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Mesenchymal stem cells from cryopreserved human umbilical cord blood

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Abstract

Umbilical cord blood (UCB) is well known to be a rich source of hematopoietic stem cells with practical and ethical advantages, but the presence of mesenchymal stem cells (MSCs) in UCB has been disputed and it remains to be validated. In this study, we examined the ability of cryopreserved UCB harvests to produce cells with characteristics of MSCs. We were able to obtain homogeneous plastic adherent cells from the mononuclear cell fractions of cryopreserved UCB using our culture conditions. These adherent cell populations exhibited fibroblast-like morphology and typical mesenchymal-like immunophenotypes ($CD73^+$, $CD105^+$, and $CD166^+$, etc.). These cells presented the self-renewal capacity and the mesenchymal cell-lineage potential to form bone, fat, and cartilage. Moreover, they expressed mRNAs of multi-lineage genes including SDF-1, NeuroD, and VEGF-R1, suggesting that the obtained cells had the multi-differentiation capacity as bone marrow-derived MSCs. These results indicate that cryopreserved human UCB fractions can be used as an alternative source of MSCs for experimental and therapeutic applications.
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Keywords: Cryopreservation; Umbilical cord blood; Mesenchymal stem cells

Bone marrow (BM) is a complex tissue containing hematopoietic progenitor cells and a connective-tissue network of stromal cells. Marrow stroma includes a subpopulation of undifferentiated cells that are capable of becoming one of a number of phenotypes, including bone and cartilage [1,2], tendon [3], muscle [4], fat [5], and marrow stromal connective tissue which supports hematopoietic cell differentiation [6,7]. Many studies have defined conditions for isolation, expansion, and in vitro and in vivo differentiation of the stromal cells. These cells are referred to as mesenchymal stem cells (MSCs), since they are known to have capacity of proliferation and differentiation into the mesenchymal lineage. Their mesenchymal differentiation potential is retained even after repeated subcultivation in vitro [8,9]. Though BM has been represented as the main available source of MSCs [10,11], the use of bone marrow-derived cells is not always acceptable owing to the high degree of viral exposure and the significant decrease in the cell number and the proliferative/differentiation capacity along with age. In addition, it requires a painful invasive procedure to obtain a BM sample. Thus, the need to find an alternative MSC source has emerged.

Umbilical cord blood (UCB) is well known to be a rich source of hematopoietic stem cells (HSCs) with practical and ethical advantages, but the issue about the existence of MSCs in UCB has not been clearly resolved. It was reported that fresh UCB-derived mononuclear cells (MNCs) seeded on flasks without supportive stromal layers did not form stroma in long-term culture conditions [12]. Wexler et al. [13] concluded that neither fresh UCB nor peripheral blood with stem cell mobilization contained MSCs. Another study also reported that fresh UCB did not produce mesenchymal progenitor cells [14]. On the contrary, fresh UCB harvests proved to give adherent expanded cells exhibiting

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phenotypes of mesenchymal progenitor cells [15]. In addition, immunophenotypes of clonally expanded cells derived from fresh UCB were similar to those of BM-derived MSCs, strongly expressing SH2, SH3, and SH4 [16]. In spite of these reported evidences, the presence of MSCs in UCB is still in controversy.

As the use of autologous or allogeneic hematopoietic stem cell transplantation in the treatment of various diseases has grown rapidly in recent years, the idea of UCB banking for future use has drawn great interest. More than 100,000 units of UCB have been collected, frozen, and stored world-wide in anticipation of their clinical use. Study on the potential of cryopreserved UCB is of great importance for its future clinical use and we have made an attempt to investigate the capabilities. Here, we report that MNC fractions from cryopreserved UCB contained stem cells with characteristics that resembled those of BM-derived MSCs.

Materials and methods

Samples: collection and cryopreservation. The Institutional Review Board of Ajou University Hospital approved this study and all samples were obtained with informed consent. Forty BM and 47 UCB samples which had undergone cryostorage for 0.1–5 years were used for the study.

Before freezing, UCB cells were separated by the Ficoll–Hypaque (Histopaque-1077; Sigma, MO, USA) density-gradient method and washed with Dulbecco's phosphate-buffered saline (DPBS; HyClone, UT, USA). Separated MNCs were cryopreserved by putting them into standard 1.8-ml cryotubes (Nalge Nunc, IL, USA) at $40–60 \times 10^6$ cells per ml with a final concentration of 10% dimethyl sulfoxide (DMSO; Sigma) in autologous plasma while being maintained at 4°C. These samples were frozen in a controlled rate freezer (Custom Biogenic System, MI, USA) and further stored in liquid nitrogen (−196 °C) until being used for the study. BM cells were processed and cryopreserved by the same method used for UCB cells except that fetal bovine serum (FBS; Gibco-BRL, NY, USA) was used instead of autologous plasma.

Cell culture and maintenance. After thawing, MNCs were used for culture without any further separation step. UCB-derived MNCs were seeded on non-coating T25 culture flasks (Nalge Nunc) at a density of 3×10^5 cells/cm^2 in low glucose Dulbecco's modified Eagle's medium (LG-DMEM; Gibco-BRL) containing 10% FBS, 100 U penicillin (Gibco-BRL), 100 U streptomycin (Gibco-BRL), and 2 mM L-glutamine (Gibco-BRL). The cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2, and the medium was changed every 7 days until the fibroblast-like cells at the base of the flask reached confluence. On reaching confluence, the adherent cells were resuspended with 0.05% trypsin–EDTA (Gibco-BRL) and reseeded at 1×10^5 cells per flask. On the next confluence, the cells were replated at 1:5 dilution under the same culture conditions. BM-derived MNCs were cultured as described by Bruder et al. [8] to obtain MSCs.

Flow cytometry. Antibodies against human antigens CD14, CD31, CD34, CD45, CD73, CD80, and CD86 were purchased from BD Sciences (San Jose, CA, USA). Antibodies against CD105 and CD166 were purchased from Ancell (Bayport, MN, USA). A total of 5×10^5 cells were resuspended in 200 μl PBS and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies for 20 min at room temperature (or for 45 min at 4 °C). The fluorescence intensity of the cells was evaluated by flow cytometry using a flow cytometer (FACScan; BD Sciences) and the data were analyzed with the CELLQUEST software (BD Sciences).

Osteogenic induction. At 50% confluence, the cells were cultured for 14–21 days in LG-DMEM containing 10% FBS, 0.1 μM dexamethasone (Sigma), 10 mM β-glycerolphosphate (Sigma), and 100 μM ascorbate-2-phosphate (Sigma) with medium changes every 3 days. Osteogenic differentiation was confirmed by the expression of alkaline phosphatase.

Adipogenic induction. The cells were cultured further for 14–21 days after confluence in LG-DMEM containing 10% FBS, 1 μM dexamethasone, 0.5 mM isobutyl methylxanthine (Sigma), 100 mM indomethacin (Sigma), and 10 μg/ml insulin (Sigma). Adipogenic differentiation was evaluated by the cellular accumulation of neutral lipid vacuoles that were stained with Oil-red O (Sigma).

Chondrogenic induction: pellet assay. At 80% confluence, the cells were trypsinized with 0.05% trypsin–EDTA and resuspended in LG-DMEM containing 1× insulin–transferrin–selenium (ITS; Gibco-BRL), 1 mM sodium pyruvate (Gibco-BRL), 0.1 μM dexamethasone, 397 μg/ml ascorbate-2-phosphate, and 10 ng/ml transforming growth factor β_1 (R&D System, MN, USA). Viable cells were counted and seeded at a density of 2×10^5 cells per pellet in 15-cm^3 conical tubes. The cells were gently centrifuged to the bottom of the tubes and allowed to form compact cell pellets in a humidified atmosphere at 37 °C with 5% CO_2. After 14–21 days in culture, chondrogenic differentiation was detected by the extracellular accumulation of chondrocyte matrix that was stained with toluidine blue (Sigma).

RNA extraction and RT-PCR analysis. Total RNA was extracted from the undifferentiated (control) and mesenchymally differentiated BM- and UCB-derived MSCs using the TRIzol Reagent (Gibco-BRL). A total of 2 μg RNA was reverse-transcribed with AMV reverse transcriptase XL (TaKaRa Shuzo, Shiga, Japan) for 90 min at 42 °C in the presence of oligo(dT) primer. PCR was performed using *Taq* polymerase (BioQuest, Seoul, Korea). The primer sequences were as follows: osteopontin (330 bp), forward: 5′-CTAGGCATCACCTGT GCCATACC-3′, reverse: 5′-CAGTGACCAGTTCATCAGATTCA TC-3′; col2a1 (399 bp), forward: 5′-CCAGGACCAAAGGGACAGA AAG-3′, reverse: 5′-TTCACCAGGTTCACCAGGATTG-3′; PP ARγ2 (352 bp), forward: 5′-GCTGTTATGGGTGAAACTCTG-3′, reverse: 5′-AT-AAGGTGGAGATGCAGGCTC-3′; stromal-derived factor-1 (SDF-1; 226 bp), forward: 5′-CGACGGGAAGCCCGTCAG C-3′, reverse: 5′-TCACATCTTGAACCTCTTG-3′; NeuroD (848 bp), forward: 5′-TGAC-CAAATCGTACAGCGAGAG-3′, reverse: 5′-AG AAGTTGCCATTGATGCTGAGCG -3′; vascular endothelial growth factor receptor-1 (VEGF-R1; 183 bp), forward: 5′-GGTCTTACGG AGTATTGCTG-3′, reverse: 5′-CTTTCTTTTGGGTCTCTGTG-3′; and GAPDH (573 bp), and forward: 5′-ATCACCATCTCCAGGAG CG-3′, reverse: 5′-GTTCTTCCACCACTTCGTCC-3′. PCR was carried out for 35 cycles, which consisted of pre-soak for 4 min at 94 °C, denaturing for 30 s at 94 °C, annealing for 30 s at 55–60 °C, and extension for 1 min at 72 °C, with additional 7 min incubation at 72 °C after completion of the cycle. The amplified cDNA fragments were electrophoresed through a 1% agarose gel, ethidium bromide stained, and photographed under an ultraviolet light transilluminator (Core-Bio, Seoul, Korea).

Results

Isolation and morphological analysis of cryopreserved UCB-derived mesenchymal stem cells

The duration of storage in frozen state for UCB was 0.1–5 years, and there were no differences in viability depending on the storage duration. When thawed, both BM- and UCB-derived cells were recovered with more than 90% viability. Frozen UCB-derived mononuclear

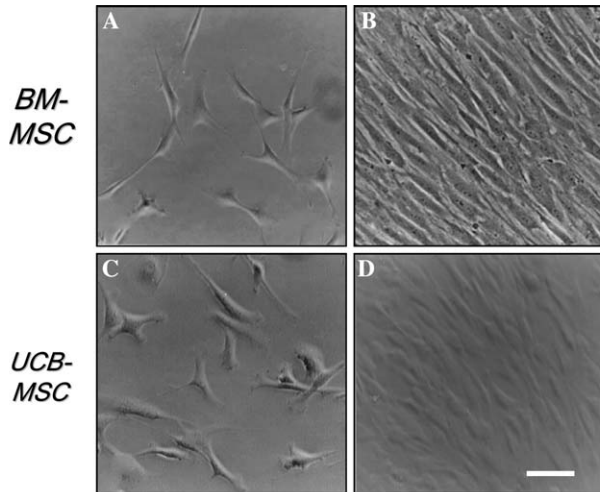

Fig. 1. Phase contrast images of MSCs from human BM and cryopreserved UCB. BM-derived cells at 17–20 days (A) and 24–27 days (B) and cryopreserved UCB-derived cells at 21–24 days (C) and 28–31 days (D). Both kinds of cells were isolated by direct adherence in our culture medium (low glucose-DMEM + 10% FBS) and pictured after two passages. Scale bar = 50 μm. All experiments were performed in triplicate.

cells were plated at a density of 3×10^5 cells/cm^2 and formed adherent heterogeneous cell populations after 4–7 days in culture, which consisted of round and spindle-shaped cells. In the initial passage of culture, the cells proliferated slowly and gave rise to confluence in 14–21 days. When subcultured, the heterogeneous cell populations changed into a homogeneous one with flat and fibroblast-like shape, the characteristics observed in BM-MSC. Fig. 1 shows that the UCB-derived cells closely resembled BM-MSCs in morphology.

Immunophenotypes and proliferation of cryopreserved UCB-derived mesenchymal stem cells

The cell-surface antigen profile of UCB-derived cells after three passages in culture was analyzed and compared with that of the UCB-MNC fraction before culture as shown in Fig. 2. The immunophenotypical profile of the MNC fraction greatly changed after the culture period, turning to typical MSC immunophenotypes. The cultured cells were strongly positive for MSC-specific surface markers such as CD105 (SH2), CD73 (SH3, SH4), and CD166 (ALCAM), while negative for CD14 (monocyte antigen), CD31 (endoglin), CD34 (HSCs antigen), CD45 (leukocyte common antigen), CD80 (co-stimulating molecule), and CD86 (co-stimulating molecule). Table 1 shows that the cell-surface antigen profile of UCB-derived cells was essentially the same as that of BM-derived MSCs.

To elucidate the proliferative capacity of UCB-derived cells, the expansion profile of the cells was compared with that of BM-MSCs. As shown in Fig. 3,

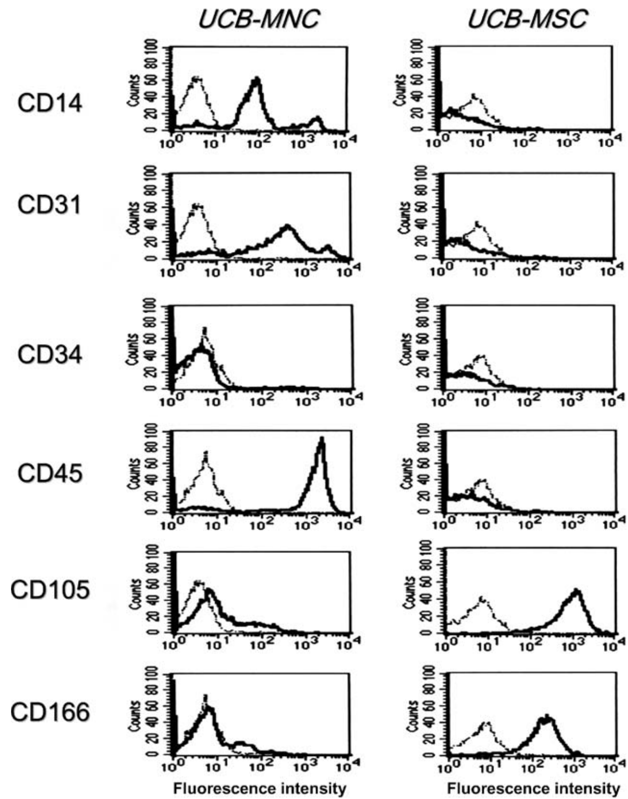

Fig. 2. Immunophenotyping of MNC fractions and MSCs from human cryopreserved UCB. Cells were labeled with FITC- or PE-conjugated antibodies and examined by flow cytometry. Histograms demonstrating the expression of surface molecules were plotted against control (anti-IgG). The immunophenotypical profile of the MNC fractions (left column) greatly changed after 4 weeks culture, changing to that of MSCs (right column). UCB-MSCs were strongly positive for MSC-specific markers such as CD105 (SH2) and CD166 (ALCAM), while negative for CD14 (monocyte antigen), CD31 (endoglin), CD34 (HSCs antigen), and CD45 (leukocyte common antigen). All experiments were performed in triplicate.

Table 1
Immunophenotypical comparison of bone marrow- and cord blood-derived MSCs

Cell surface marker	BM-MSC	UCB-MSC
CD14	−	−
CD31 (endoglin)	−	−
CD34	−	−
CD45	−	−
CD73 (SH3, SH4)	+	+
CD80 (B7-1)	−	−
CD86 (B7-2)	−	−
CD105 (SH2)	+	+
CD166 (ALCAM)	+	+

Values of immunophenotyping were determined by flow cytometry on a FACScan (BD Sciences). CD14, monocyte; CD31 (endoglin), endothelial progenitor; CD34, hematopoietic stem cell; CD45, leukocyte common; CD73 (SH3, SH4), mesenchymal stem cell; CD80 (B7-1), co-stimulating molecule; CD86 (B7-2), co-stimulating molecule; CD105 (SH2), mesenchymal stem cell; CD166, ALCAM (activated leukocyte cell adhesion molecule); +, strong positive; −, strong negative.

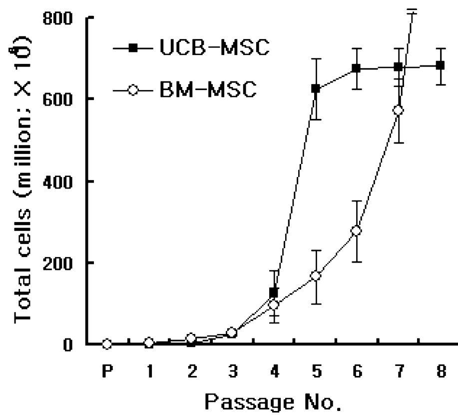

Fig. 3. The growth curves of MSCs from human BM and cryopreserved UCB. The initial culture was generated by plating 7.5×10^6 cells on a 25-cm^2 flask. After trypsinization of the initial culture, obtained MSCs were replated on 25-cm^2 flasks at a density of 1×10^5 cells. The number of total cells was assessed by trypan blue dye exclusion method every passage. The results are expressed as means ± SD of the number of total cells from three separate experiments.

UCB-derived cells were highly proliferative until passage 6 and resulted in approximately 1250-fold expansion in the cell number, yielding a minimum of 6.8×10^8 cells from one flask with the first seeding. Although proliferative rate of UCB-derived cells slowed down significantly after passage 6, the rapid expansion of the UCB-derived cells during the early phases of the culture would allow these cells to produce a sufficient quantity for therapeutic application.

Differentiation of cryopreserved UCB-derived mesenchymal stem cells

To assess the ability of UCB-derived cells to differentiate into mesenchymal derivatives, we grew those cells in osteogenic, adipogenic, and chondrogenic induction media for 14–21 days and probed for activity of alkaline phosphatase, deposition of neutral lipid vacuoles, and accumulation of chondrocyte matrix, respectively. As shown in Fig. 4, mesenchymally induced UCB-derived cells were positive in all the specific markers tested in the same manner as BM-derived MSCs. UCB-derived cells also expressed mRNA of osteopontin (osteoblast), PPARγ2 (adipocyte), and Col2al (chondrocyte) and the expression pattern was very similar to that observed in BM-MSCs (Fig. 5).

To further define the characteristics of UCB-derived cells, we investigated multi-lineage differentiation markers. UCB-derived cells expressed mRNA of multi-lineage genes including stromal-derived factor-1 (SDF-1), NeuroD, and vascular endothelial growth factor receptor-1 (VEGF-R1) as BM-MSCs did (Fig. 6). These results indicated that MSCs derived from thawed UCB also had the multi-differentiation capacity, which is regarded as one of the characteristics for MSCs derived from BM.

Discussion

UCB as the power of HSCs has been increasingly used for various clinical settings since 1988 [17]. Since then, hundreds of thousands of UCB collections have been frozen and stored throughout the world, in anticipation of their potential use to treat various disorders. Thus, study on the long-term storage of UCB-derived stem cells including HSCs and MSCs is of critical importance. We have investigated whether MNC fractions from cryopreserved UCB contained stem cells which represented typical characteristics of MSCs. When thawed, the UCB-derived cells were recovered with

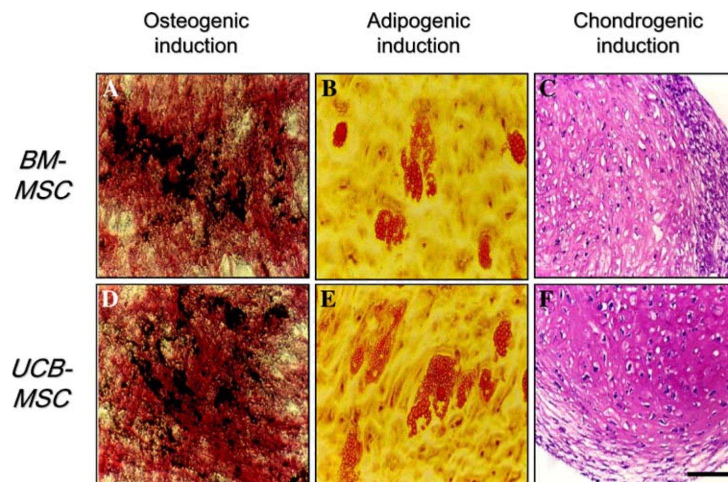

Fig. 4. Mesenchymal differentiation potential of MSCs from human BM and cryopreserved UCB. After incubation for 2–3 weeks in respective induction media, BM-MSCs and UCB-MSCs expressed alkaline phosphatase activity (A,D) and were stained positively for lipid vacuole with Oil-red O (B,E) and for chondrocyte matrix with toluidine blue (C,F), indicating osteogenic, adipogenic, and chondrogenic differentiation, respectively. Scale bar = 50 μm. All experiments were performed in triplicate.

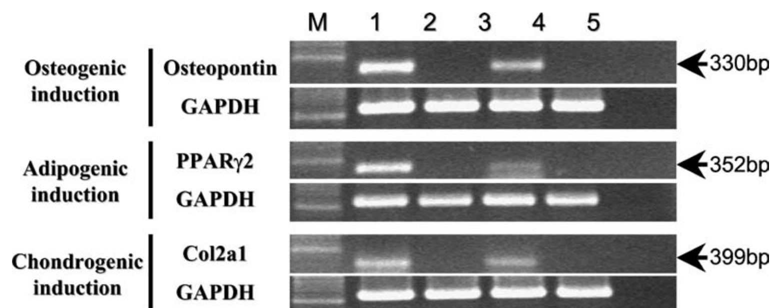

Fig. 5. RT-PCR analysis of mesenchymal tissue specific genes in differentiated BM- and UCB-MSCs. cDNAs prepared from the total RNA of mesenchymally differentiated BM-MSCs and UCB-MSCs were put into PCRs together with primers corresponding to mesenchymal lineage specific markers (osteogenesis, osteopontin; adipogenesis, PPARγ2; chondrogenesis, col2a1; and GAPDH, RT-PCR internal control). Mesenchymally differentiated UCB-MSCs expressed lineage specific markers in the same manner as differentiated BM-MSCs. M, size marker; lane 1, differentiated BM-MSCs; lane 2, undifferentiated BM-MSCs; lane 3, differentiated UCB-MSCs; lane 4, undifferentiated UCB-MSCs; and lane 5, negative control. All experiments were performed in triplicate.

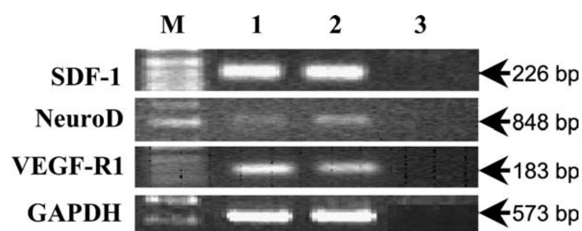

Fig. 6. RT-PCR analysis of lineage specific markers expressed in human BM-MSCs and cryopreserved UCB-MSCs. cDNAs prepared from the total RNA of BM-MSCs and UCB-MSCs were put into PCRs together with primers corresponding to SDF-1, NeuroD, VEGF-R1 (Flt-1), and GAPDH (RT-PCR control). Both BM-MSCs and UCB-MSCs expressed lineage specific markers in the same manner. M, size marker; lane 1, BM-MSCs; lane 2, UCB-MSCs; lane 3, negative control; SDF-1, stromal-derived factor-1; and VEGF-R1, vascular endothelial growth factor-receptor 1. All experiments were performed in triplicate.

more than 90% viability, regardless of the storage duration within 0.1–5 years. The adherent fibroblast-like cell populations obtained in this study gave rise to MSC characteristics in regard to morphology, immunophenotypes, and proliferation and differentiation potential.

The primary culture initially consisted of two main populations such as round and spindle-shaped cells. As the culture progressed with trypsinization, the CD14, CD31, and CD45-positive round and spindle-shaped cells gradually decreased (64 and 2% at week 2 and 4, respectively; data not shown) and eventually disappeared by week 4–6. Fibroblast-like cells remained homogeneous in morphology and they were CD73 (SH3, SH4)-, CD105 (SH2)-, and CD166 (ALCAM; activated leukocyte cell adhesion molecule)-positive (Fig. 2, Table 1). These cells showed prolonged proliferative capacity without any morphological changes for more than 6 passages (over 3 months) and had a differentiation potential to mesenchymal derivatives including osteoblast, adipocyte, and chondrocyte (Figs. 4 and 5). These characteristics were consistent with the results from BM-MSCs originally described by Fridenstein et al. [18]. BM-MSCs have already been shown to give rise to skeletal muscle, hepatocytes, glia, and neurons in addition to the mesenchymal derivatives [19]. The obtained cells also expressed mRNA of multi-lineage genes such as SDF-1, NeuroD, and VEGF-R1 as BM-MSCs did (Fig. 6). These observations elucidated that specific genes for multi-lineages are present in the UCB-derived cells, allowing these cells to multi-lineage differentiation.

In this study, we measured the growth rate of MSCs derived from cryopreserved UCB. As shown in Fig. 3, UCB-MSCs were more proliferative than BM-MSCs in early passages, while the total cell number of expanded UCB-MSCs in long-term culture was lower than that from BM-MSCs. These phenomena can be explained by intrinsic and/or extrinsic factors. The report that UCB had more primitive stem cells than BM [20] may explain the phenomenon that UCB-MSCs were more proliferative than BM-MSCs in early stages. The difference in total cell numbers in long-term culture may also relate to other inherent characteristics of UCB and BM samples including the frequency of MSC. The frequency of MSC in cryopreserved UCB units has not been clearly defined. Previous studies reported that the frequency of BM-MSC in adults was one in 3.4×10^4 cells, using the culture of colony forming unit-fibroblast (CFU-F) as a surrogate assay, while fresh UCB and PBSC did not produce CFU-F at all [13,21]. It is possible that MSCs in cryopreserved UCB units are present at a very low frequency relative to MSCs in BM in our samples. Since a few UCB-MSCs were more proliferative than BM-MSCs in early passages, doubling potential of UCB-MSCs may decrease in a faster manner compared to that of BM-MSCs after a finite range of doublings as shown in Fig. 3. Extrinsic factors that may affect the different growth would include differences in the culture conditions required for UCB-MSCs and BM-MSCs (e.g., medium composition, serum, pH, positive or negative selection, or trypsinization effectiveness, etc.) and the

human factor, which determined when and how cultures would be passaged. However, the cause of these differences in the growth rate between BM-MSCs and UCB-MSCs in this study requires further study.

Our results, together with other studies including Erices et al. [15] and Rosada et al. [22] 's work, provide strong evidence for the presence of circulating non-hematopoietic stem cells including MSCs in human UCB units. The assessment of the growth factors, and prolonged proliferation and differentiation of these circulating stem cells remain to be investigated, and further studies are necessary to explore the full potential of these cells. Also, the origin and the mechanisms of homing of the cells to various stromal cell compartments such as bone marrow remain to be determined.

In summary, this is the first report to show MSCs with the high proliferative and differentiation potential present in the cryopreserved human UCB. The in vitro isolation, expansion, and characterization of UCB-MSCs can be used for the development of basic research and therapeutic strategies such as cellular and genetic therapy.

Acknowledgments

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