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Mesenchymal stem/progenitor cells developed in cultures from UC blood

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Background

Whether umbilical cord blood (UCB) serves as a source of mesenchymal stem/progenitor cells (MSPC) is controversial. MSPC are the best candidates for cellular therapy of orthopedic skeletal tissues. In order to explore the possibility of UCB as a useful source of MSPC, we identified, expanded in culture, and characterized MSPC from UCB harvests on a large scale.

Methods

Mononuclear cells isolated from UCB harvests (n = *411) were cultured in media supplemented with 10% FBS. MSPC-like cells cultured from each UCB harvest were expanded* ex vivo *by successive subcultivation. UCB harvests with a more than 1000-fold expanding capacity* (n = *9) were examined for surface Ag phenotypes and* in vitro *differentiation potentials into osteogenic, chondrogenic and adipogenic lineages.*

Results

Ninety-five out of a total of 411 UCB units (23.1%) generated MSPC-like cells during cultivation. Nine UCB units (2.2%) yielded MSPC with more than 1000-fold expansion capacity. These cells positively expressed MSPC-related Ag, but did not express myeloid, histocompatibility or endothelial Ag. These cells also possessed multiple capacities for osteogenic, chondrogenic and adipogenic differentiation.

Discussion

Although the incidence of UCB harvests producing MSPC in culture was low, some of them showed a more than 1000-fold expanding capacity, which is enough in cell numbers to be an allogeneic source for cellular therapy. Our results may encourage the use of UCB as an attractive target for allogeneic cellular therapeutic options in tissue engineering.

Keywords

adipogenic differentiation, chondrogenic differentiation, mesenchymal stem/progenitor cells, osteogenic differentiation, UC blood.

Introduction

Umbilical cord blood (UCB) is a rich source of hematopoietic stem/progenitor cells (HSPC) [1]. More than 3000 cases of UCB HSPC transplantation have been performed world-wide for patients with various hematologic and genetic disorders, such as lymphoid and myeloid leukemia, Fanconi anemia, aplastic anemia, Hunter syndrome, Wiskott–Aldrich syndrome, β-thalassemia and neuroblastoma [2–8]. UCB contains a higher proportion of primitive hematopoietic cells, including multipotent colony-forming cells as well as *in vivo* repopulating cells, than adult BM [9–11]. Furthermore, UCB-derived HSPC possess higher proliferation and expansion potentials than their adult BM counterparts [11–14].

Hematopoiesis is a complex process where hematopoietic stem cells self-replicate and differentiate into committed progenitors and mature cells. This occurs in close proximity with stromal cells and extracellular matrix molecules in the BM microenvironment [15,16]. Two main cellular components are involved in hematopoiesis:

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the HSPC and the mesenchymal stem/progenitor cells (MSPC) [15,16]. MSPC are different from typical BM stromal cells and proliferate well in culture in the form of well-attached fibroblast-like adherent cells [17]. They are multipotent, and serve as long-lasting precursors that contribute to the regeneration of mesenchymal tissues such as BM stroma, bone, cartilage, adipose, muscle and tendon [18]. Interestingly, MSPC can be transplantable between unrelated individuals without immunosuppressive treatment [19].

MSPC are the best candidates for tissue engineering and cellular therapy of orthopedic musculoskeletal tissues [20], and for promoting the engraftment of $CD34^+$ hematopoietic cells in HSPC transplantation [21]. Although BM is the most common source of MSPC, the collection procedure of BM from the donor is invasive and unfeasible as a routine method. UCB is relatively easy to collect; however, it is controversial as to whether UCB serves as a source of MSPC [22–24].

This study assessed the MSPC yield from a great number of UCB harvests on a large scale. About a quarter of the UCB harvests yielded MSPC-like adherent cells under the culture conditions described. Some of them, with more than 1000-fold expanding capacity, showed multiple differentiation potentials *in vitro*. Although the incidence of UCB producing MSPC in culture is low, we have shown the presence of circulating MSPC in UCB and encourage the use of UCB as an attractive target for allogeneic cellular therapeutic options in tissue engineering.

Methods

UCB harvest

UCB samples ($n = 411$) were collected from the umbilical vein of deliveries with informed maternal consent. A 16-gauge needle from a UCB collection bag containing 23 mL of CPDA-1 anticoagulant (Greencross Co., Yongin, Kyungki-do, Korea) was inserted into the umbilical vein, and UCB was allowed to flow by gravity. In all cases, UCB harvests were processed within 24 h of collection, with viability of more than 90%.

Isolation and expansion in culture of MSPC

Mononuclear cells were isolated by centrifugation in a Ficoll–Hypaque gradient (density 1.077 g/cm^3, Sigma, St Louis, MO, USA). The separated mononuclear cells were washed, suspended in α-minimum essential medium (α-MEM, Gibco BRL, Carlsbad, CA, USA), supplemented with 10% FBS (HyClone, Logan, UT, USA), and seeded at a concentration of 5×10^6 cells/cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 with a change of culture medium twice a week. UCB-derived mononuclear cells were set in culture, and the onset of fibroblast-like adherent cells was observed. One to 3 weeks later, when the monolayer of MSPC colonies reached 80% confluence, cells were trypsinized (0.25% trypsin, HyClone), washed, resuspended in culture medium (α-MEM supplemented with 10% FBS) and subcultured at a concentration of 5×10^4 cells/cm^2. MSPC of each UCB harvest were expanded *ex vivo* by successive subcultivation under the same condition. Neither recombinant cytokine nor growth factor was used in the culture medium. The 5–8th passage cells of UCB harvests with more than 1000-fold expanding capacity ($n = 9$) were examined for surface Ag phenotypes and *in vitro* differentiation potentials.

Immunophenotyping of UCB-derived MSPC

For cell-surface Ag phenotyping, cells were detached and stained with FITC- or PE-coupled Ab. Labeled cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the CELLQUEST software (Becton Dickinson).

To detect surface Ag, aliquots of the UCB-derived MSPC after treatment with 0.25% trypsin were washed three times with PBS, pH 7.4. For direct assays, cells were immunolabeled with the anti-human Ab CD45–FITC, CD34–FITC, CD14–FITC, HLA-DR–FITC, CD13–PE (Becton Dickinson) and CD29–PE, CD44–PE, CD31–FITC, CD106–PE, CD90–FITC, CD51/61–FITC, CD64–FITC (Pharmingen, Los Angeles, CA, USA). As isotypic controls, mouse IgG_1–FITC, IgG_1–PE, IgG_{2a}–FITC (Becton Dickinson) and mouse IgG_{2b}–PE, IgG_1–FITC, IgG_1–PE (Pharmingen) were used. For indirect assays, cells were immunolabeled with anti-human Ab SH2, SH3 and SH4. SH2, SH3 and SH4 Ab were made from each hybridoma cell line (American Type Culture Collection, Rockville, MD, USA). As a secondary Ab, anti-mouse IgG–FITC (Jackson ImmunoResearch, West Grove, PA, USA) was used.

Osteogenic differentiation

To induce osteogenic differentiation, cells were cultured in osteogenic medium for 3 weeks. The medium was

composed of α-MEM supplemented with 10% FBS, 0.1 μm dexamethasone (Sigma), 10 mm β-glycerol phosphate (Sigma) and 50 μm L-ascorbic acid 2-phosphate (Sigma) [25]. The medium was changed twice a week. The onset of osteoblasts was evaluated by the expression of alkaline phosphatase (ALP), using an ALP staining kit (85L-3R, Sigma). Mineralized matrix was also stained with silver nitrate (Sigma) by the method of von Kossa.

Chondrogenic differentiation

To induce chondrogenic differentiation, approximately 2×10^5 cells were placed in a 15-mL polypropylene tube and centrifuged at 500 *g* for 5 min at room temperature, to form a pellet micromass. Then, the micromass was treated with chondrogenic medium for 6 weeks. Medium was replaced twice a week. Chondrogenic medium consisted of high-glucose DMEM (Gibco BRL) supplemented with 100 nm dexamethasone (Sigma), 50 μg/mL L-ascorbic acid (Sigma), 100 μg/mL sodium pyruvate (Sigma), 40 μg/mL L-proline (Sigma), 10 ng/mL transforming growth factor β3 (TGF-β3, Sigma), 500 ng/mL bone morphogenic protein 6 (BMP-6, R&D Systems, Minneapolis, MN, USA) and 50 mg/mL ITS$^+$ premix (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL BSA and 5.35 mg/mL linoleic acid, Becton Dickinson) [26].

For histologic and immunohistochemical analyses, the pellet aggregates were frozen in OCT compound (Tissue-Tek, Torrance, CA, USA) and 5-μm sections were cut. For histologic evaluation, sections were stained with Safranin-O (Sigma). For immunohistochemistry, the frozen sections were fixed for 10 min in methanol after a brief immersion in distilled water to remove the OCT compound. Non-specific Ab binding sites were blocked by incubating the slides in PBS with 5.0% BSA for 1 h. Slides were then incubated for 1 h at room temperature with primary Ab, with epitope in type II collagen (Oncogene, San Diego, CA, USA) diluted in 0.05 M Tris–HCl with 1.0% BSA (TBS). Reactivity was detected by using the DAKO EnVision™+ System Peroxidase (DAB) kit (K4006, DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions.

Adipogenic differentiation

To induce adipogenic differentiation, cells were treated with adipogenic medium for 4 weeks. Medium changes were carried out twice a week. Adipogenic medium consisted of DMDM (Gibco BRL) supplemented with 10% FBS (Hyclone), 0.5 mm 3-isobutyl-1-methylxanthine (IBMX, Sigma), 1 μm dexamethasone (Sigma), 0.2 mm indomethacin (Sigma) and 10 μm h-insulin (Sigma) [27]. The onset of adipocytes was evaluated with Oil-red O (Sigma) stain.

RT-PCR for *in vitro* differentiation

Total RNA was extracted from each differentiated cell with a modification of the method of Chomczynski and Sacchi [28]. Cells were lysed using TRIzol Reagent (Invitrogen, La Jolla, CA, USA), and RNA was prepared as per the kit instructions. The mRNA was reverse transcribed (RT) to cDNA in a 20-μL reaction volume containing 1 μg RNA, 1 μL 500 μg/mL Oligo dT primer (Invitrogen), 1 μL (200 U) SuperScript™ II reverse transcriptase (Invitrogen), 4 μL 5 × First-Strand buffer (250 mm Tris–HCl, pH 8.3, 375 mm KCl, 15 mm $MgCl_2$, Invitrogen), 2 μL 0.5 mm dNTP, 1 μL 10 mm dTT and 1 μL RNaseOUT ribonuclease inhibitor (Invitrogen).

PCR reactions were performed in 25-μL reaction volumes containing 2 μL cDNA, 2 μL each 10 pm primer, 0.5 μL AccuPrime™ *Taq* DNA polymerase (Invitrogen), 2.5 μL 10 × AcuuPrime™ PCR buffer (Invitrogen). Primers used for amplification are listed in Table 1. cDNA was amplified using a ABI GeneAmp PCR System 9600 (Perkin Elmer, Applied Biosystems, Boston, MA, USA) at 95°C for 30 s, the annealing temperature for each target gene as described in Table 1 for 30 s, and 72°C for 30 s for 35 cycles, after initial denaturation at 95°C for 5 min. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

Results

Identification and expansion of mesenchymal cells from UCB culture

When set in culture in the presence of 10% FBS, UCB-derived low-density mononuclear cells gave rise to a population of fibroblast-like adherent cells. After 1 week in culture, UCB-derived MSPC-like cells began to be identifiable as colonies of adherent cells with fibroblast-like appearance. By day 21 of primary culture, 95 out of 411 UCB harvests (23.1%) produced MSPC-like cells that remained as such even after regular changes of the medium. No adherent or loosely adherent small round cells were present in the primary cultures, but the latter disappeared soon after the second or third passage.

Table 1. RT-PCR primers used for the evaluation of osteogenic, chondrogenic and adipogenic differentiation

Target gene	Primer sequence	Annealing temperature (°C)	PCR product size (bp)
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	55	452
Osteogenic markers			
Osteocalcin	5′-CATGAGAAGCCCTCACA-3′ 5′-AGAGCGACACCCTAGAC-3′	55	310
Alkaline phosphatase	5′-TGGAGCTTCAGAAGCTCAACACCA-3′ 5′-ATCTCGTTGTCTGAGTACCAGTCC-3′	55	453
Osteopontin	5′-CTAGGCATCACCTGTGCCATACC-3′ 5′-CAGTGACCAGTTCATCAGATTCATC-3′	55	347
Collagen type I	5′-CCCCCTCCCCAGCCACAAAGA-3′ 5′-TCTTGGTCGGTGGTGGACTCT-3′	60	360
Cbfa-I	5′-CAGTAGATGGACCTCGGGAA-3′ 5′-GAGGCAGAAGTCAGAGGTGG-3′	60	395
Chondrogenic markers			
Aggrecan	5′-TCAGGAGGGCTGGAACAAGTACC-3′ 5′-GGAGGTGGTAATTGCAGGGAACA-3′	57	392
Collagen type II	5′-TGGAGCTTCAGAAGCTCAACACCA-3′ 5′-ATCTCGTTGTCTGAGTACCAGTCC-3′	55	225
Collagen type II	5′-TTTCCCAGGTCAAGATGGTC-3′ 5′-CTTCAGCACCTGTCTCACCA-3′	55	377
Collagen type IX	5′-CCCCCTCCCCAGCCACAAAGA-3′ 5′-TCTTGGTCGGTGGTGGACTCT-3′	57	159
Sox-9	5′-GGTTGTTGGAGCTTTCCTCA-3′ 5′-TAGCCTCCCTCACTCCAAGA-3′	57	401
Adipogenic markers			
PPARγ	5′-TTCAGAAATGCCTTGCAGTG-3′ 5′-GGGCTCCATAAAGTCACCAA-3′	57	599
C/EBPα	5′-CTGGAGCTGACCAGTGACAA-3′ 5′-CCAAGAATTCTCCCCTCCTC-3′	57	374
Leptin	5′-ACAGAAAGTCACCGGTTTGG-3′ 5′-TGGCTTAGAGGAGTCAGGGA-3′	57	481
Lipoprotein lipase	5′-TCAATCACAGCAGCAAAACC-3′ 5′-CCACATCTCCAAGTCCTCTC-3′	57	575
αP2	5′-TACTGGGCCAGGAATTTGAC-3′ 5′-TCAATGCGAACTTCAGTCCA-3′	57	240

MSPC-like colonies derived from UCB culture mainly consisted of bipolar fibroblast-like cells (Figure 1a). At day 21 of primary culture, the mean value for the total adherent cell number was 2.6×10^6 ($n = 95$, range $0.04 \times 10^5 - 22.0 \times 10^6$). After *ex vivo* expansion, nine (9.5%) out of 95 UCB (9/411, 2.2%) harvests producing MSPC-like colonies showed a more than 1000-fold increase in cell number. These cells could be passaged up to 18 times without displaying significant changes in morphology (Figure 1b).

Immunophenotypic characterization of UCB-derived MSPC

Immunophenotyping of UCB-derived MSPC with more than 1000-fold expanding capacity ($n = 9$) showed positive expression of the MSPC-related Ag, SH2, SH3 and SH4

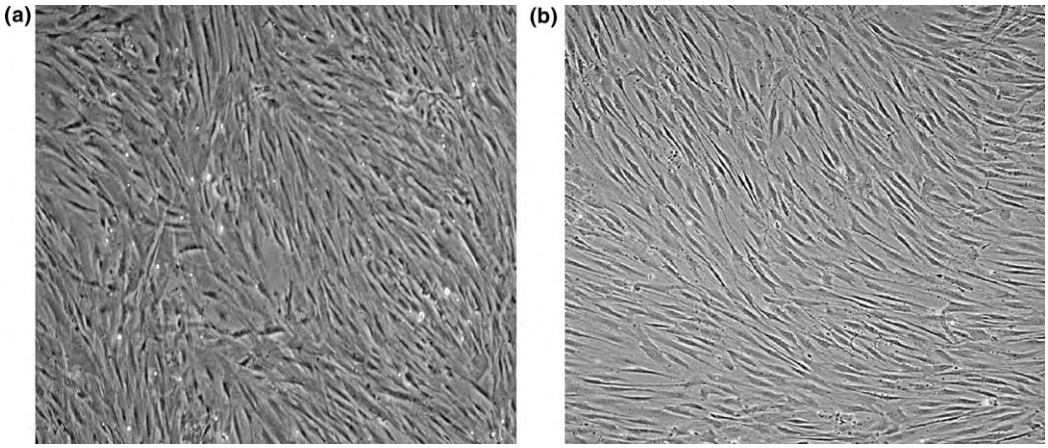

Figure 1. *Photomicrographs showing MSPC from primary cultures of UCB at day 21 (a) and advanced passage 15 (b). UCB-derived MSPC colonies mainly consist of bipolar fibroblast-like cells. No significant morphologic changes were observed at the advanced passage (original magnification × 200).*

(Figure 2). These cells expressed Ag CD13, CD29, CD44 and CD90, but did not express Ag CD14, CD31, CD34, CD45, CD51/61, CD64, CD106 and HLA-DR (Figure 2).

Osteogenic, chondrogenic and adipogenic differentiation of UCB-derived MSPC

Further characterization studies on the UCB-derived MSPC revealed their potential to differentiate into osteoblasts, chondrocytes and adipocytes. Under the influence of dexamethasone, β-glycerol phosphate, ascorbic acid and 10% FBS, the cultured MSPC formed aggregates and increased their ALP expression (Figure 3a). These cells also formed extracellular calcium matrix, as demonstrated by von Kossa's staining method (Figure 3b). Differentiated osteoblasts expressed mRNA of ALP, osteopontin (OP), osteocalcin (OC), collagen type I (Col I) and core binding factor α1 (Cbfa-I) during osteogenic induction (Figure 3c).

To demonstrate the chondrogenic potential of UCB-derived mesenchymal cells, the cells were centrifuged to form pellet micromass, and cultured in serum-free chondrogenic medium containing TGF-β3 and BMP-6. The cell pellets histologically showed an increased proteoglycan-rich extracellular matrix during culture. After 2 weeks of differentiation, the accumulation of sulfated proteoglycans was visualized by Safranin-O staining (Figure 4a). Chondrocyte-like lacunae were also evident in the histologic sections. Extensive matrix was rich in type II collagen, which is typical of articular cartilage, at the sixth week (Figure 4b). mRNA of collagen type II (Col II), aggrecan, collagen type IX (Col IX) and Sox-9, marker genes for chondrocytes, was detected by RT-PCR at 4 weeks (Figure 4c).

To assess the adipogenic potential, cells were cultured by treatment with 10% FBS, IBMX, dexamethasone, insulin and indomethacin. Induction was apparent by the accumulation of lipid-rich vacuoles within cells, which were visualized by staining with Oil red O (Figure 5a, b). Multiple induction treatments resulted in more than 50% of the cells committing to this lineage, and the lipid vacuoles continued to develop over time, coalesced, and eventually filled the cells. These adipocytes expressed mRNA of peroxisome proliferation-activated receptor γ (PPARγ), lipoprotein lipase (LPL), fatty acid-binding protein aP2 (αP2), CCAAT/enhancer-binding protein α (C/EBPα) and leptin during adipogenic induction (Figure 5c).

Of the nine UCB harvests with more than 1000-fold expanding capacity, nine underwent osteogenic differentiation, eight underwent chondrogenic differentiation, and five underwent adipogenic differentiation. These results indicated that the cultured UCB-derived MSPC possess multiple capacities for each lineage differentiation.

Discussion

The present study was performed focusing on the identification, expansion and phenotypic characterization of the MSPC developed in cultures from human UCB in the absence of growth factors and any recombinant cytokines. Approximately one-quarter of the UCB harvests

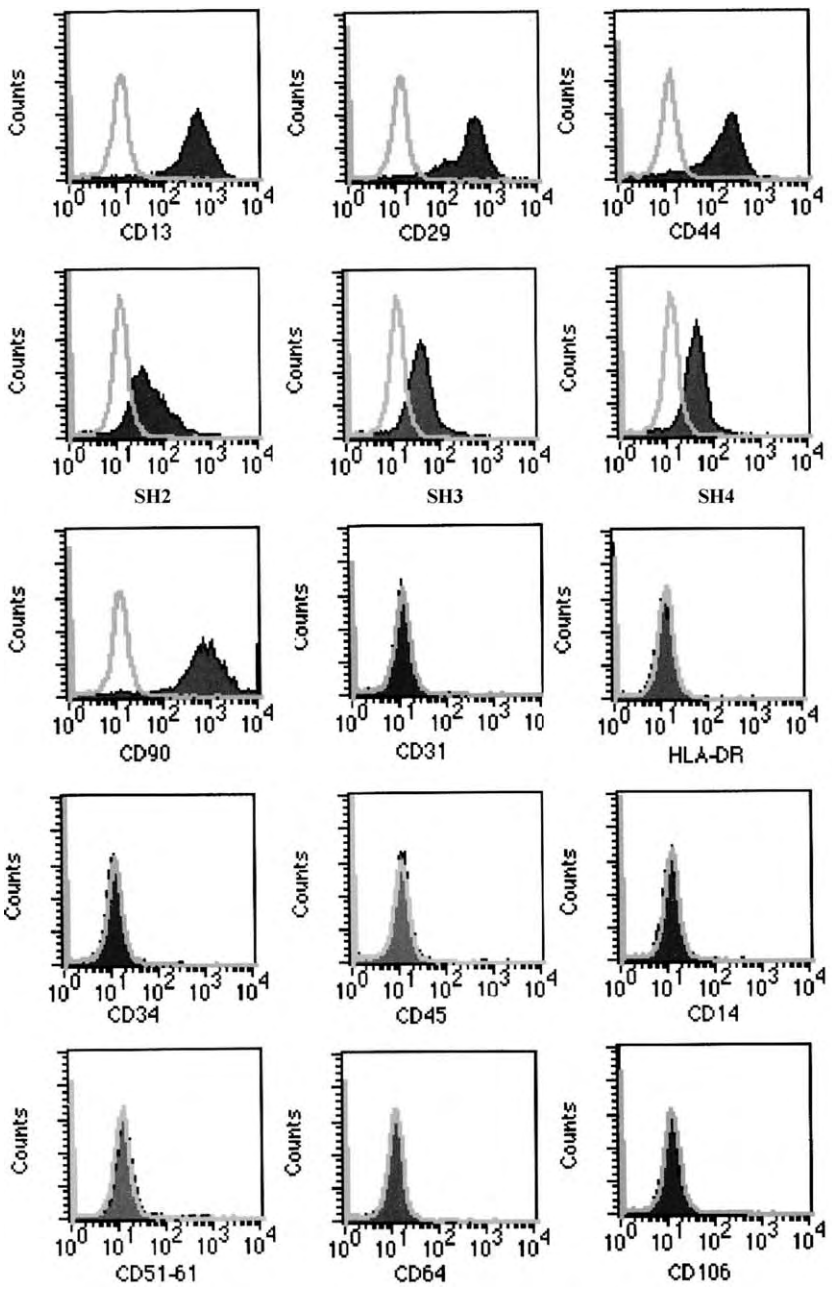

Figure 2. *Immunophenotypic characterization of MSPC derived from UCB. These cells positively expressed antigens CD13, CD29, CD44, CD90, SH2, SH3 and SH4. These cells did not express antigens CD31, HLA-DR, CD34, CD45, CD14, CD51/61, CD64 and CD106.*

(95 out of 411 harvests, 23.1%) generated MSPC-like adherent cells. Colonies of the mesenchymal cells mainly consisted of bipolar fibroblast-like cells. Nine of the 411 UCB samples (2.2%) showed a large *ex vivo* expansion capacity (>1000). These cells positively expressed MSPC-related Ag, but did not express myeloid, histocompatibility or endothelial Ag. In addition, these cells possessed multiple capacities for osteogenic, chondrogenic and adipogenic lineage differentiations. These results indicated that characteristics of the *ex vivo* expanded UCB-derived mesenchymal cells are similar to the features of BM-derived MSPC [17,18].

So far, it is controversial regarding whether UCB serves as a source of MSPC. Results of the present study correspond with an earlier study that reported that almost 25% of UCB harvests generate, when set in culture,

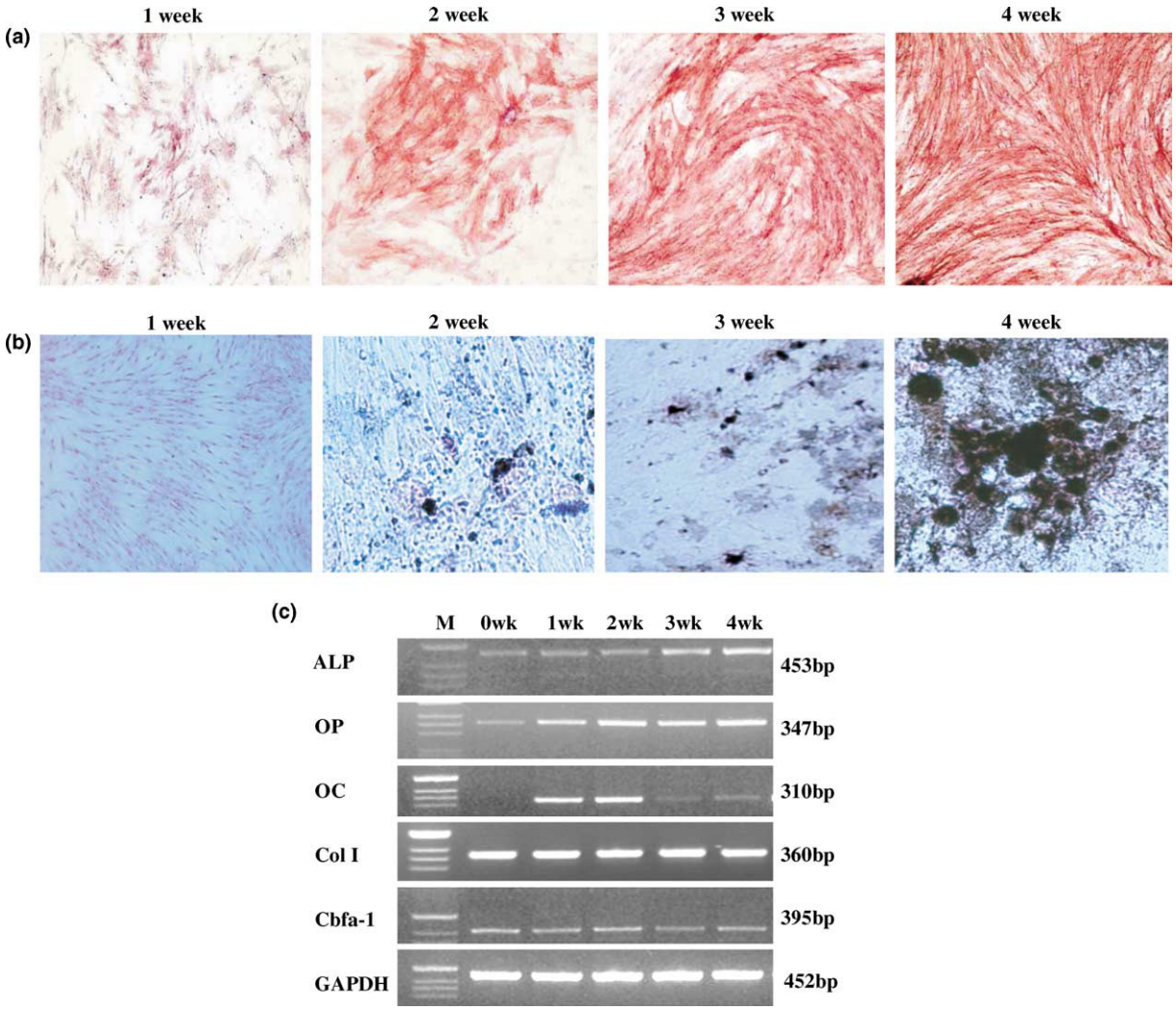

Figure 3. *Osteogenic differentiation potential of UCB-derived MSPC. These cells formed aggregates and increased their expression of ALP (a, original magnification × 100). They also formed extracellular calcium matrix as demonstrated by von Kossa's stain (b, original magnification × 100). Differentiated osteoblasts expressed mRNA of ALP, OP, OC, Col I and Cbfa-I during osteogenic induction (c).*

mesenchymal progenitor cells with a large *ex vivo* expansion capacity, as well as osteogenic and adipogenic differentiation potentials [24]. Recently, this group also reported that UCB-derived mesenchymal stem cells (MSC) home and survive in the marrow of immunodeficient mice after systemic infusion [29]. They also showed that UCB-derived human MSPC are detected in other mice tissues, such as cardiac muscle, teeth and spleen [29]. On the other hand, Wexler *et al.* [22] and Mareschi *et al.* [23] asserted that adult BM is a reliable source of functional cultured MSPC, but UCB and mobilized peripheral blood are not. Actually, there are few reports in relation to the non-hematopoietic cell components in UCB. Based on the cell-surface Ag phenotypes, MSPC, myelosupportive stromal cells, endothelial precursor cells and DC were reported as non-hematopoietic cell populations residing in human UCB [24,29–32]. In this study, we examined the MSPC yield from a great number of UCB harvests via *ex vivo* culture expansion with only FBS. Our results showed that only 23.1% of UCB harvests generated MSPC-like cells. This result suggests that the frequency of MSPC residing in UBC may be lower than that of BM. The number of MSC in BM is 2–5 MSC/10^6 mononuclear cells [33]. In general, many investigators can observe reliable generation of MSC from relatively small volumes of BM. On the other hand, Goodwin *et al*. [34] showed that UCB multilineage cells are slower to establish in culture and have a lower precursor frequency (0.05–2.8

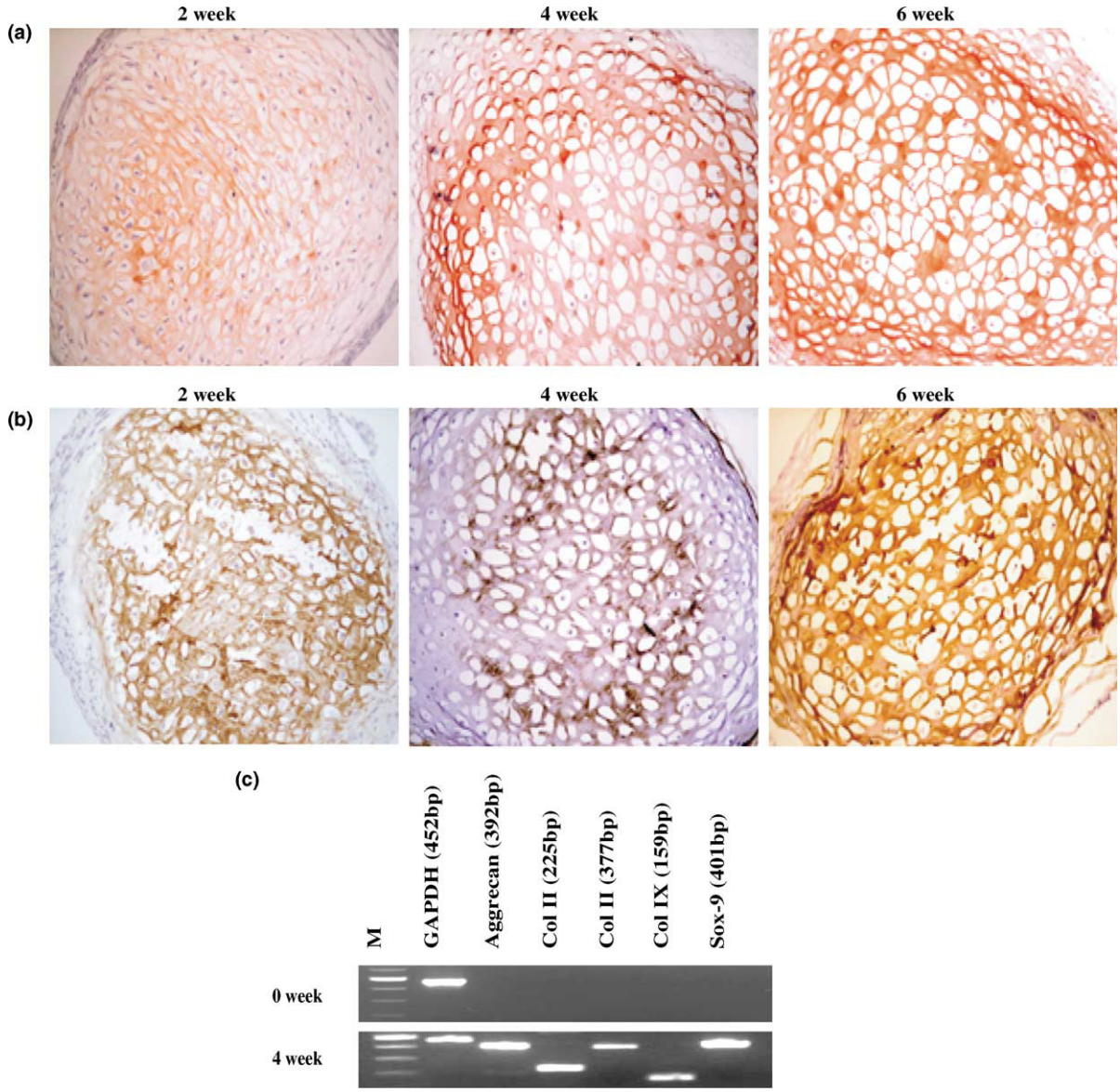

Figure 4*. Chondrogenic differentiation potential of UCB-derived MSPC. After 2 weeks of differentiation, the accumulation of sulfated proteoglycans was visualized by Safranin-O stain (a, original magnification × 200). Chondrocyte-like lacunae were also evident in the histologic sections. Extensive matrix was rich in Col II, which is typical of articular cartilage, at week 6 (b, original magnification × 200). mRNA of Col II, aggrecan, Col IX and Sox-9, marker genes for chondrocytes, was detected by RT-PCR (c).*

non-hematopoietic precursors/10^6 mononuclear cells) compared with BM. Because the MSPC frequency in UCB is low, these cells cannot be easily detected in the conventional culture conditions described. In addition, for more than 50% of the UCB harvests that generated MSPC-like colonies, the cells were detected during approximately the third week of the culture period (data now shown). We assume that because of the lower MSPC frequency in UCB, these cells were detected at a much later time point. Recently, Lee *et al*. [35] reported a novel method for obtaining single cell-derived, clonally expanded MSC from UCB by negative immunoselection and limiting dilution. Like this, further studies will be needed to develop a new technique to detect MSPC contained in UCB.

The immunophenotype of UCB-derived MSPC was nearly consistent with that reported for BM-derived MSC [17,18]. These cells expressed Ag CD13, CD29, CD44 and CD90, but did not express Ag CD14, CD31, CD34, CD45, CD51/61, CD64, CD106 and HLA-DR. They also showed

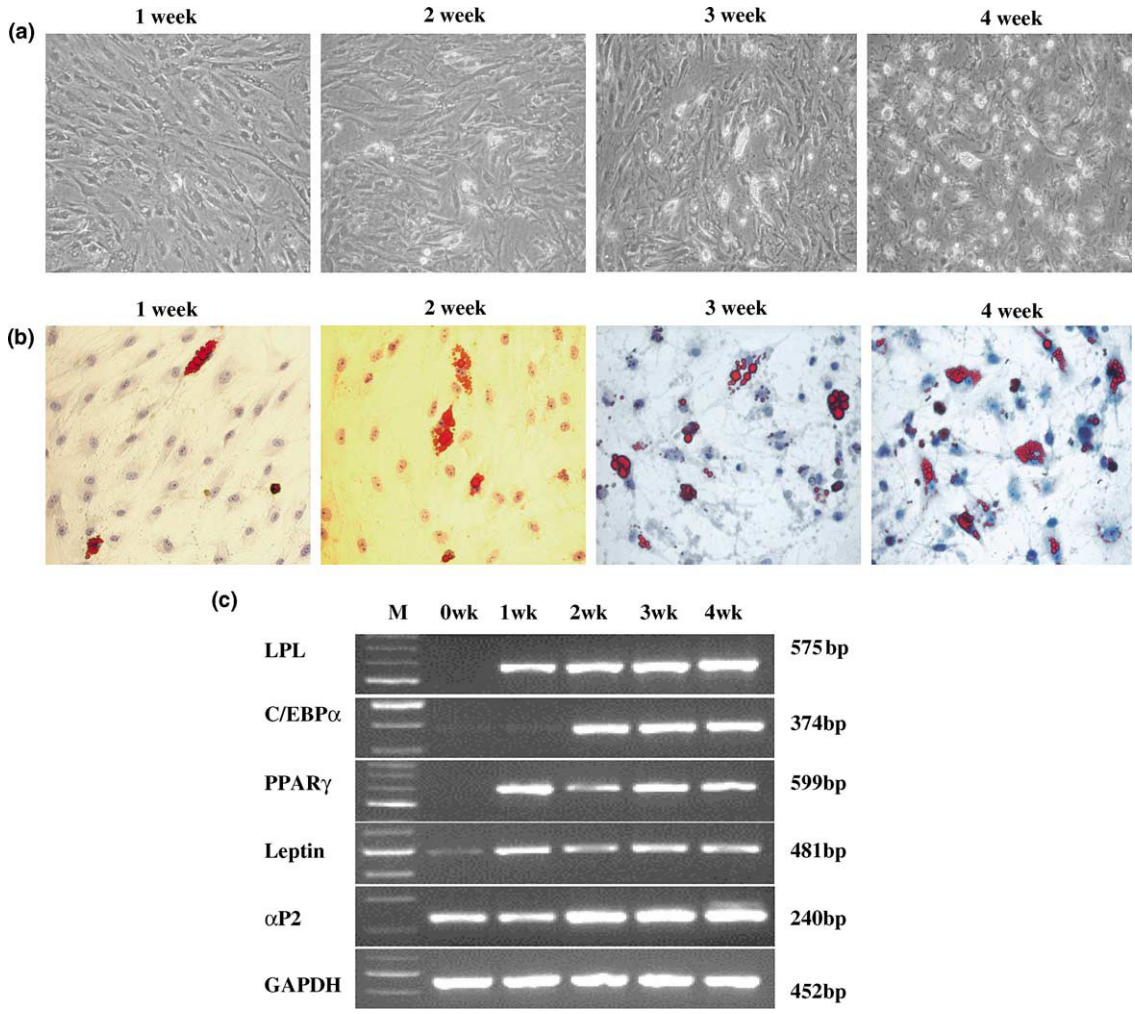

Figure 5. *Adipogenic differentiation potential of UCB-derived MSPC. Induction was apparent by the accumulation of lipid-rich vacuoles within cells, which were visualized by staining with Oil red O (a, original magnification × 200; b, original magnification × 200). Differentiated adipocytes expressed mRNA of PPARγ, LPL, αP2, C/EBPα and leptin during adipogenic induction (c).*

positive expression of the MSPC-related Ag, SH2, SH3 and SH4. SH2, SH3 and SH4 are the putative markers for human MSC [18]. Although the expression of SH2 and SH3 on the cells was relatively weak in our study, we could not find any correlation between SH2 and SH3 intensities of UCB-derived MSPC and their *ex vivo* expansion capacity and differentiation potentials. This finding raises the questions as to whether the expression pattern of SH2 and SH3 on the cells during successive cultivation is variable and, if so, whether it has an effect on the functions of UCB-derived MSPC. CD106 (VCAM-1) was completely negative in our study, but is often positive with BM-derived MSC [18]. Goodwin *et al*. [34] and Erices *et al*. [24] showed that MSPC derived from UCB are CD106-negative, thus it will be important to cultivate MSPC from BM and UCB under the same conditions and to directly compare the two cell preparations.

After *ex vivo* expansion, nine out of 95 UCB harvests showing MSPC-like colonies exhibited a more than 1000-fold increase in cell number. These cells could be passaged up to 18 times without displaying significant changes in morphology. In addition, we performed further characterization studies on these nine UCB harvests to reveal their potential to differentiate into osteoblasts, chondrocytes and adipocytes. Of the nine UCB harvests with more than 1000-fold expanding capacity, all underwent osteogenic differentiation, eight underwent chondrogenic differentiation, and five underwent adipogenic differentiation. These findings correspond with earlier reports demonstrating that MSC derived from human BM can be induced to

differentiate into the adipocytic, chondrocytic and osteocytic lineages [36]. Lee *et al.* [35] reported that UCB contains MSC that can differentiate into bone, cartilage and fat. They showed that the UCB-derived MSC are also able to differentiate into neuroglial- and hepatocyte-like cells under appropriate induction conditions. Thus, further studies will be needed to induce the neurogenic and endodermal lineage differentiations using our MSPC.

In recent years, the use of MSPC has been highlighted in osteobiology and applied in regeneration medicine. Because MSPC is thought to be the origin of mesenchymal tissues during the process of normal growth, remodeling and repair [37,38], various tissues have been examined as a source for MSPC. Although the incidence of UCB harvest producing MSPC in culture was low in our study, some of the harvests showed a more than 1000-fold expanding capacity, which is sufficient in cell number to be the source of tissue regeneration. In addition, the collection procedure of UCB is easy and not harmful to the donor baby and mother, thus UCB can be a useful candidate for MSPC sources. Interestingly, MSPC can be transplantable between HLA-incompatible individuals, because they do not elicit alloreactive lymphocyte proliferate responses and modulate immune responses [19,39–41]. Therefore, UCB can be used as a useful source for MSPC as an allogeneic cellular therapy agent, and further studies will be needed to make the criteria for selecting optimal UCB harvests before and/or during MSPC cultivation.

In conclusion, approximately one-quarter of UCB harvests developed MSPC-like cells during cultivation, and a small fraction (2.2%) of them yielded MSPC with a more than 1000-fold expansion capacity as well as multiple differentiation potentials. Although the incidence of UCB harvests producing MSPC in culture is low, the results may encourage the use of UCB as an attractive target for allogeneic cellular therapeutic options in tissue engineering.

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