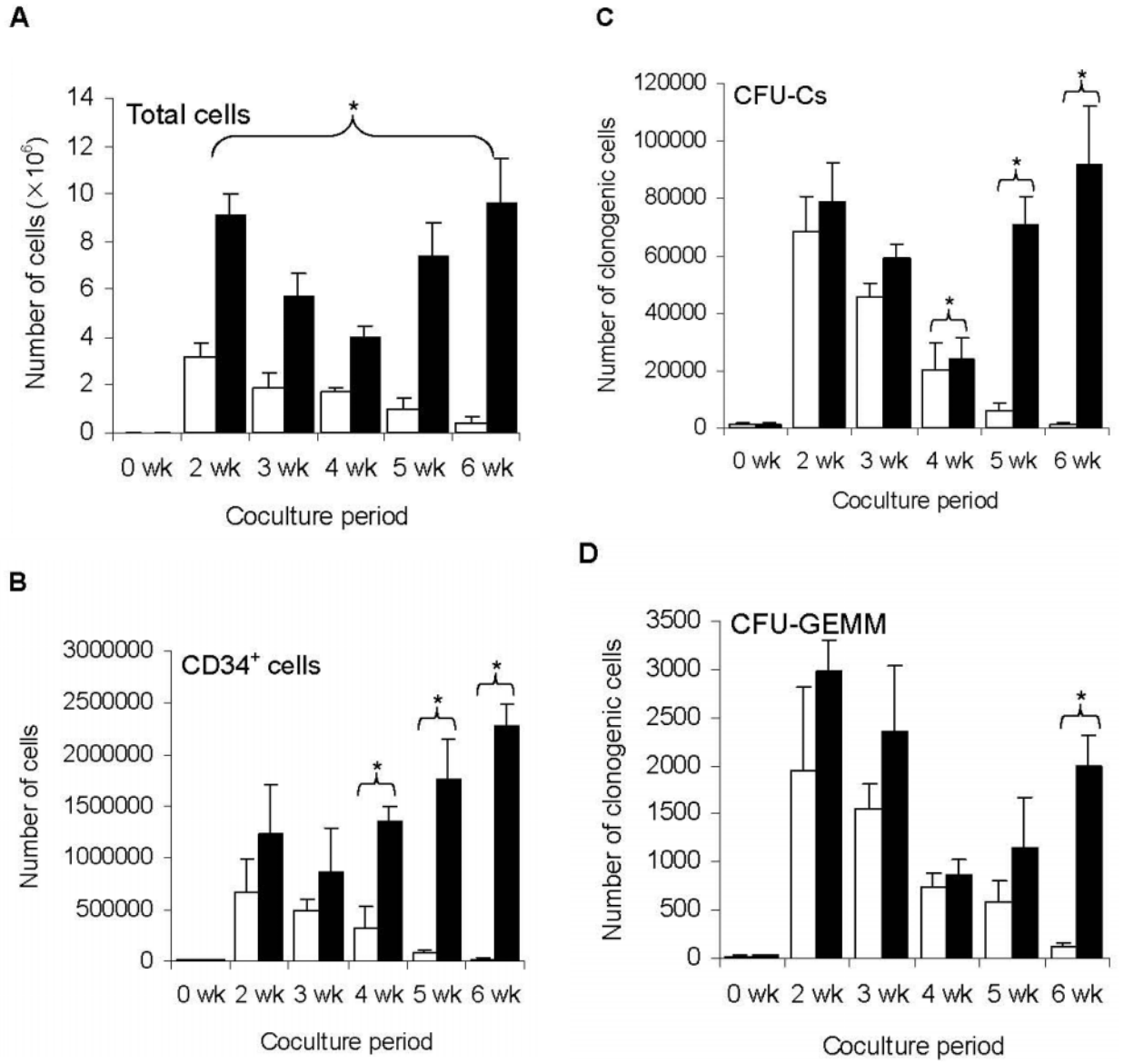

Figure 5. Production of primitive HPCs. Production of primitive hematopoietic cells from cobblestone-forming cells beneath the primary hMSCs layer (open bars) or TPO/FL-transduced hMSC layer (closed bars) over 6 weeks. Expanded hematopoietic cells were harvested each week and analyzed. The x-axis indicates the period of cell expansion, and the y-axis indicates the number of cells. (A) Total number of nucleated cells. (B) Number of CD34+ cells. The number of CD34+ cells was calculated from the percentage of CD34+ cells, which was determined by flow cytometric analysis. (C) Total number of clonogenic cells (CFU-Cs). (D) Number of CFU-GEMM cells. *$p < 0.05$ versus primary hMSCs (Student's t test).

ent passages (p2, p6 and p12) of hMSCs transduced with GFP were tested for GFP expression by flow cytometry. No decrease in GFP fluorescence was observed over this time period, illustrating the maintenance of transduced cells and transgene expression after prolonged culturing in vitro (fig. 3).

As an additional demonstration of maintenance of transgene expression from TPO/FL-transduced hMSCs, cultures were maintained and expanded further for up to 8 passages. Twenty-four hours after each replating, an aliquot of supernatant was removed to measure the secreted TPO and FL (fig. 4). The secretion value obtained was normalized to the secretion value obtained for endogenous human IL-6 measured in the same sample aliquot. The absolute values for each cytokine are shown in figure 4. We chose IL-6 as the control cytokine because expression was in a range similar to that of the transduced gene product and previous data showed similar expression of IL-6 from hMSCs over time in hMSC cultures [35]. We observed TPO transgene expression from TPO/FL-transduced hMSCs and primary hMSCs averaging 175 ± 85 and 25 ± 11ng/10^5 cells per 24 hours, respectively (fig.

Table 1. Ex vivo expansion of UCB CD34+ cells over 2 weeks.

	hMSCs free	Primary hMSCs	TPO/FL hMSCs
Total cells	55 ± 7	320 ± 40*	910 ± 120*
CD34+ cells	7 ± 2	67 ± 12*	121 ± 15*
CFU-Cs	5 ± 1	52 ± 10*	62 ± 15*
CFU-GEMM	4 ± 0	32 ± 6*	90 ± 12*

Values indicate the fold increase compared with the initial number of cells. Data shown are from a representative experiment of three showing similar results. The results are expressed as the mean fold increase ± SD (n = 3). *p < 0.05 versus hMSCs free (n = 3) (Student's t test).

4A), and FL transgene expression averaging 190 ± 99 and 31 ±12 ng/10^5 cells per 24 hours, respectively (fig. 4B), and endogenous IL-6 protein levels averaging 6 ± 2 ng/ 10^5 cells per 24 hours (fig. 4). The cytokine ratio (transduced/endogenous) of TPO/FL-transduced hMSCs demonstrated a 14- to 36-fold and 22- to 41-fold increase, respectively, in TPO and FL secretion over endogenous IL-6 expression. The results demonstrate that in vitro transgene expression from TPO/FL-transduced hMSCs was maintained for at least 3 months in culture.

Hematopoietic support by TPO/FL-transduced hMSCs in vitro. The HPC support by TPO/FL-transduced hMSCs was examined. UCB CD34+ cells were cultured in medium containing the three cytokines described above in the following three conditions for 2 weeks (first period): (i) without hMSCs, (ii) with primary hMSCs or (iii) with TPO/FL-transduced hMSCs. The total number of nucleated cells, CD34+ cells, and clonogenic cells was evaluated. The total number of cells and the number of CD34+ cells, CFU-Cs, and CFU-GEMM at 2 weeks after the start of coculture with primary hMSCs were remarkably increased in comparison with the respective initial cell number (table 1). When UCB CD34+ cells were cocultured with TPO/FL-transduced hMSCs for 2 weeks, the expansion magnitude of total number of nucleated cells, CD34+ cells, and CFU-GEMM were much higher than that by the other two culture systems (table 1). A low level of expansion of hematopoietic cells was observed, owing to the presence of cytokines even in the absence of both stromal cell lines. Noticeably, the total number of nucleated cells and the number of CFU-GEMM in the TPO/FL-transduced hMSCs coculture system were three times higher than that in primary hMSCs, and the number of CD34+ cells was twofold higher than the latter. However, the expansion magnitude of CFU-Cs was similar between the two coculture systems.

We also examined the expression of CD34 and CD38 on the expanded HPCs by flow cytometry. The ratio of CD34+CD38– cells in HPCs expanded on TPO/FL-transduced hMSCs was remarkably increased in comparison with that on primary hMSCs or in the hMSC-free system (data not shown). These findings suggested that TPO/FL-transduced hMSCs could effectively support hematopoietic cells during this period. Starting at the beginning of the third week of culture, we studied the number of HPCs generated from cobblestone-forming hematopoietic cells beneath the hMSC layer each week. The number of HPCs in the coculture with the primary hMSCs decreased through 6 weeks (fig. 5). The HPC expansion on TPO/FL-transduced hMSCs showed a tendency similar to that on primary hMSCs up to 4 weeks. However, as TPO/FL-transduced hMSCs were able to survive longer than the primary hMSCs, the number of HPCs expanded on TPO/FL-transduced hMSCs was significantly higher than that on primary hMSCs during the fourth, fifth, and sixth weeks. In addition, cobblestone-area-forming cells were observed in the culture with TPO/FL-transduced hMSCs even in the sixth week (data not shown). These results demonstrate that TPO/FL-transduced hMSCs may be a better feeder layer for long-term coculture of UCB cells than primary hMSCs and other reported hu-

Table 2. Results of LTC-IC assay using UCB CD34+ cells expanded for 2 or 4 weeks.

	LTC-IC frequency		Fold LTC-IC amplification
	pre-expansion	post-expansion	
hMSC free, 2 weeks	1/625.38 ± 154.26	1/84,112.78 ± 10,867.64	2.58 ± 1.48
Primary hMSCs, 2 weeks	1/619.57 ± 120.89	1/102,258.56 ± 9,568.49	5.79 ± 0.56
TPO/FL hMSCs, 2 weeks	1/617.43 ± 119.76	1/24,140.96 ± 7,586.62	10.23 ± 2.89*
Primary hMSCs, 4 weeks	1/672.43 ± 215.53	1/93,462.57 ± 9,758.63	3.26 ± 1.47
TPO/FL hMSCs, 4 weeks	1/635.57 ± 129.75	1/6,035.84 ± 587.67	25.37 ± 12.52**

CD34+ cells were isolated from UCB and cocultured for 2 or 4 weeks on primary hMSCs or TPO/FL-transduced hMSCs. LTC-IC frequency was compared between UCB CD34+ cells (pre-expansion) and those generated by culture systems (post-expansion). The fold increase in LTC-IC was determined by the LTC-IC frequency. Results represent mean ± SD of three different experiments. *p < 0.05; **p < 0.01 versus hMSC free (n = 3) (Student's t test).

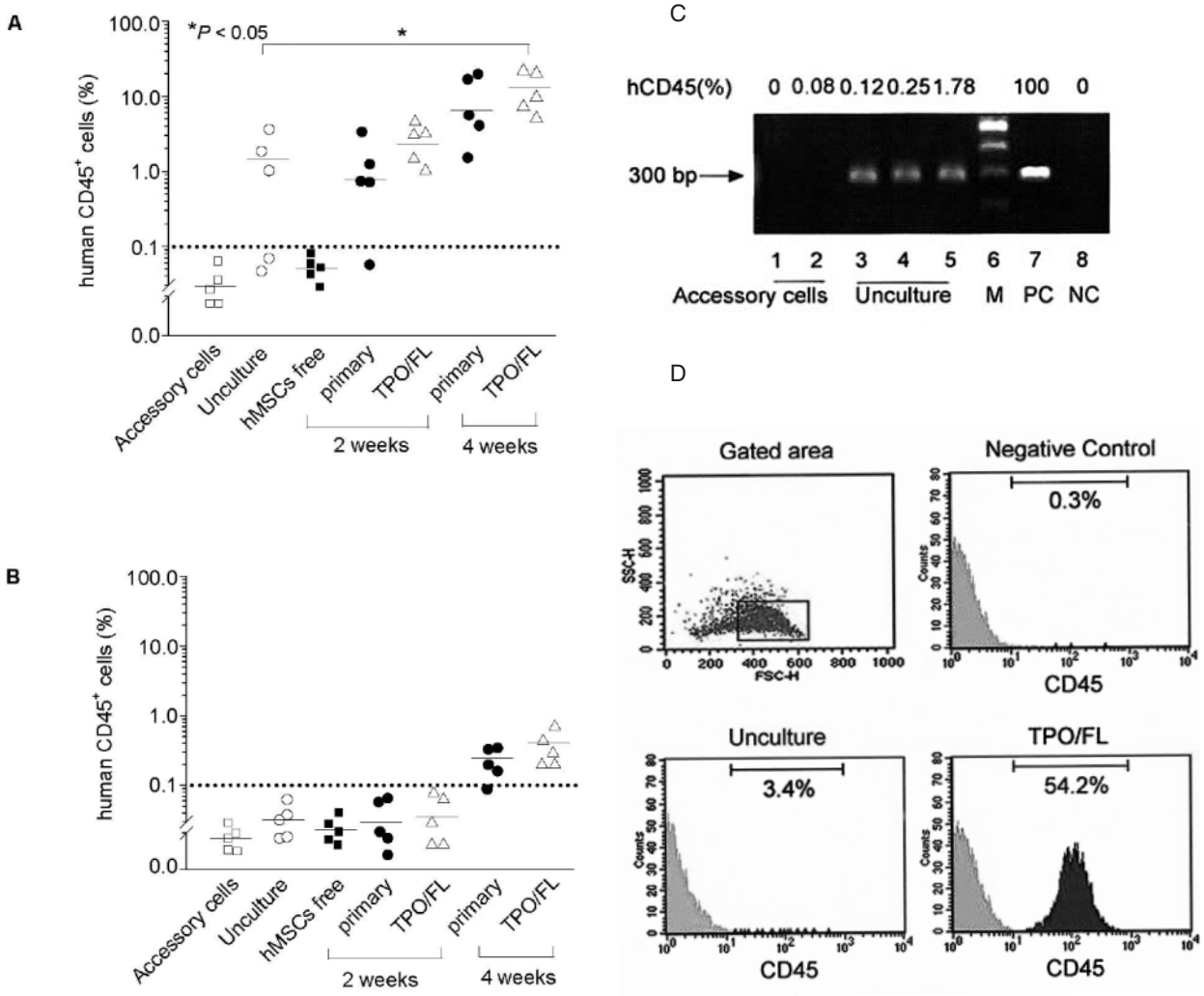

Figure 6. Analyses of human CD45+ (hCD45+)cells in the BM and PB of NOD/SCID mice that had received transplants. NOD/SCID mice received irradiated accessory cells**,** uncultured CD34+ cells, or HPCs expanded for 2 or 4 weeks on TPO/FL-transduced hMSCs or primary hMSCs. Mice were killed 7 weeks after transplantation, and the BM (A) and PB (B) were analyzed by flow cytometry. □, accessory cells; ○, uncultured CD34+ cells; ■, HPCs expanded ex vivo in the absence of primary hMSCs for 2 weeks; ●, HPCs expanded on primary hMSCs for 2 or 4 weeks; △, HPCs expanded on TPO/FL-transduced hMSCs for 2 or 4 weeks. The horizontal lines indicate the mean level of engraftment. The dotted lines indicate the cutoff level (0.1%) of successful engraftment of human hematopoietic cells. (C) PCR amplification of human Alu sequences and hCD45 percentages of the BM of NOD/SCID mice. Lanes 1, 2, mice (n = 5) receiving transplants of accessory cells only; lanes 3–5, mice (n = 5) receiving transplants of uncultured CD34+ cells, lane 6, M indicates DNA marker (100-bp DNA Ladder); lane 7, PC indicates positive control (human PB MNCs); lane 8, NC indicates negative control mouse (n = 1) without transplants. hCD45%, the percentage of the human CD45+ hematopoietic cells in the BM of mice that had received transplants. (D) Representative data of flow cytometric analysis of the BM MNCs of NOD/SCID mice, using antihuman CD45 antibody. Cells in the gated area (upper left) were analyzed by flow cytometry. NOD/SCID mice received transplants of either uncultured CD34+ cells (lower left) or hematopoietic cells that had been expanded on TPO/FL-transduced hMSCs (lower right) for 4 weeks. BM cells from an untransplanted mouse (upper right) were used as negative control.

man stromal cell lines such as hTERT-transfected human stromal cells.

Analysis of LTC-ICs using expanded HPCs. We next determined whether HPCs generated in the three culture systems described above could preserve the ability to sustain long-term hematopoiesis in vitro. The LTC-IC frequency in HPCs cultured by these systems was quantified. Initially, isolated UCB CD34+ cells were cultured for 2 or 4 weeks using three culture systems. The expanded HPCs were harvested and subjected to a second CD34+ cell purification by sorting. An LTC-IC assay was performed using sorted CD34+ cell populations, as well as those initially prepared from UCB (control samples). The LTC-IC frequency was determined as previously described [33]. The output of LTC-IC expansion in the

	Mice with reconstituted cells/transplant recipients	CD45+ cells in BM of recipients (%)	CD45+ cells in PB of recipients (%)
Accessory cells	0/5	ND	ND
Unculture	3/5	1.83 ± 1.98	ND
hMSC free, 2 weeks	0/5	ND	ND
Primary 2 weeks	4/5	0.85 ± 1.22	ND
TPO/FL 2 weeks	5/5	2.52 ± 1.98	ND
Primary 4 weeks	5/5	6.76 ± 7.25	0.28 ± 0.17
TPO/FL 4 weeks	5/5	12.67 ± 5.83*	0.45 ± 0.21

Table 3. Engraftment of hCD45+ cells in BM and PB in NOD/SCID mice. ND indicates hCD45% values below the cutoff level (0,1%) ± SD. *p < 0,05 as compared with unculture group.

TPO/FL-transduced hMSC culture system was remarkably increased in comparison with that in the primary hMSC system or hMSC-free system after 2 weeks culture (table 2). A low level of expansion of LTC-ICs was also observed, owing to the presence of cytokines even in the absence of both stromal cell lines (table 2). When UCB CD34+ cells were cocultured with TPO/FL-transduced hMSCs for 4 weeks, the fold increase of LTC-IC was significantly higher than that with primary hMSCs (table 2).

Expansion of SRCs. The results described above strongly suggest that TPO/FL-transduced hMSCs can support PPCs. Hence, we examined the engraftment of SRCs as a substitute for the in vivo human stem cell assay to evaluate the expansion of HSCs. Irradiated NOD/SCID

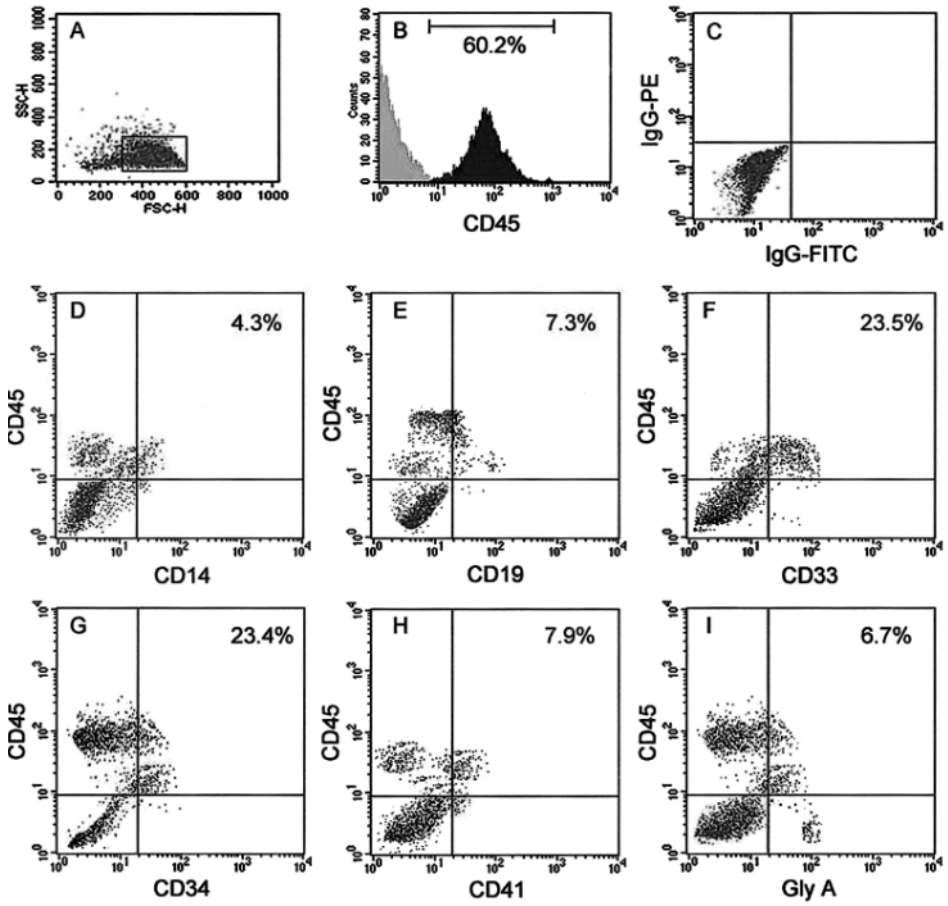

Figure 7. Flow cytometric analysis of lineage markers on human hematopoietic cells engrafted in NOD/SCID mice**.** CD34+ cells were expanded on TPO/FL-transduced hMSCs, followed by transplantation into NOD/SCID mice. Bone marrow MNCs of NOD/SCID mice were immunolabeled with PE-conjugated hCD45 antibody to ensure human origin and with FITC-conjugated antibody specific for the indicated lineage marker. Data shown are from 1 experiment representative of 5 showing similar results.

mice received either the uncultured UCB CD34+ cells (1 x 10^5 cells) or the expanded HPCs (1 x 10^5 cells) that had been generated from cocultures with the three culture systems for 2 or 4 weeks. Simultaneously, irradiated nonrepopulating $CD34^-$-cells were cotransplanted into NOD/SCID mice as accessory cells. Mice were killed 7 weeks after transplantation, and the presence of human cells in the BM and PB of the NOD/SCID mice was evaluated by flow cytometry and ALU PCR analysis (fig. 6). The cutoff level of the presence of human cells in NOD/SCID mice was arbitrarily determined at an hCD45 of 0.1%, because the human ALU sequence gene could be detected by PCR amplification when the hCD45 percentage was more than 0.12% (fig. 6C, lanes 3–5), whereas it was not detectable at an hCD45 of 0.08% (fig. 6C, lane 2). The hCD45+ cells were detected in the BM of mice receiving transplants of HPCs expanded with the primary hMSCs or TPO/FL-transduced hMSCs for 2 weeks (fig. 6A). However, there was no significant difference in the percentage of hCD45+ cells between mice receiving transplants of uncultured CD34+ cells and transplants of HPCs expanded with the primary hMSCs or TPO/FL-transduced hMSCs, suggesting that SRCs had not expanded at 2 weeks although the number of clonogenic cells was remarkably increased (table 1). The hCD45+ cells were not detected in the BM of mice receiving transplants of HPCs expanded only with cytokines for 2 weeks. The hCD45+ cells were also detected in the BM and PB of mice receiving transplants of HPCs expanded with hMSCs for 4 weeks (fig. 6A, B, D), and the percentage of hCD45+ cells in the BM of mice receiving transplants of HPCs expanded with TPO/FL-transduced hMSCs was significantly higher than those of mice receiving transplants of uncultured CD34+ cells or transplants of HPCs expanded with primary hMSCs (table 3). These results suggested that the amplification of SRCs with TPO/FL-transduced hMSCs for a longer time might be superior to that with primary hMSCs.

Next, we examined the surface markers on HPCs that had differentiated from SRCs expanded on TPO/FL-transduced hMSCs (fig. 7). There were marked differences in the percentage of chimerism between BM cells in mice transplanted with cultured cells and those transplanted with control samples. Human CD45+ cells in the murine BM were further subjected to flow cytometric analysis to determine multilineage reconstitution. As a result, human CD45+ cells were positive for CD34, CD33, CD14, CD41, glycophorin A or CD19 (fig. 7).

Discussion

Many approaches have been developed to expand HSPCs from UCB in vitro. However, progress to develop these approaches has been impeded by the absence of an effective culture system that can maintain HSPC activity in long-term cultures. The TPO/FL-transduced hMSCs we have developed here provide a new feeder layer for ex vivo expansion of HSPCs from UCB. Our results indicate that the TPO/FL-transduced hMSCs could effectively support ex vivo expansion of HSPCs from UCB in synergy with exogenic human cytokines. We assessed the supportive effects of three culture systems on proliferation of HSPCs. Although ex vivo expansion of HSPCs on the primary hMSCs was enhanced during the first period (2 weeks), TPO/FL-transduced hMSCs could support ex vivo expansion of HSPCs for a relatively long time, up to 6 weeks, with no decrease in the number of primitive progenitors. More important is that TPO/FL-transduced hMSCs were capable of supporting expansion of LTC-ICs. The SRC assay also demonstrated the extensive ability of the expanded HSPCs to sustain and reconstitute long-term human hematopoiesis in vivo. These results suggest that TPO/FL-transduced hMSCs may be a novel feeder for ex vivo expansion of HSPCs.

We introduced TPO and FL into primary hMSCs for the following reasons. Maximum proliferation in vitro of the hematopoietic cell types depends on stimulation by the highest concentrations of cytokines [30]. In vitro transgene expression from TPO/FL-transduced hMSCs was maintained for at least 3 months in culture, and the level of expression of TPO and FL did not decrease. This can maintain a higher concentration in culture medium. Previous investigations have indicated that TPO and FL, the two early acting cytokines, could effectively support ex vivo expansion of HSPCs from UCB for a relatively short period in synergy with other exogenous cytokines [6, 7, 9]. However, they could not support hematopoiesis for a long time. The reason may be the exhaustion of TPO or FL during the culture period. The TPO/FL-transduced hMSCs we developed here could overcome this problem.

In ex vivo expansion of HSPCs from human UCB, there are several benefits from using TPO/FL-transduced hMSCs as the feeder: (i) TPO/FL-transduced hMSCs, like the primary hMSCs, can also be easily maintained and expanded in vitro; (ii) consistent hematopoietic-supportive effects are repeatedly obtained, and (iii) additive TPO and FL are not needed in the culture system. The effect of TPO/FL-transduced hMSCs on the expansion of HSPCs was comparable with that obtained from primary hMSC or hMSC-free culture systems. However, in the stroma-contact culture, the possibility of TPO/FL-transduced hMSC contamination in harvested HSPCs is an important problem for clinical application. Another problem is the difficulty to harvest cultured hematopoietic cells completely since some of the cultured HSPCs migrate under feeder layers. To overcome these problems, a noncontact culture for the coculture system with TPO/FL-transduced hMSCs might be suggested,

so that HSPCs could be physically separated from the feeder layer by a polyethylene terephthalate track-etched membrane [36, 37]. If these problems could be solved, the TPO/FL-transduced hMSCs coculture system we have developed here could be considered a suitable system for ex vivo manipulation of HSPCs. Moreover, TPO/FL-transduced hMSCs could be expanded and cryopreserved without transformation (data not shown). Thus, we could prepare a large quantity of these human TPO/FL-transduced hMSCs at any time. Taking advantage of this coculture system, clinical research on ex vivo expansion could be facilitated. For example, progenitor cells such as CD34+ cells and CFU-Cs were extensively expanded more than 1000-fold in this system in 6 weeks, and it may be possible to use these expanded cells as a new source of blood transfusion after differentiation of the expanded cells into megakaryocytes or erythroblast progenitor cells [38].

Although the present study and others [5, 8, 30] have demonstrated that addition of exogenic TPO and FL in culture systems can lead to significant expansion of HSPC populations (including LTC-ICs), the TPO/FL-transduced hMSC coculture system, which has no added of TPO and FL, also manifested higher expansion of LTC-ICs. The mechanism of the supportive effect of TPO/FL-transduced hMSCs remains unclear.

Previous findings showed that UCB LTC-ICs were present among the CD34+ cell fraction [39, 40]. Furthermore, Bhatia et al. [34] identified the SRCs that were capable of multilineage reconstitution of human hematopoiesis in the bone marrow of NOD/SCID mice. Therefore, the expanded hematopoietic progenitors were expected to sustain long-term hematopoiesis. In the present study, the amplification of LTC-ICs at 2 and 4 weeks in the culture system with TPO/FL-transduced hMSCs was 5- and 12-fold higher than that in the culture system without hMSCs, suggesting that TPO/FL-transduced hMSCs could support the amplification of LTC-ICs efficiently.

The SRC assay indicated the reconstituting ability of these cultured human HSPCs. Although we could not perform a quantitative SRC assay, the difference in the percentage of chimerism of human CD45+ cells between BM cells of mice transplanted with cultured cells and those transplanted with control samples strongly suggested the extensive ability of these ex-vivo-generated HSPCs to sustain and reconstitute long-term human hematopoiesis in vivo. Furthermore, PCR amplification using the human ALU repetitive sequence gene confirmed flow cytometric results.

In conclusion, we successfully established a TPO/FL-transduced hMSC line that could effectively support expansion of HSPCs by introducing TPO and FL cDNA into primary BM-derived MSCs, and these expanded HSPCs could sustain and reconstitute long-term human hematopoiesis in NOD/SCID mice. The extent of LTC-IC expansion shown here has important practical implications in terms of clinical HSC transplantation.

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