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Mesenchymal stem cells feeder layer from human umbilical cord blood for ex vivo expanded growth and proliferation of hematopoietic progenitor cells

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Abstract Ex vivo expansion of hematopoietic stem cells was suggested as the best way of overcoming problems caused by limited hematopoietic cell number for cord blood transplantation. In this study, we quantified and characterized an ex vivo expansion capacity of umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) as a cell feeder layer for support of UCB-derived committed hematopoietic progenitor cells (HPCs) in the absence or presence of recombinant cytokines. The UCB-derived MSCs used in the study differentiated into osteoblast, chondrocytes, and adipocytes under proper conditions. Frequencies in colony forming unit-granulocyte, macrophage, colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte, burst forming unit-erythrocyte, and colony forming unit-erythrocyte increased to 3.46-, 9.85-, 3.64-, and 2.03-folds, respectively, only in culture supplemented by UCB-derived MSCs as a cell feeder layer without recombinant cytokines (culture condition C). Identified expansion kinetics in all kinds of committed HPCs showed plateaus at 7 culture days, suggesting some consumable components were required for the expansion. Physiological importance and different roles for different committed HPCs of UCB-derived MSCs as a cell feeder layer were revealed by a distinguished expansion capacity for colony forming unit-megakaryocyte. The preferred maintenance of $CD33^{-}CD34^{+}$ in culture condition C was also identified. The presence of cobblestone-like areas as hematopoietic microenvironment and various cell feeder layer-originated hematopoietic cytokines including interleukin-1β and granulocyte, macrophage-colony stimulating factor were suggested as underlying mechanisms for the identified expansion capacity. The present numeric and biological information about intrinsic expansion capacity for UCB-derived committed HPCs will increase further biological and clinical applications of UCB-derived MSCs.

Keywords Umbilical cord blood · Mesenchymal stem cells · Hematopoietic stem cells · Hematopoietic transplantation · Cell expansion · Cytokine

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Introduction

Although the human bone marrow (BM) has been the most well-known source for hematopoietic stem cell (HSC) transplantation, the difficulty in finding appropriate human leukocyte antigen (HLA)-matched donors and the invasiveness of the BM aspiration procedure led many researchers to investigate alternative sources for HSCs. The human umbilical cord blood (UCB) has gained tremendous importance over the last decade as a valid source of transplantable HSCs [1]. Previous studies have carefully compared frequencies of committed hematopoietic progenitor cells (HPCs) in UCB and BM, and several important differences were identified: (1) significantly higher number of colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM) per 10^5 mononucleated cells (MNCs) in UCB than in BM [2]; (2) higher frequency of burst forming unit-erythroid (BFU-E) in UCB than in BM [2–4]; and (3) higher frequency of colony forming unit-megakaryocyte (CFU-Mk) in UCB than in BM [3]. Thus, it was generally accepted that UCB possesses a higher proportion of immature and committed HPCs than adult BM [5]. Also, HSCs from UCB showed greater tolerable HLA-disparity with a decreased frequency of acute or chronic graft-

versus-host disease between donor and recipient in unrelated UCB transplantation than in BM transplantation [6, 7].

However, the total number of UCB-derived HSCs harvested from one donor's UCB is limited and majority of recipients belong to the pediatric group. So, ex vivo expansion was suggested as a major means of obtaining lager cell numbers to improve outcomes from cord blood transplantation (CBT) in younger patients with higher body weight or in adult patients [8]. Many studies reported for this purpose have used and focused on synergic effects obtained by a combined usage of recombinant cytokines and mesenchymal stem cells (MSCs)-derived cell feeder layer. Although, it was reported that UCB-derived HSCs and HPCs could be synergistically expanded over a cell feeder layer in the presence of recombinant cytokines [9–11], enhanced differentiation into subset lineages and reduced therapeutic potential could be evoked by the recombinant cytokines [12]. Recombinant cytokines previously used as various combinations include Flk-2/Flt-3 ligand (FL), stem cell factor (SCF), thrombopoietin (TPO), granulocyte-colony stimulating factor, megakaryocyte growth and development factor, interleukin-3 (IL-3), and interleukin-6 (IL-6) [13–16].

Several research groups including our group have successfully isolated and cultivated human UCB-derived MSCs capable of differentiating into various cell types such as osteoblast, chondrocytes, adipocytes, stromal cells, skeletal cells, neural cells, and endothelial cells [17–21]. So, the novel application of human UCB-derived MSCs as a cell feeder layer for ex vivo expansion of allogeneic UCB-derived transplantable HSCs and HPCs will be highlighted. Even though, it was found that human placenta-, lung- or UCB-derived MSCs could serve as a cell feeder layer supporting ex vivo expansion of HSCs and HPCs collected from the UCB in the presence of recombinant cytokines [22, 23], unfavorable differentiation of immature and committed HPCs into subset lineages [12] and reported concerns in exhausting long-term engrafting cells by recombinant cytokines [11] raise a question about using recombinant cytokines in cultures for ex vivo expansion. Meanwhile, a recent report showed data revealing no major numeric advantage in culture only supplemented with a cell feeder layer in recombinant cytokines-free medium [24]. So, an intrinsic ex vivo expansion capacity of UCB-derived MSCs as a cell feeder layer is still controversial and should be answered for improved clinical applications of UCB-derived MSCs such as cotransplantation with HSCs and ex vivo expansion of HSCs.

In the present study, we have evaluated whether or not ex vivo expansion of committed HPCs could be expanded over a cell feeder layer composed of UCB-derived MSCs in the absence or presence of recombinant cytokines. We present a series of data showing that committed HPCs and $CD34^+$ cells can be maintained and significantly augmented in a culture condition only supplemented with a cell feeder layer in the absence of recombinant cytokines; and the cell feeder layer play important roles in the expansion by providing hematopoietic microenvironment and cytokines.

Materials and methods

UCB-derived HSCs and MSCs derivation

Human UCBs were obtained after written informed consent from normal full-term pregnant women. This study was approved by the Institutional Review Board of Samsung Medical Center, Seoul, Korea. To get UCB-derived HSCs, MNCs were isolated by density gradient centrifugation at 400×*g* for 30 min using Histopaque (1.077 g/ml, Sigma-Aldrich, St. Louis, MO, USA) and then incubated with anti-human CD34 conjugated with MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min at 4°C. $CD34^+$-enriched cells were isolated using automagnetic-activated cell separation (MACS) system (Miltenyi Biotec). The averaged percentage of $CD34^+$ cells in the $CD34^+$-enriched cells was more than 95% by flow cytometric analysis (BD Bioscience, San Jose, CA, USA). MSCs were isolated and cultivated from human UCB as previously reported [18]. Poietics BM-derived human MSCs were purchased from Cambrex Inc. (Walkersville, MD, USA) and were used for comparison as BM-derived MSCs. To be used as a cell feeder layer, $1\times10^4/cm^2$ UCB- or BM-derived MSCs were plated and cultured in 6-well plates. When the cells reached more than 90% confluence in alpha-minimum essential medium (alpha-MEM) (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 100 unit/ml of penicillin-streptomycin (Gibco), the cells were treated with 10 μg/ml of mitomycin C (Sigma-Aldrich) for 2.5 h at 37°C. The cell feeder layer was washed two times with serum-free Iscove's modified Dulbecco's media (IMDM) (Gibco).

Multilineage differentiation of UCB-derived MSCs

The differentiation into various cell types including osteoblast, chondrocytes, and adipocytes of UCB-derived MSCs were examined as previously reported [18]. After differentiation under proper stimuli, the multilineage potential was evaluated by the expression of alkaline phosphatase (ALP) and von Kossa's staining for osteoblast by the expression of type II collagen and safranin O staining for chondrocyte, and by accumulation of lipid-rich vacuoles and Oil Red O staining for adipocytes.

Ex vivo expansion of total and $CD34^+$ cells

Initial 1.2×10^5 $CD34^+$-enriched cells were cultured in various culture conditions at 1×10^4 cells/ml for 14 days. The culture conditions for the present ex vivo expansion study were classified into six conditions:

- Culture condition A, both without a cell feeder layer and recombinant cytokines consisting of 100 ng/ml recombinant human (rh) SCF, 100 ng/ml rh IL-6, 50 ng/ml rh FL, and 10 ng/ml rh TPO.
- Culture condition B, only supplemented with recombinant cytokines without a cell feeder layer.

- Culture condition C, only supplemented with UCB-derived MSCs as a cell feeder layer without recombinant cytokines.
- Culture condition D, supplemented with UCB-derived MSCs as a cell feeder layer and recombinant cytokines.
- Culture condition E, only supplemented with BM-derived MSCs as a cell feeder layer without recombinant cytokines.
- Culture condition F, supplemented with BM-derived MSCs as a cell feeder layer and recombinant cytokines.

A quarter amount of fresh culture medium, IMDM containing 10% FBS and penicillin-streptomycin with or without recombinant cytokines, was exchanged to all cultures every 3 or 4 days. Before culture initiation or after 4, 7, 10, and 14 culture days, cells were counted for total cell number or were sorted for $CD34^+$ cells using MACS system. After sorting, the averaged percentage of $CD34^+$ cells was 98.7% as a mean value. Expansion folds were calculated by comparing cell numbers obtained after indicated culture days with cell number before culture initiation.

Ex vivo expansion of CFC

To verify the ex vivo expansion of committed HPCs, 1.5×10^3 $CD34^+$-enriched cells were cultured in various culture conditions and were taken before culture initiation or after 4, 7, 10, and 14 days. The cells were induced to various types of committed HPCs in 35-mm tissue culture dishes supplemented by methylcellulose medium containing 30% FBS, 1% bovine serum albumin (BSA), 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml rh SCF, 10 ng/ml rh granulocyte, macrophage-colony stimulating factor (GM-CSF), 10 ng/ml rh IL-3, and 3 units/ml rh erythropoietin (Stem Cell Technologies, Vancouver, Canada) for 14 days at 37°C under 5% CO_2 in a humidified atmosphere. The formed colony forming unit-granulocyte, macrophage (CFU-GM), CFU-GEMM, BFU-E, and colony forming unit-erythrocyte (CFU-E) colonies were scored using a light microscope and considered as colony number per 1×10^4 cells. To compare frequencies in megakaryocytic colonies, 1.25×10^3 $CD34^+$-enriched cells were cultured in various culture conditions and were taken before culture initiation or after 4, 7, 10, and 14 days. The cells were incubated in serum-free Iscove's MEM containing 1.1 mg/ml collagen, 1% BSA, 10 μg/ml rh insulin, 2 mM L-glutamine, 10^{-4} M 2-mercaptoethanol, 50 ng/ml rh TPO, 10 ng/ml rh IL-6, and 10 ng/ml rh IL-3 (Stem Cell Technologies). After 12 days of culture, the formed CFU-Mk colonies were identified using anti-human glycoprotein (GP)IIb/IIIa according to manufacture's instruction. The formed CFU-Mk colonies were scored and regarded as colony number per 1×10^4 cells. The expansion folds were calculated by comparing the colony forming cells (CFCs) obtained after indicated culture days with the CFCs before culture initiation.

Cobblestone-like areas

Cell feeder layer-adherent cells were washed five times with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS) supplemented by 2 mM EDTA and then were microscopically examined. Flow cytometric analysis of the cell feeder layer-adherent cells was conducted after 0.025% trypsin/EDTA isolation at 7 culture days. Isolated 2×10^5 cells were incubated with fluorescein isothiocyanate (FITC)-conjugated CD45 and phycoerythrin (PE)-conjugated CD44 (BD Bioscience) at 4°C for 30 min. After washing with PBS, cells were fixed with 1% paraformaldehyde (Sigma-Aldrich). FACSCaliber (BD Bioscience) was used for analysis. The expression of megakaryocyte/platelet precursor-related antigen in the cell feeder layer-adherent cells was examined by immunocytochemistry. For a brief period, the cell feeder layer was sequentially incubated with GPIIb/IIIa antibody for 30 min and with biotin-conjugated secondary antibody for 30 min at room temperature. Then the cell feeder layer was made to react with avidin-conjugated ALP and ALP substrate, and then counterstained with Evan's blue solution (Stem Cell Technologies).

FACS analysis of ex vivo expanded HSCs

Flow cytometric analyses of ex vivo expanded HSCs in culture conditions B, C, and D were conducted at 7 and 14 culture days to evaluate preferred maintenance of $CD33^-CD34^+$ and $CD38^-CD34^+$ cells and at 14 culture days for $CD13^+CD34^+$ cells. Then 2×10^5 cells were incubated with FITC-conjugated CD34 and PE-conjugated CD33, CD38, and CD13 (BD Bioscience) at 4°C for 30 min. After washing with PBS, the cells were fixed with 1% paraformaldehyde (Sigma-Aldrich). FACSCaliber was used for analysis and at least 10,000 events were collected for each analysis.

RT-PCR and immunoblotting

To identify expression profile of hematopoietic cytokines from UCB- or BM-derived MSCs as a cell feeder layer, RNA was extracted using Trizol reagent (Gibco). Each 0.5 μg RNA was reverse transcribed with oligo-dT primer and reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for cDNA synthesis and then was subjected to polymerase chain reaction (PCR) with *Taq* polymerase (Invitrogen). Each cytokine-specific primers used in the present study are listed in Table 1. RT-PCR products were analyzed in 1.5% agarose gel under UV light. For immunoblotting analysis of interleukin 1β (IL-1β) and GM-CSF, each 30 μg of protein from UCB- or BM-derived MSCs was run on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred to nitrocellulose (NC) membrane. Immunoblotting analysis using each specific primary antibody (Abcam Ltd, Cambridge, UK) was conducted as general laboratory protocols.

Table 1 Primer sequences specific for hematopoietic cytokines and receptors

Primer	Sequences	bp
GAPDH	5'–ACCACAGTCCATGCCATCAC–3'	452bp
	5'-TCCACCACCCTGTTGCTGTA-3'	
IL-1alpha	5'-GTCTCTGAGTATCTCTGAAACCTC-3'	513bp
	5'-AAGAGGAGGTTGGTCTCACTAC-3'	
IL-beta	5'-AGATGATAAGCCCACTCTACAGC-3'	500bp
	5'-CTTTAAGTGAGTAGGAGAGGTGAG-3'	
IL-2	5'-CTCACATTAACCTCAACTCCTG-3'	442bp
	5'-CTACAATGGTTGCTGTCTCATC-3'	
IL-3	5'-AAGGACCAGAACAAGACAGAGT-3'	443bp
	5'-CAGATAGAACGTCAGTTTCCTC-3'	
IL-5	5'-TTGAGTTTGCTAGCTCTTGGAG-3'	442bp
	5'-GCAGTAAAATGTCCTTCTCCTC-3'	
IL-6	5'-GGTATACCTAGAGTACCTCCAGAA-3'	648bp
	5'-AGTCTACTCCCTGCTGTCTGAATA-3'	
IL-7	5'-GAGTGTTCTAATGGTCAGCATC-3'	533bp
	5'-GGTGCATTCAGTAACTTCTAGG-3'	
IL-11	5'-GTCATACATATCCACTTGAGGG-3'	574bp
	5'-GTACTGTTGATCACAGGGTGACT-3'	
LIF	5'-CTAGGTGAGATATAGGGGTGTG-3'	608bp
	5'-CTCTATCTCTTCCTTCCCTAGTG-3'	
FL	5'-TGG AGC CCA ACA ACC TAT CTC-3'	333bp
	5'-TGG TTT ACA CGG AAA GTC GGG-3'	
TPO	5'-GTGGACTTTAGCTTGGGAGAAT-3'	570bp
	5'-CAAGAGTTCGTGTATCCTGTTC-3'	
SCF	5'-GAGACAGCCAAGTCTTACAAGG-3'	437bp
	5'-ATGGTACATGCAGTCTGAGACAC-3'	
C-kit	5'-CTCTTCTCAACCATCTGTGAGT-3'	667bp
	5'-CGTTGAGTACACAGAACTAGACAC-3'	
IL-6 receptor	5'-CCTGGAACTCATCTTTCTACAG-3	394bp
	5'-TATCTGGGGAAGAAGTAGTCTG-3'	
GM-CSF	5'-CCTGAACCTGAGTAGAGACACT-3'	382bp
	5'-CCATCCTGAGTTTCTAGCTCTT-3'	
M-CSF	5'-CAGTGTAGAGGGAATTCTAAGACC-3'	648bp
	5'-GACTGAAAAGACAGTCAGAAGG-3'	

Statistical analysis

Results of experimental points are reported as mean±standard deviation (SD). Paired *t* test was used for comparison of the significance of different experimental groups. A 95% confidence interval was chosen and $P<0.05$ was considered statistically significant by ANOVA analysis.

Results

Multilineage differentiation of UCB-derived MSCs

To verify differentiation potential of UCB-derived MSCs, cells were culture expanded and stimulated under target tissue-specific conditions, and morphologically or immunohistochemically analyzed (Fig. 1). The MSCs showed a bipolar fibroblast-like shape and there were no significant differences after 21 culture days (Fig. 1a) and at advanced passage 15 (Fig. 1b). Under stimulation by dexamethasone, β-glycerol phosphate, ascorbic acid, and 10% FBS, the cultured MSCs showed an osteogenic differentiation with increased ALP expression (Fig. 1c) and with formation of extracellular calcium matrix (Fig. 1d) at 4 weeks of differentiation. To investigate differentiation potential into chondrocyte, the cultured cells were centrifuged to form pellet micromass, and cultured in serum-free medium containing transforming growth factor-β3 and bone morphogenetic protein-6. At 6 weeks of differentiation, accumulations of sulfated proteoglycans and extensive matrix were visualized by safranin O staining and collagen type II immunostaining, respectively (Fig. 1e, f). Adipogenic differentiation potential was tested after stimulation with 3-isobutyl-1-methyl xanthine, dexamethasone, insulin, and indomethacin in the presence of 10% FBS. Adipogenic differentiation was apparent by an accumulation of lipid-rich vacuoles within cells visualized by staining with Oil Red O (Fig. 1g). These results clearly demonstrated that the used UCB-derived MSCs for the present ex vivo expansion study possessed a multilineage differentiation potential.

Ex vivo expansion of total and $CD34^+$ cells over UCB-derived MSCs

To answer whether or not UCB-derived MSCs can support ex vivo expansion of total and $CD34^+$ cells, we cultured the isolated $CD34^+$-enriched cells in various culture conditions (see Materials and methods for classification) and also compared the expansion capacity of UCB-derived MSCs with that of BM-derived MSCs. For 14 culture days with initial 1.2×10^5 $CD34^+$-enriched cells, using recombinant cytokines regardless of a cell feeder layer in culture conditions B, D, and F significantly increased the total cell number up to 300-fold when compared with culture conditions A, C, and E (Fig. 2j). For better evaluation of culture conditions without recombinant cytokines (culture conditions A, C, and E), Fig. 2k, having a different range in

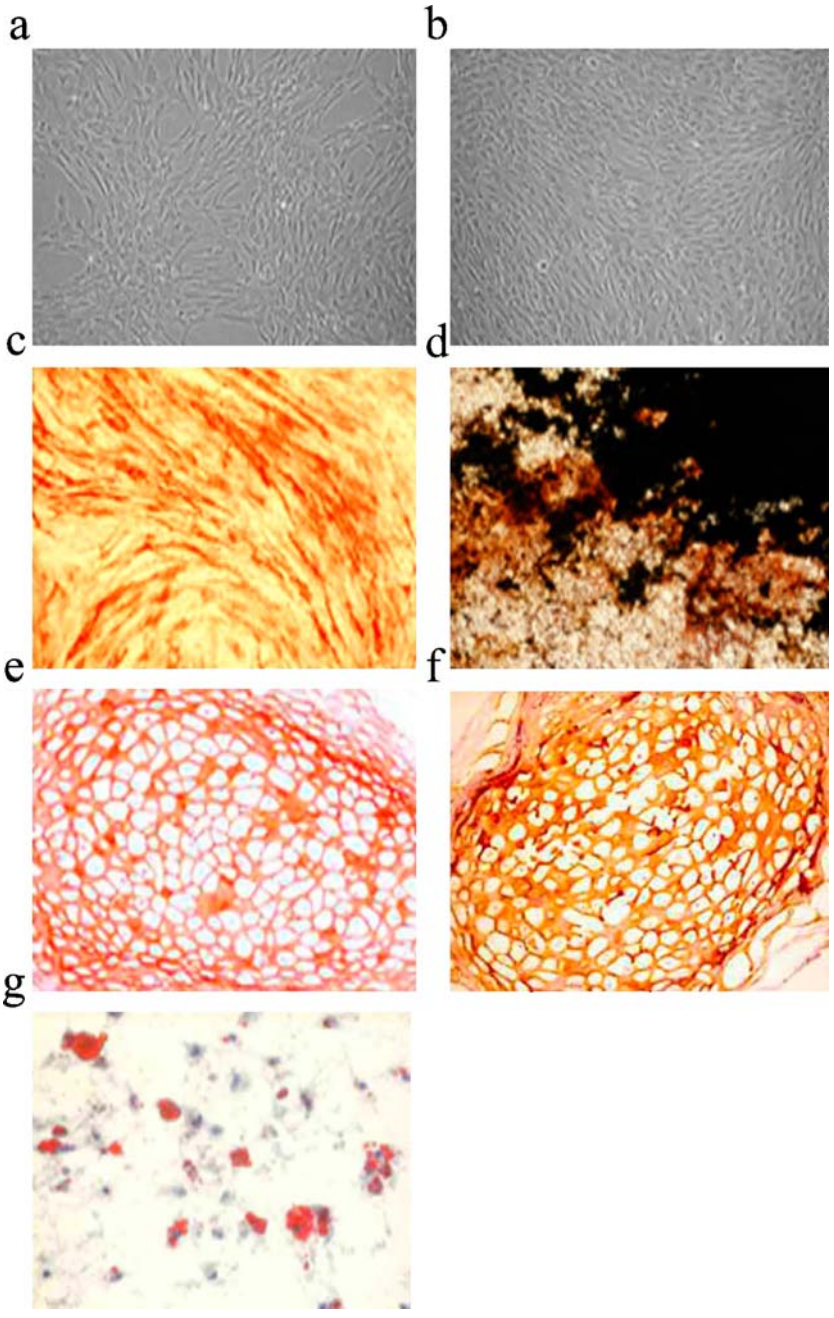

Fig. 1 Morphology and differentiation characteristics of UCB-derived MSCs. Morphology of UCB-derived MSCs after 21 culture days (**a**) and advanced passage 15 (**b**) (magnification 200×). Osteogenic differentiation of the MSCs was proven by the expression of ALP (*red color*) (**c**) and by the formed extracellular calcium matrix in von Kossa's staining (*black color*) (**d**) after 4 weeks of differentiation (magnification 100×). Chondrogenic differentiation of the MSCs was proven by the accumulation of sulfated proteoglycan in safranin O staining (*red color*) (**e**) and type II collagen immunostaining (*orange color*) (**f**) after 6 weeks of differentiation (magnification 200×). Adipogenic differentiation of the MSCs was proven by the accumulation of lipid-rich vacuoles within cells in Oil Red O staining (*red color*) (**g**) after 4 weeks differentiation (magnification 200×)

y-axis, was graphed from Fig. 2j. Despite the tremendous fold increase in total cell number by using recombinant cytokines, it should not be missed that cell feeder layers composed of UCB- or BM-derived MSCs without recombinant cytokines in culture conditions C and E also significantly increased the total cell numbers to 15.2 (± 2.5)$\times 10^5$ (n=6) and 8.6(± 0.8)$\times 10^5$ (n=6) cells when compared with culture condition A (Fig. 2a, d, g: culture condition A; b, e, h: culture condition E; c, f, i: culture condition C; k). For 14 culture days, $CD34^+$ cell numbers counted after MACS system with CD34 antibody were increased up to 2.25- (n=2) and 2.0-fold (n=2) in culture conditions C and E, respectively. Also, the fold increases of $CD34^+$ cells in culture conditions B, D, and F were higher than that in culture conditions C and E (Fig. 2l). Without a cell feeder layer and recombinant cytokines in culture condition A, total and $CD34^+$ cells continuously decreased with culture days (n=6) (Fig. 2a: at 0 day; d: at 7 days; g: at 14 days; j, k, l). It is interesting to note that the huge effect of recombinant cytokines on total cell number regardless of a cell feeder layer was not observed in the fold increase of $CD34^+$ cells (Fig. 2).

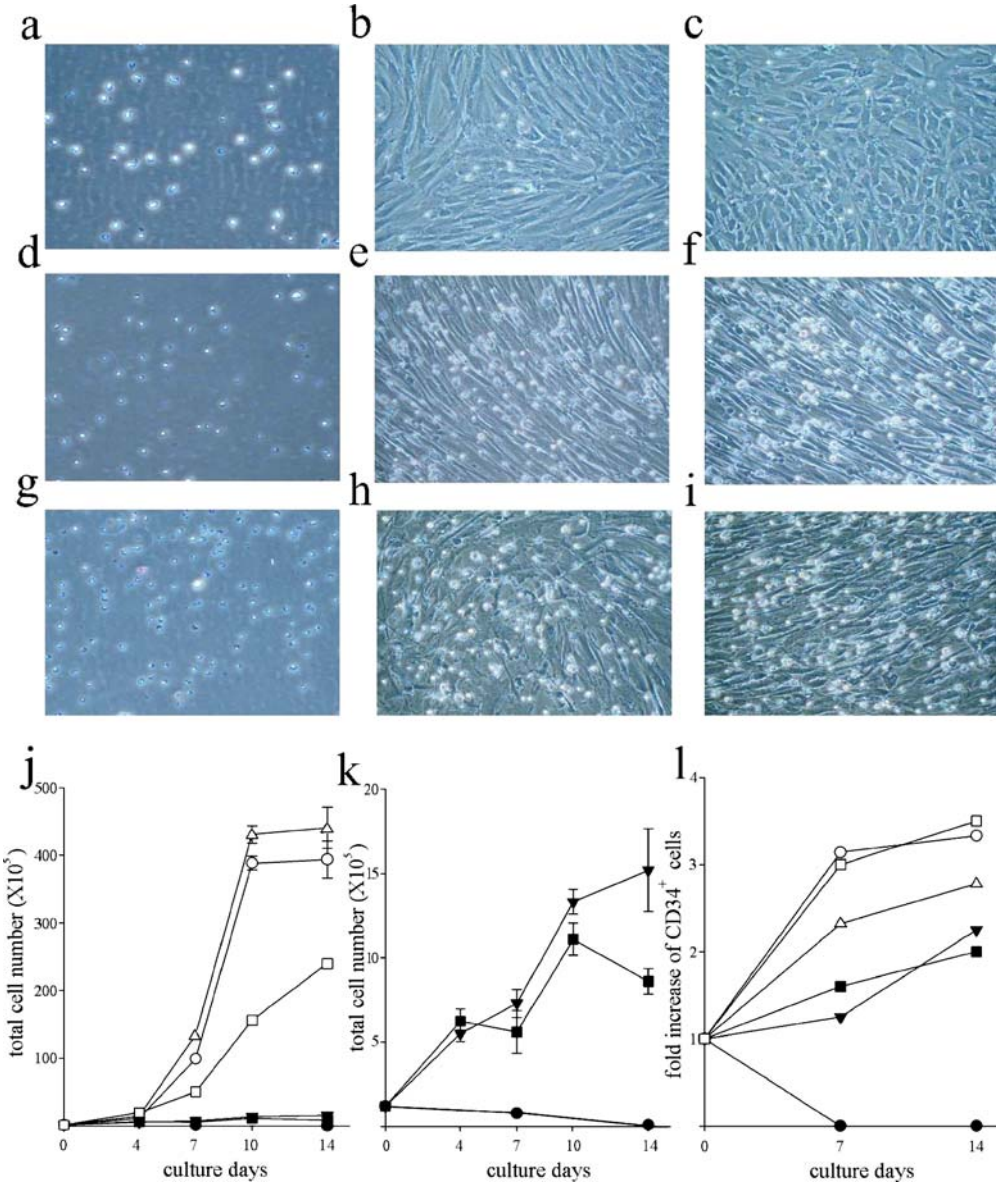

Fig. 2 Ex vivo expansion of total and CD34$^+$ cells over UCB-derived MSCs. After 0 (**a**, **b**, and **c**), 7 (**d**, **e**, and **f**) and 14 (**g**, **h**, and **i**) culture days, total cells were examined by an inverted microscopy with 200× magnification. Cells in culture conditions A (**a**, **d**, and **g**), E (**b**, **e**, and **h**), and C (**c**, **f**, and **i**) were visualized and counted after trypan blue staining (see Materials and methods for classification). The total cells were counted and presented as mean values±SD (**j** and **k**) (culture conditions B, D, and F: n=3, culture conditions A, C, and E: n=6) but CD34$^+$ cells obtained by MACS system with CD34 antibody were counted and presented as mean values (**l**) (n=2) (•: culture condition A, ○: culture condition B, ▾: culture condition C, △: culture condition D, ▪: culture condition E, □: culture condition F). For better evaluation of culture conditions without recombinant cytokines (culture conditions A, C, and E), subpanel **k**, having a different range in the y-axis, was graphed from subpanel **j**

Ex vivo expansion of committed HPCs over UCB-derived MSCs

To further confirm intrinsic ex vivo expansion capacity of UCB-derived MSCs in the absence or presence of recombinant cytokines, we performed various kinds of CFC assays before culture initiation or after 4, 7, 10, and 14 culture days. CD34$^+$-enriched cells generated 2.27× 10^3±69.3 CFU-GM colonies per 10^4 cells before culture initiation (Fig. 3a). In culture condition A, CFU-GM colonies dramatically decreased with culture days and showed no CFU-GM colony at 10 and 14 culture days (Fig. 3a). It is interesting to note that CFU-GM colony number in culture condition C significantly increased to 7.87×10^3±256.2 colonies per 10^4 cells at 7 culture days and then gradually decreased with prolonged culture days (n=4) (Fig. 3a). The corresponding values in culture conditions B and D were 1.60×10^3±187.4 and 2.20× 10^3±149.4 colonies per 10^4 cells, respectively (Fig. 3a). It should be noted that the huge effect of recombinant cytokines on total cell number was completely useless for CFU-GM (Figs. 2j and 3a). As a more primitive progenitor cells, the frequency of CFU-GEMM was investigated. The 46.6± 14.9 CFU-GEMM colonies per 10^4 cells before culture initiation did not completely grow in culture condition A even at 4 culture days. But, CFU-GEMM colony number in culture condition C significantly increased to 458.9±47.0 colonies per 10^4 cells at 7 culture days and then gradually decreased with prolonged culture days (n=4) (Fig. 3b). The corresponding values in culture conditions B and D were 26.6± 13.3 and 16.6±14.5 colonies per 10^4 cells, respectively (Fig. 3b). The 379.1±66.1 BFU-E colonies per 10^4 cells before culture initiation decreased to 0 in culture condition A at 7 culture days. But, it increased to 1.38× 10^3±124.5 colonies per 10^4 cells and remained at 748.1±33.1 and 103.1± 5.8 colonies per 10^4 cells at 10 and 14 culture days in culture condition C, respectively (Fig. 3c). The corresponding values in culture conditions B and D were 69.8± 19.7 and 93.1±18.8 colonies per 10^4 cells at 7 culture days,

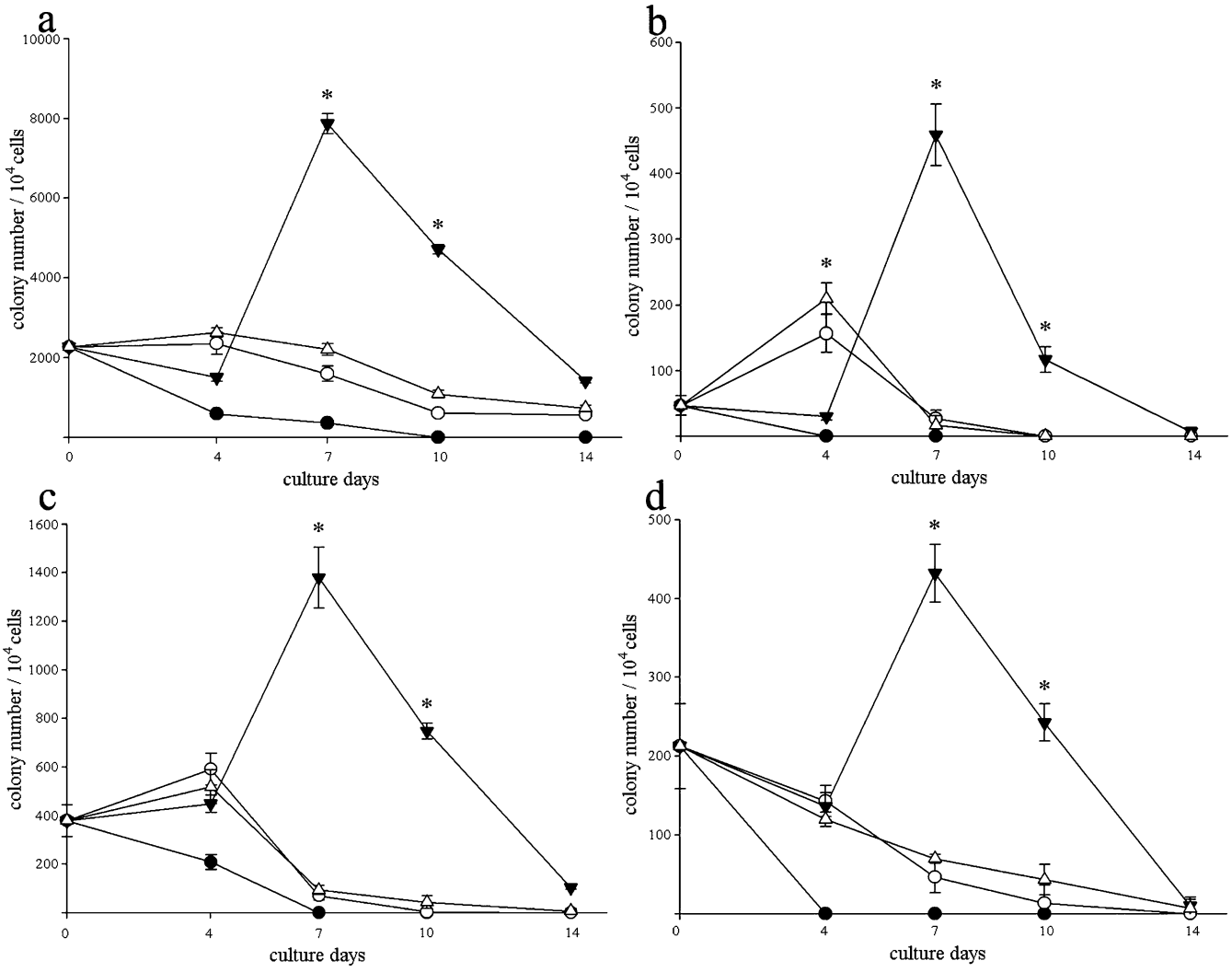

Fig. 3 Ex vivo expansion of committed HPCs over UCB-derived MSCs. Various CFC colonies were evaluated in culture conditions A (•, n=4), B (○, n=4), C (▾, n=4), and D (△, n=4) before culture initiation or after 4, 7, 10, and 14 culture days. To count formed CFC colonies, taken cells were maintained in 35 mm tissue culture dishes supplemented by methylcellulose medium containing several cytokines (Stem Cell Technologies) for 14 days at 37°C under 5% CO_2 in a humidified atmosphere. The counted CFU-GM (**a**), CFU-GEMM (**b**), BFU-E (**c**), and CFU-E (**d**) colony numbers per 10^4 cells were presented as mean values±SD (n=4). The * indicates statistically significant increase vs initial CFC colonies examined before culture initiation at each culture day (P<0.05, ANOVA analysis)

respectively (n=4) (Fig. 3c). The 212.8±54.2 CFU-E colonies per 10^4 cells before culture initiation rapidly decreased to 0 in culture condition A at 4 culture days. However, it increased to 432.3±36.4 colonies per 10^4 cells in culture condition C at 7 culture days (Fig. 3d). The corresponding values in culture conditions B and D were 46.6±20.0 and 69.8±5.8 colonies per 10^4 cells at 7 culture days, respectively (n=4) (Fig. 3d). Unexpectedly, frequencies of all committed HPCs in culture condition C reached to maximal levels at 7 culture days through the lag period around 4 culture days and decreased to basal level with prolonged culture days.

Ex vivo expansion of CFU-Mk over UCB-derived MSCs

Ex vivo expanded megakaryocyte progenitor cells in various culture conditions supplemented with or without UCB-derived MSCs as a cell feeder layer and recombinant cytokines were compared from 0 to 14 culture days. The taken cells from before culture initiation or after 4, 7, 10, and 14 culture days were assayed as a CFU-Mk colony number in a collagen-based medium. CFU-Mk colonies were stained with red color but non-CFU-Mk colonies were counter stained with blue color (Fig. 4a–h). The 80.0±26.9 colonies per 10^4 cells before culture initiation rapidly decreased to near 0 colony in culture condition A at 7 culture days

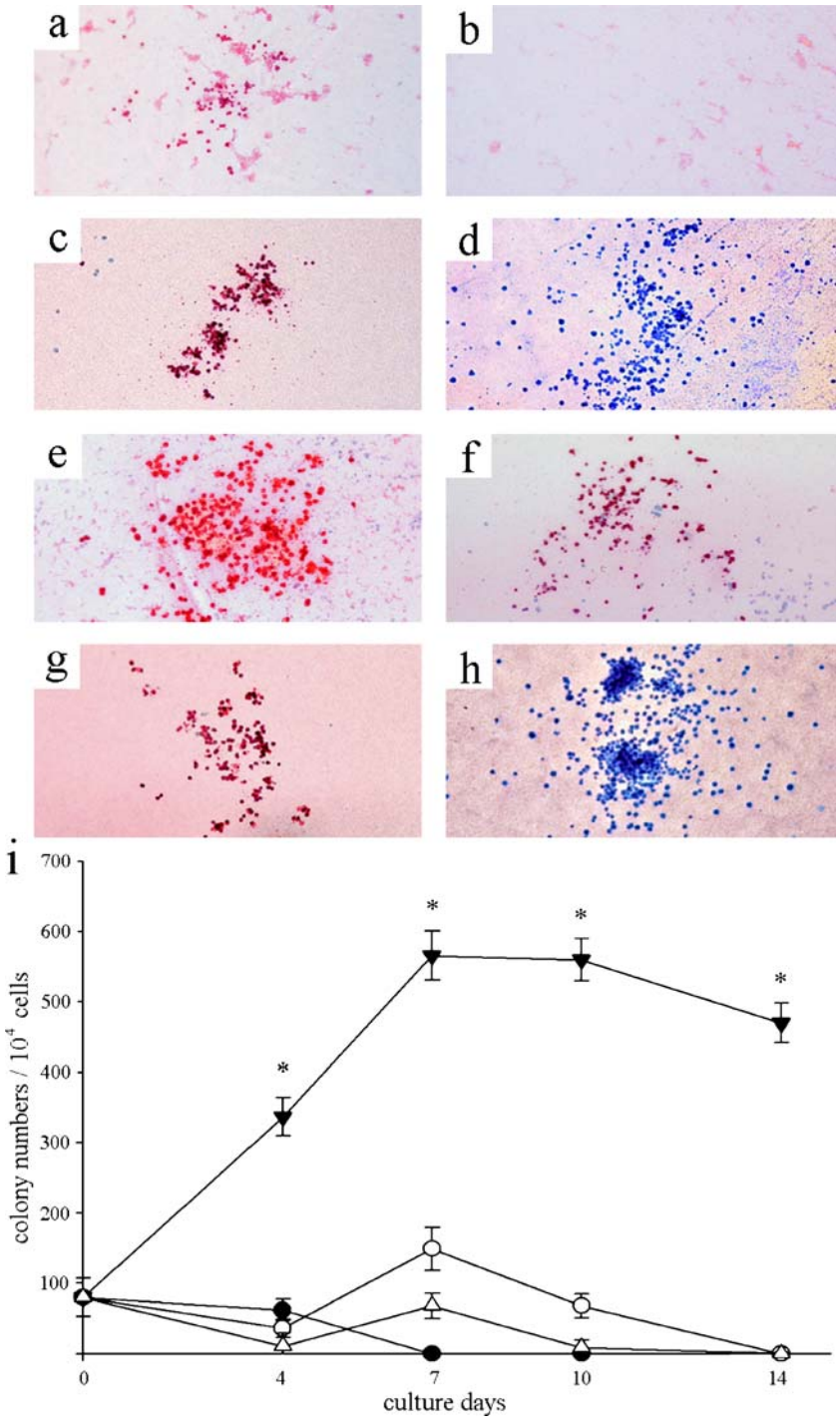

Fig. 4 Ex vivo expansion of CFU-Mk over UCB-derived MSCs. CFU-Mk colonies were evaluated in culture conditions A (**a**: 7 days, **b**: 14 days) (•, *n*=4), B (**c**: 7 days, **d**: 14 days) (○, *n*=4), C (**e**: 7 days, **f**: 14 days) (▾, *n*=4), and D (**g**: 7 days, **h**: 14 days) (△, *n*=4) before culture initiation or after 4, 7, 10, and 14 culture days. The results were presented as colony numbers per 10^4 cells in a graph (**i**). To count formed CFC colonies, taken cells were incubated in serum-free Iscove's MEM containing several cytokines (Stem Cell Technologies) for 12 days at 37°C under 5% CO_2 in a humidified atmosphere. The formed CFU-Mk colonies were identified using anti-human GPIIb/IIIa according to manufacture's instruction (*red color*). Magnification 200×. The * indicates statistically significant increase vs initial CFC colonies examined before culture initiation at each culture day (P<0.05, ANOVA analysis)

(Fig. 4a: at 7 days, b: at 14 days, i). But, the CFU-Mk colony number in culture condition C significantly increased to 566.0±35.5 colonies per 10^4 cells at 7 culture days and then was relatively well maintained up to 14 culture days (*n*=4) (Fig. 4e: at 7 days, f: at 14 days, i). The corresponding values in culture conditions B and D were 149.0±30.5 and 68.0±17.4 colonies per 10^4 cells, respectively (Fig. 4).

Cell growth in cobblestone-like areas over UCB-derived MSCs

During experiments investigating the intrinsic ex vivo expansion capacity of UCB-derived MSCs, we found that the cell feeder layer composed of UCB-derived MSCs physically and functionally interacted with the ex vivo expanding HSCs and formed previously reported cobble-

stone-like areas without recombinant cytokines (culture condition C) (Fig. 5a). The interaction was not easily disrupted by repeated washing with Ca^{2+}, Mg^{2+}-free PBS supplemented by 2 mM EDTA. Microscopic examination showed that round-shaped $CD34^{+}$-enriched cells were evenly embedded in and associated with bipolar fibroblast-shaped UCB-derived MSCs (Fig. 5a). Expectedly, when a fresh culture media was added after removing suspended cells from the culture condition C, new cells were continually proliferated from the cell feeder layer-adherent cells in repeated trials (data not shown). To characterize the cell feeder layer-adherent cells, these cells were isolated by 0.025% trypsin/EDTA solution and were analyzed with flow cytometry after labeling with FITC-conjugated CD45 and PE-conjugated CD44 antibodies (Fig. 5b, c). The ratio of $CD44^{+}CD45^{+}$ HSCs in the cell feeder layer-adherent cells was 28.8% in FACS analysis (Fig. 5b, c). In addition, we incubated the cell feeder layer-adherent cells with GPIIb/IIIa antibody at 7 and 14 culture days to seek a possible reason why CFU-Mk colonies were better maintained from 7 to 14 culture days (Fig. 4) than other committed HPCs (Fig. 3). It is interesting to note that the presence of megakaryocyte/platelet precursor cells in the cell feeder layer-adherent cells at 14 culture days was significantly higher than that at 7 culture days, suggesting a sustained ex vivo expansion capacity especially for the megakaryocyte/platelet precursor cells (Fig. 5d: at 7 days, e: at 14 days, f). Using CFC analysis, other committed HPCs in the cell feeder layer-adherent cells showed similar expansion kinetics observed in the suspended cells showing plateaus at 7 culture days and decrease with prolonged culture days (Fig. 3) (data not shown).

Preferred maintenance of $CD33^{-}CD34^{+}$ cells over UCB-derived MSCs

To further confirm the identified beneficial effect on ex vivo expansion capacity especially for primitive progenitor cells in culture condition C, we analyzed $CD33^{-}CD34^{+}$, $CD38^{-}CD34^{+}$, and $CD13^{+}CD34^{+}$ cells at 7 and 14 culture days (Fig. 6a–f: CD33/CD34, g–l: CD38/CD34, m–o:

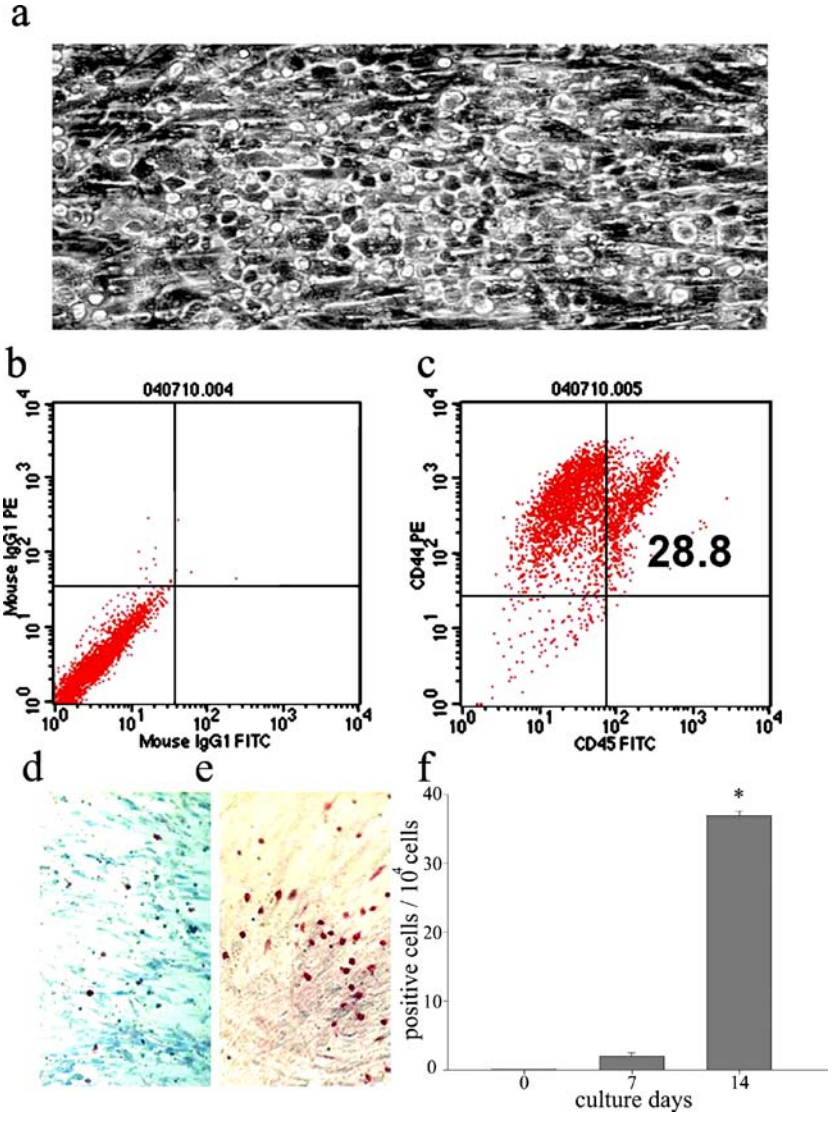

Fig. 5 Characterization of cobblestone-like areas and cell feeder layer-adherent cells over UCB-derived MSCs. Cobblestone-like areas found in culture condition C (**a**) (magnification 400×). Round shaped HSCs physically interact with bipolar fibroblast-shaped MSCs. Isolated, the cell feeder layer-adherent cells were conducted by flow cytometric analysis after labeling with FITC-conjugated CD45 and PE-conjugated CD44 antibodies (**b**, **c**). The ratio of $CD44^{+}CD45^{+}$ HSCs in the cell feeder layer-adherent cells was 28.8% in FACS analysis. The cell feeder layer-adherent cells were incubated with GPIIb/IIIa antibody at 7 (**d**) and 14 (**e**) culture days to compare frequencies of megakaryocyte/platelet precursor cells (magnification 200×). The positive cell number per 10^4 cells was presented as a *bar graph* (n=3) (**f**). The * indicates statistically significant increase vs at 7 culture days (P<0.05, ANOVA analysis)

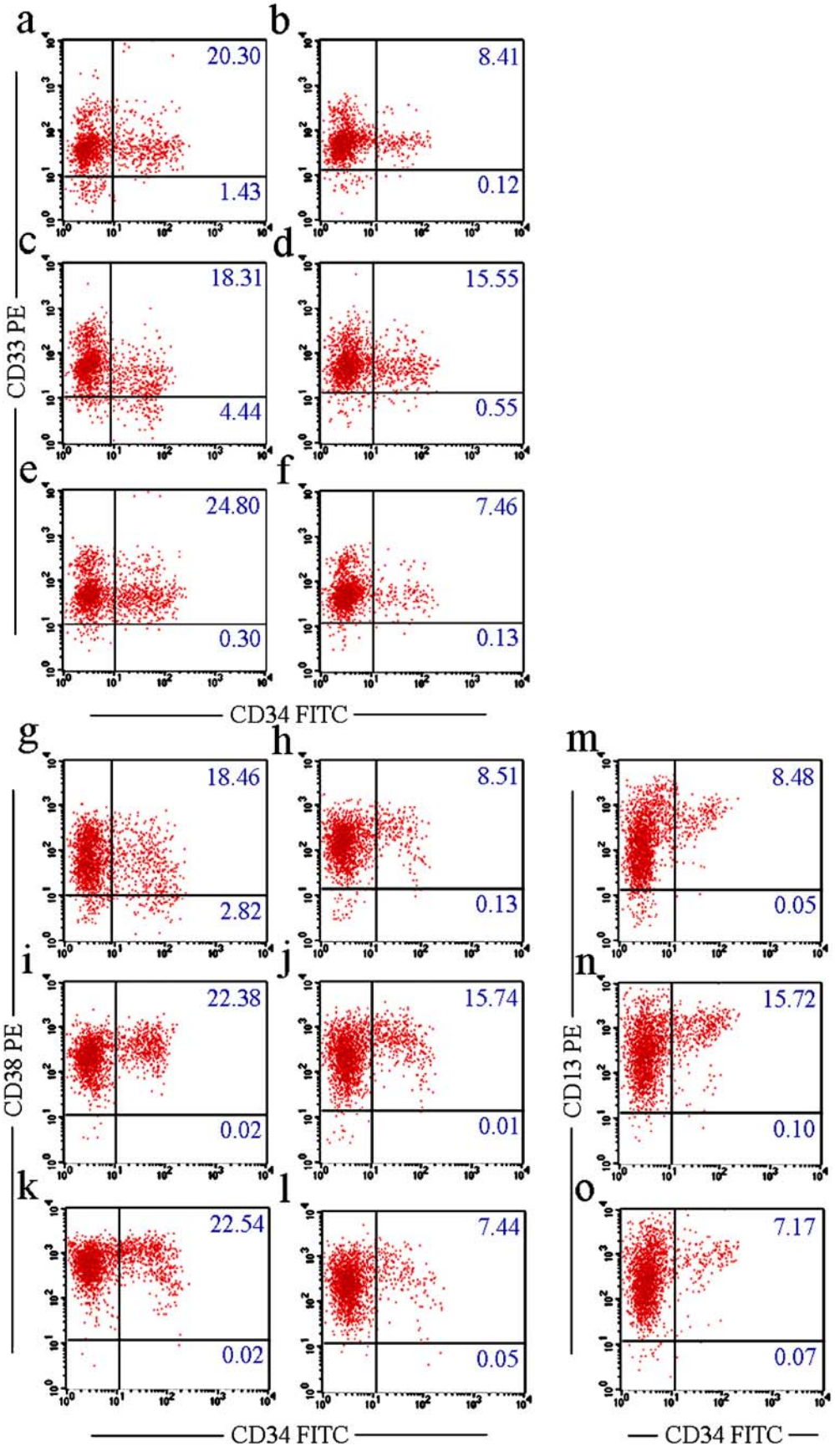

Fig. 6 FACS analysis of ex vivo expanded HSCs over UCB-derived MSCs. Flow cytometric analyses of ex vivo expanded HSCs in culture conditions B (**a** and **b** for CD33/CD34, **g** and **h** for CD38/CD34, **m** for CD13/CD34), C (**c** and **d** for CD33/CD34, **i** and **j** for CD38/CD34, **n** for CD13/CD34), and D (**e** and **f** for CD33/CD34, **k** and **l** for CD38/CD34, **o** for CD13/CD34) were conducted. Then 2×10^5 cells were incubated with FITC-conjugated CD34 and PE-conjugated CD33, CD38, or CD13 at 4°C for 30 min. After washing with PBS, cells were fixed with 1% paraformaldehyde (Sigma-Aldrich). FACSCaliber was used for analysis and at least 10,000 events were collected and analyzed. The ratio of $CD33^{-}CD34^{+}$, $CD38^{+}CD34^{+}$, and $CD13^{+}$ $CD34^{+}$ cells were better maintained in culture condition C than in other culture conditions when those were examined at 7 (**a**, **c**, **e**, **g**, **i**, and **k**) and 14 (**b**, **d**, **f**, **h**, **j**, **l**, **m**, **n**, and **o**) culture days for CD33/CD34 and CD38/CD34 and at 14 culture days for CD13/CD34, respectively

CD13/CD34). The used $CD34^+$-enriched cells showed 1.21% $CD33^-CD34^+$, 93.86% $CD33^+CD34^+$, 0.73% $CD38^-CD34^+$, 93.28% $CD38^+CD34^+$, and 94.41% $CD13^+CD34^+$ cells before culture initiation (data not shown). $CD33^-CD34^+$ cells observed in culture condition C were highest as 4.44% and 0.55% at 7 and 14 culture days, respectively (Fig. 6c, d). Although $CD38^-CD34^+$ cells were not preferred, $CD38^+CD34^+$ cells were better maintained up to 14 culture days as 15.74% in culture condition C (Fig. 6i, j) than in culture conditions B (Fig. 6g, h) and D (Fig. 6k, l). The ratio of $CD13^+CD34^+$ cells was also highest as 15.72% in culture condition C at 14 culture days (Fig. 6m–o).

Expression of hematopoietic cytokines from UCB-derived MSCs as a cell feeder layer

Then we investigated expression profile of hematopoietic cytokines from UCB-derived MSCs by RT-PCR and immunoblotting analysis (Fig. 7). Also, the expression profile was compared with that of BM-derived MSCs. The specific RT-PCR products generated by cytokine-specific PCR primers (Table 1) were separated in 1.5% agarose gel and visualized by EtBr staining. As in Fig. 7a, b, the UCB- or BM-derived MSCs both expressed IL-1α, IL-6, IL-7, leukemia inhibitory factor (LIF), FL, TPO, SCF, C-kit, and macrophage colony stimulating factor (M-CSF) in common. It is interesting to note that IL-1β and GM-CSF were only expressed in UCB-derived MSCs by RT-PCR. The UCB-derived MSCs specific expression of IL-1β and GM-CSF was also confirmed by immunoblotting analysis (Fig. 7c). These results suggested that UCB-derived MSCs expressed various intrinsic hematopoietic cytokines supporting ex vivo expansion of UCB-derived HSCs and HPCs. Even though global profiles in hematopoietic cytokines were similar between UCB- and BM-derived MSCs, UCB-derived MSCs could possess different roles or activities through cytokines unique for UCB-derived MSCs such as IL-β and GM-CSF.

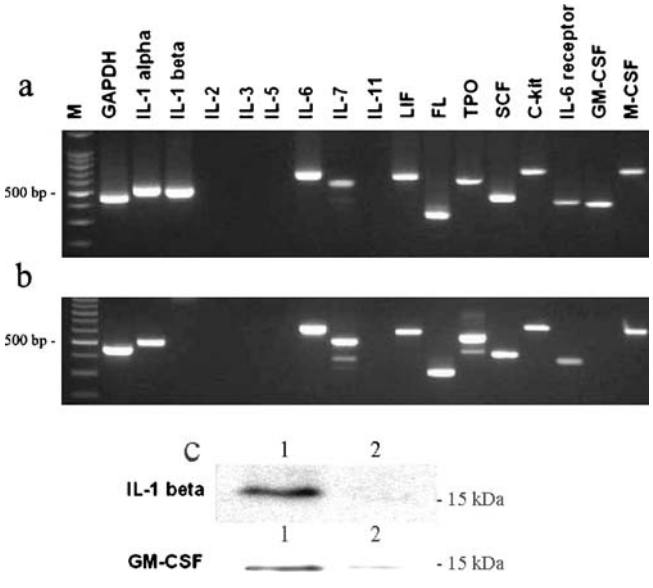

Fig. 7 Expression profile of hematopoietic cytokines from UCB-derived MSCs. RT-PCR was performed to identify hematopoietic cytokine profiles in UCB-derived MSCs (**a**) and BM-derived MSCs (**b**) with each cytokine-specific PCR primers (Table 1). RT-PCR products obtained from each optimized PCR conditions were run on 1.5% agarose gel and visualized by EtBr staining. All RT-PCR products migrated to the expected sizes. IL-1β and GM-CSF were only expressed in UCB-derived MSCs. Immunoblotting for UCB-derived MSCs specific expression of IL-1β and GM-CSF was conducted with each 30 μg of protein on 15% SDS-PAGE. After electric transfer to NC membrane, IL-1β (*upper blot*) and GM-CSF (*lower blot*) specific antibodies were used to detect each cytokines from UCB- (*lane 1*) or BM-derived (*lane 2*) MSCs (**c**)

Discussion

Among possible clinical and biological usages of UCB-derived MSCs, ex vivo expansion of transplantable HSCs derived from human UCB is a very promising approach for different clinical applications, ranging from rescue after myeloablative therapy to other graft engineering techniques [25, 26]. Because almost all previous studies focused on synergic effects for maximal expansion capacities by using a combined usage of recombinant cytokines over a cell feeder layer [22, 23], intrinsic ex vivo expansion capacity, underlying mechanisms, and clinical implications of UCB-derived MSCs were not fully understood. In this report, we present clear data supporting ex vivo expansion capacity of UCB-derived MSCs for allogeneic HSCs and HPCs when used as a cell feeder layer without recombinant cytokines and underlying phenomena explaining the observed expansions. We carefully tested and quantified ex vivo expansion capacity of UCB-derived MSCs in the absence or presence of recombinant cytokines such as SCF, IL-6, FL, and TPO and tried to compare the capacity with that of BM-derived MSCs. Most of all, we used highly purified UCB-derived MSCs capable of multilineage differentiation. The used UCB-derived MSCs were functionally differentiated into various mesoderm-associated cell types as in our previous report [18] and in the present study (Fig. 1).

As a cell feeder layer, UCB-derived MSCs expressed various hematopoietic cytokines also seen in BM-derived MSCs, but they expressed two unique cytokines considered characteristic for UCB-derived MSCs, e.g., IL-1β and GM-CSF (Fig. 7). Our result showing unique expression of GM-CSF in UCB-derived MSCs but not in BM-derived MSCs was comparable to the no expression of GM-CSF for human placental mesenchymal progenitor cells and adult BM-derived MSCs at basal level [22, 27]. Because the GM-CSF reported not to be expressed on BM-derived MSCs appeared to be expressed after a treatment with IL-1α [22, 27] and is required for growth and development of various committed granulocyte and macrophage progenitor cells, the unique and constitutive expression of GM-CSF in UCB-derived MSCs may participate in the reported differences such as higher numbers of committed HPCs in UCB than in BM [2–4]. But, the exact roles of GM-CSF and IL-1β only found in UCB-derived MSCs must be

addressed in future studies. Together with these two cytokines, it should be noted that other hematopoietic cytokines also observed in BM-derived MSCs could be hypothesized as underlying mechanism for the observed ex vivo expansion of UCB-derived HSCs and committed HPCs in the present study.

When purified $CD34^+$-enriched cells were cultured over UCB-derived MSCs as a cell feeder layer in the absence of recombinant cytokines (culture condition C) for 14 culture days, total and $CD34^+$ cells increased about 12.6- and 2.25-fold, respectively (Fig. 2). As expected, the results of the expansion folds in total and $CD34^+$ cells were lower than those in cultures supplemented with a cell feeder layer and recombinant cytokines (culture condition D) in the present study and in previous reports [22, 23]. To examine expected advantages such as reduction in unfavorable differentiation of HSCs into non-$CD34^+$ cells [12] in culture condition C and to evaluate unfavorable effect of recombinant cytokines used in culture condition D, we newly considered a percentage of fold increase in $CD34^+$ cells in the fold increase in total cell number, suggesting how well $CD34^+$ cells were proliferated when compared with total cells. The percentages were calculated and compared with each other from previous and present studies. We found out that the values 14.05% (105.92-fold increases in total cells and 14.89-fold increases in $CD34^+$ cells) for placenta-derived MSCs and 11.43% (65.25-fold increases in total cells and 7.46-fold increases in $CD34^+$ cells) for BM-derived MSCs with recombinant cytokines increased to 21.2% (23.1-fold increases in total cells and 4.9-fold increases in $CD34^+$ cells) and 19.1% (17.8-fold increases in total cells and 3.4-fold increases in $CD34^+$ cells) without recombinant cytokines, respectively [22]. The calculated value of 0.76% (367-fold increases in total cells and 2.78-fold increases in $CD34^+$ cells) in culture condition D was dramatically increased to 17.8% (12.6-fold increases in total cells and 2.25-fold increases in $CD34^+$ cells) in culture condition C (Fig. 2). Although the identified 12.6 and 2.25-folds increases in total and $CD34^+$ cells in the present study were lower than the corresponding values in previous reports using placenta- and BM-derived MSCs, the increase in calculated percentage by the absence of recombinant cytokines was consistent with the previous results, suggesting a restricted differentiation of non-$CD34^+$ cell populations during ex vivo expansion without recombinant cytokines (Fig. 2). Clinical advantages of this restriction in ex vivo expansion of transplantable HSCs without recombinant cytokines should be further investigated in CBT and other applications of UCB-derived MSCs.

We mainly considered how much committed HPCs could be ex vivo expanded and which culture condition is most suitable for ex vivo expansion of committed HPCs using various CFC assays. Chivu et al. [24] recently reported that mouse BM-derived feeder layer induce about 2.2-fold increase of $CD34^+HLA\text{-}DR^-$ cells and about 1.8-fold increase of $CD34^+CD71^-$ cells in the absence of recombinant cytokines. Also, in a previous study without recombinant cytokines, CFC and long-term culture initiating cells were expanded to 11.5- and 2.2-folds for placenta-derived mesenchymal progenitor cells and 7.7- and 1.7-folds for BM-derived mesenchymal progenitor cells, respectively [22]. Our fold increases from 2.03- to 9.85-folds in committed HPCs obtained in culture condition C (CFU-GM: 3.46-fold; CFU-GEMM: 9.85-fold; BFU-E: 3.64-fold; CFU-E: 2.03-fold; and CFU-Mk: 7.08-fold) relatively matched well with the reported fold increases (Fig. 3). Also, present results were relatively similar with the corresponding values (about 8.3-fold increase in CFU-GM and 8.6-fold increase in high proliferate potential colony-forming cells) reported in culture with UCB-derived MSCs using IMDM culture media containing 20% FBS in the absence of recombinant cytokines [23].

In addition to the identification and quantification of ex vivo expansion capacity of UCB-derived MSCs, several valuable findings were also identified in the present study. It is interesting to note that the colony numbers in all tested CFU-GM, CFU-GEMM, BFU-E, CFU-E, and CFU-Mk assays increased and reached to plateaus at 7 culture days and then decreased with the prolonged culture days (Fig. 3) except for CFU-Mk (Fig. 4). Especially at 14 culture days, the CFU-GM, CFU-GEMM, BFU-E, and CFU-E colonies even in culture condition C were lower than the corresponding values before culture initiation. Because total and $CD34^+$ cells continuously increased till 14 culture days, it could be assumed that these committed HPCs, except for CFU-Mk, were induced to differentiate into subset or non-$CD34^+$ lineages by consumption or dilution of some factors stimulating self-renewal activity or inhibiting differentiation of primitive and immature HPCs after 7 culture days. Also, this unexpected expansion kinetics by UCB-derived MSCs suggests that ex vivo expansion capacity should not be simply concluded by a direct comparison of CFC colony number obtained before the culture with the corresponding value obtained at the end of the culture.

During the present studies, we could see an active growth and proliferation of HSCs from cobblestone-like areas previously reported in studies with UCB-derived total cover slip-adherent cell layer [12] and BM-derived MSCs [25]. Although the exact roles of interaction between HSCs and MSCs in the cobblestone-like areas still need to be addressed, it can play important roles in supporting growth, maintenance, differentiation, and possibly self-renewal of immature and committed HPCs. To answer a question "Are there different ex vivo expansion activities or roles for different kinds of committed HPCs in the cobblestone-like areas?", we investigated committed HPCs in the cell feeder layer-adherent cells. Because the CFU-Mk colony number was better maintained than other committed HPCs after 7 culture days till 14 culture days (Figs. 3 and 4), we tried to answer the above question by finding out possible dependency of expansion capacity for CFU-Mk on the cell feeder layer-adherent cells using immunostaining for megakaryocyte/platelet progenitor cells. Unlikely other committed HPCs such as CFU-GM, CFU-GEMM, BFU-E, and CFU-E, the megakaryocyte/platelet progenitor cells in the cell feeder layer-adherent cells were higher at 14 culture days than at 7 culture days by immunostaining using CD41

GPIIb/IIIa antibody (Fig. 5d–f). Other committed HPCs such as CFU-GM, CFU-GEMM, BFU-E, and CFU-E in the same cell feeder layer-adherent cells were significantly higher at 7 culture days than at 14 culture days and there were no remarkable differences in between suspended cells and the cell feeder layer-adherent cells by CFC assays (data not shown). The present data suggest that the expansion capacity was strongly correlated with the cell feeder layer-adherent cells and there can be different roles for growth, proliferation, and differentiation of different committed HPCs. Although prolonged expansion of CFU-Mk colonies till 14 culture days could be further related in the functional expression of CFU-Mk colony stimulating cytokines such as GM-CSF, TPO, IL-6, SCF from the feeder layer (Fig. 7) [27, 28], it remains to be addressed by further studies. The $CD33^{-}CD34^{+}$ cells have been suggested as cells capable of giving rise to CFC in long-term marrow culture but are mainly depleted of progenitor cells that directly form colonies in semisolid media such as $CD38^{+}CD34^{-}$ cells [29]. FACS analysis of ex vivo expanded cells in various culture conditions showed that the culture condition C could better maintain $CD33^{-}CD34^{+}$ and $CD38^{+}CD34^{-}$ cells than other culture conditions up to 14 culture days, suggesting the ex vivo expansion capacity especially for immature and committed progenitor cells (Fig. 6).

The goal of this study was to investigate an intrinsic ex vivo expansion capacity of UCB-derived MSCs as a cell feeder layer for UCB-derived committed HPCs in the absence of recombinant cytokines. In this report, we present a series of data supporting and quantifying an ex vivo expansion capacity of UCB-derived MSCs for transplantable HSCs and committed HPCs derived from UCB. The various hematopoietic cytokines from UCB-derived MSCs as a cell feeder layer as well as cell-to-cell and cell-to-extracellular matrix interactions were hypothesized as underlying mechanisms for this ex vivo expansion.

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Yun Kyung Jang and Dai Hyun Jung contributed equally to this work.

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