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In vitro mesengenic potential of human umbilical cord blood-derived mesenchymal stem cells

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Abstract

Human mesenchymal stem cells (hMSCs) have been paid a great deal of attention because of their unprecedented therapeutic merits endowed by powerful ex vivo expansion and multilineage differentiation potential. Umbilical cord blood (UCB) is a convenient but not fully proven source for hMSCs, and hence, greater experience is required to establish UCB as a reliable source of hMSCs. To this end, we attempted to isolate hMSC-like adherent cells from human UCB. The isolated cells were highly proliferative and exhibited an immunophenotype of $CD13^+$ $CD14^-$ $CD29^+$ $CD31^-$ $CD34^-$ $CD44^+$ $CD45^-$ $CD49e^+$ $CD54^+$ $CD90^+$ $CD106^-$ $ASMA^+$ $SH2^+$ $SH3^+$ $HLA\text{-}ABC^+$ $HLA\text{-}DR^-$. More importantly, these cells, under appropriate conditions, could differentiate into a variety of mesenchymal lineage cells such as osteoblasts, chondrocytes, adipocytes, and skeletal myoblasts. This mesengenic potential assures that the UCB-derived cells are multipotent hMSCs and further implicates that UCB can be a legitimate source of hMSCs.
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Human mesenchymal stem cells (hMSCs) hold much promise as therapeutics for degenerative diseases or damages in connective tissues such as bone, cartilage, tendon, and muscle [1]. Unlike most other human adult stem cells, techniques for isolation from bone marrow (BM) and ex vivo expansion of hMSCs have been well established so that they can be obtained in quantities appropriate for clinical applications. In addition, in vitro and in vivo multi-lineage differentiation capability of BM-derived hMSCs has been rigorously demonstrated in the past few years [2,3].

In adult, hMSCs are prevalent in BM and dispersed over a variety of mesenchymal tissues. Of a particular concern, however, is the presence of hMSCs in the circulating blood system that provides a convenient way of cell collection. Several attempts to isolate hMSCs from either mobilized [4–6] or elutriated [7] adult peripheral blood have been only partly successful. Likewise, neonatal umbilical cord blood (UCB) has not been universally accepted as a reliable source of hMSC. Some investigators have suggested that full-term UCB lacks hMSC [6,8,9], whereas others have reported findings indicating that hMSCs reside in blood [10–12] as well as venous endothelium [13,14] of newborn umbilical cord. On the other hand, the presence of hMSC in prenatal blood has been least debated and the cells were shown to be rich in first and mid-trimester fetal blood [9,15].

Over other sources of stem cells, UCB has a number of advantages in cell procurement, such as vast abundance, lack of donor attrition, and low risk of transmission of herpes family viruses. Moreover, stem cells in this neonatal blood are less mature than other adult cells so that they do not produce strong immune rejection in unrelated donor transplantation. It is now known that the UCB graft can tolerate 1–2 mismatch in human

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leukocyte antigen (HLA) types between a donor and recipient, which expands significantly the available donor pool. More importantly, a large quantity of UCB units can be cryopreserved and maintained in public or private banks so that the unrelated donor UCB can be instantly available from the stored pool. Therefore, UCB-derived hMSCs, provided that they can be routinely isolated and expanded in culture, would be the most practical tool for stem cell-based therapy and transplantation.

In the present study, we have attempted to isolate adherent cells of a mesenchymal phenotype from human UCB sample and assessed the identity of cells by inducing them to differentiate into various cell types of a mesenchymal lineage, including osteocytes, chondrocytes, adipocytes, and skeletal myoblasts.

Materials and methods

Isolation and culture of UCB-derived adherent cells

A full-term UCB sample containing about 80 ml of blood was obtained with mother's consent and processed within 24 h of collection. Mononuclear cells (MNCs) were separated from UCB using Ficoll–Paque PLUS (Amersham Biosciences, Uppsala, Sweden) by centrifugation at 3000 rpm for 20 min and suspended in cell culture medium comprising LG (low glucose)-DMEM (Life Technologies, Gaithersburg, MD, USA), 15% fetal bovine serum (FBS, RH Biosciences, Lenexa, KS, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1% antibiotics–antimycotics (Life Technologies) after washing with HF2 (Hanks' balanced salt solution, Jeil Biotechservices, Daegu, Korea). The cells were plated onto Falcon flasks (Becton–Dickinson, San Jose, CA, USA) at a density of $1 \times 10^6/cm^2$. Suspended cells were removed after 5 days of culture, and adherent cells were continued to culture. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and culture medium was changed every 5 days. Cells were detached with 0.1% trypsin–EDTA (ethylenediaminetetraacetic acid, Sigma–Aldrich, St. Louis, MO, USA) when they reached their 50–60% confluence and replated at a density of $2 \times 10^3/cm^2$ in Falcon culture flasks. Cells cultured for 4–7 passages were used for further cellular analyses and differentiation experiments.

Cell cycle analysis and proliferation studies

For cell cycle analysis, cells were trypsinized, washed with cold PBS (phosphate-buffered saline, Gibco, Grand Island, NY, USA), fixed with 70% ethanol/PBS overnight at 4 °C, and centrifuged. The pellets were resuspended in 500 μl PBS in the presence of 50 μg/ml propidium iodide and 1 mg/ml DNase-free RNase A and incubated in the dark for 30 min at room temperature. Cell cycle status was determined using flow cytometry (Beckman Coulter Epics XL, Miami, FL, USA) and analyzed with MultiCycle software for the proportions of cells in G_1, S, and G_2/M phases. For proliferation studies, cells were detached and replated at a density of 1×10^4/ml in culture medium. Cell viability was measured every 24 h after seeding by trypan blue exclusion assay.

Immunophenotyping of UCB-derived adherent cells by flow cytometry

For flow cytometric analysis, the cells were harvested by treatment with 0.1% trypsin–EDTA, and detached cells were washed with PBS and incubated at 4 °C for 20 min with the following cell-specific antibodies; CD13, CD14, CD29, CD31, CD34, CD44 (β_1 integrin), CD45, CD49e (α_5-integrin), CD54 (ICAM-1), CD90 (Thy-1), CD106, α-smooth muscle actin (ASMA), SH2 (CD105, endoglin), SH3 (CD73), HLA-ABC, and HLA-DR, all of which were conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Becton–Dickinson). For staining with a monoclonal mouse anti-human ASMA antibody, cells were first permeabilized with cold methanol/PBS for 2 min at −20 °C. After a wash with cold PBS, cells were incubated with mouse anti-AMSA antibody at 4 °C for 30 min followed by staining with a secondary antibody, anti-mouse-IgG-FITC (Becton–Dickinson), for another 20 min. Mouse IgG_1-FITC and IgG_1-PE were used as isotype controls. Labeled cells were assayed by flow cytometry and analyzed with System II Software.

Differentiation of UCB-derived hMSCs

Osteogenic differentiation and evaluation. Adherent cells were cultured in osteogenic medium consisting of LG-DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.1 μM dexamethasone (Sigma–Aldrich), and 50 μM ascorbate for 2 weeks. Osteogenic differentiation was evaluated by alkaline phosphatase (ALP) staining and calcium deposition. For ALP staining, the mono-layered cells were prefixed with 4% formaldehyde and added with Western blue stabilized substrate (Promega, Madison, WI, USA) for 30 min at room temperature. Deposited calcium was stained for 15 min at room temperature with 1% alizarin red S (Sigma–Aldrich). Expression of osteoblast-specific genes, such as osteopontin and ALP, was also measured by the reverse transcriptase-polymerase chain reaction (RT-PCR).

Chondrogenic differentiation and evaluation. For chondrogenic differentiation, about 5×10^6 cells in the 15 ml polypropylene tube were centrifuged at 1000 rpm for 5 min to form a pelleted micromass in the bottom of the tube and incubated for up to 5 weeks with chondrogenic medium consisting of LG-DMEM supplemented with 1 mM pyruvate, 0.1 mM ascorbate 2-phosphate, 100 nM dexamethasone, ITS^+ premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenious acid, 5.35 μg/ml linoleic acid, and 1.25 mg/ml bovine serum albumin), and 10 ng/ml recombinant human TGFβ1, TGFβ2, and TGFβ3 (Sigma–Aldrich). Chondrogenic differentiation was verified by histochemical staining of micromasses with safranin red O (Sigma–Aldrich). Expression of chondrocyte-specific genes, such as Runx2, SOX9, and aggrecan, was also measured by RT-PCR.

Adipogenic differentiation and evaluation. To induce adipogenic differentiation, cells were incubated for 2 weeks in adipogenic medium consisting of LG-DMEM supplemented with 0.5 mM 3-isobutyl-1-methylxantine (IBMX, Sigma–Aldrich), 1 μM hydrocortisone (Sigma–Aldrich), 0.1 mM indomethacine (Sigma–Aldrich), and 10% rabbit serum (Sigma–Aldrich). Cell morphology was examined under a phase-contrast microscope in order to confirm the formation of neutral lipid vacuoles. The presence of neutral lipids was visualized by staining with oil-red O. Adipogenic differentiation of the cells was also investigated by the RT-PCR analysis of PPARγ, a specific transcription factor involved in adipogenesis.

Myogenic differentiation and evaluation. For skeletal myogenic differentiation, cells were cultured for up to 6 weeks in the myogenic medium consisting of LG-DMEM supplemented with 5% horse serum, 0.1 μM dexamethasone, and 50 μM hydrocortisone. Myogenic differentiation was analyzed by the flow cytometric analysis for myosin heavy chain (MyHC) and RT-PCR analysis for MyoD1 and myogenin. For detection of MyHC, an intracellular protein, cells were permeabilized with cold methanol/PBS for 2 min at −20 °C followed by staining with mouse anti-myosin antibody (Sigma) and FITC-conjugated rat anti-mouse IgG_1 (Becton–Dickinson) at 4 °C for 20 min.

Total RNA extraction and RT-PCR

To detect mRNA levels of specific genes related to each differentiation event, about 1×10^6 differentiated and undifferentiated

Table 1
Pimers and annealing temperatures used for RT-PCR

Primer	Sequence	Annealing temperature (°C)
Osteopontin	F 5′-CAC ATC GGA ATG CTC ATT GC-3′ R 5′-ATC ACC TGT GCC ATA CCAGT-3′	56
ALP	F 5′-CTG GTA GGC GAT GTC CTT A-3′ R 5′-ACG TGG CTA AGA ATG TCA TC-3′	56
SOX9	F 5′-CAG GAG AAC ACG TTC CCC AAG-3′ R 5′-CAG CGC CTT GAA GAT GGC GTT-3′	55
Runx2	F 5′-CGC TCC GGC CCA CAA ATC TC-3′ R 5′-CCG CAC GAC AAC CGC ACC AT-3′	56
Aggrecan	F 5′-GCC TTG AGC AGT TCA CCT-3′ R 5′-CTC TTC TAC GGG GAC AGC-3′	55
MyoD	F 5′-AAG CGC CAT CTC TTG AGG TA-3′ R 5′-GCG CCT TTA TTT TGA CC-3′	60
Myogenin	F 5′-TAA GGT GTG TAA GAG GAA GTC G-3′ R 5′-CCA CAG ACA CAT CTT CCA CTG T-3′	60
PPARγ	F 5′-GCT GTT ATG GGT GAA ACT CT-3′ R 5′-ATA AGG TGG AGA TGC AGG CT-3′	55
GAPDH	F 5′-CCC ATC ACC ATC TTC CAG GA-3′ R 5′-TTG TCA TAC CAG GAA ATG AGC-3′	57

(control) cells were harvested and washed once in cold PBS. Total RNA was extracted using RNEasy Mini isolation kit (Qiagen, Valencia, CA, USA) according to the provided protocol. The first strand complementary DNA (cDNA) was synthesized using RNA PCR kit (Takara Bio, Shiga, Japan). The initial denaturation was performed at 95 °C for 5 min. PCR amplification was carried out at 95 °C for 30 s, at annealing temperature for 30 s, and 72 °C for 1 min for a total of 35 cycles and final extension at 72 °C for 7 min using DNA Engine Dyad Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). The sense and antisense primers and annealing temperature were listed in Table 1. The PCR products were fractionated by 1% GenproTech (Genpro Technologies, Pleasant, PA, USA) agarose gel electrophoresis and the bands were visualized by ethidium bromide (EtBr) staining and photographed with Chemi Doc XRS (Bio-Rad Laboratories, Hercules, CA, USA).

Results

Morphological and proliferative characteristics of human UCB-derived adherent cells

A MNC fraction obtained from the full-term UCB harvest yielded an adherent layer of heterogeneous cells in the primary culture, but upon the first passage of culture, highly proliferative fibroblast-like cells arose and became predominant over cells of other types. Between 2 and 3 passages, the adherent cells were composed almost of bipolar fibroblast-like cells that could later grow to confluence (Fig. 1A). The cell cycle studies revealed that approximately 14% of cells were actively involved in proliferation while the rest of cells were in the phase of G_0/G_1 (Fig. 1B). Cell proliferation studies indicated that the cells doubled themselves every ~60 h (Fig. 1C). These characteristics of cells closely resemble those of hMSCs isolated from BM and UCB by other investigators [10,16].

Immunophenotypes of UCB-derived adherent cells

To verify the nature of these adherent cells, cells were culture expanded, labeled against a variety of cell surface and intracellular antigens, and analyzed by flow cytometry. As results, it was found that the cells expressed neither hematopoietic lineage markers such as CD14, CD34, and CD45, nor endothelial markers such as CD31 and CD106 (V-CAM) (Fig. 2A). Instead, they were all positive for MSC-related antigens such as CD13, CD29 (β_1-integrin), CD44, CD49e (α_5-integrin), CD54 (ICAM-1), CD90 (Thy-1), AMSA, SH2 (CD105/endoglin), and SH3 (CD73) (Fig. 2B). And the cells were positive for HLA class I but negative for HLA-DR (Fig. 2A). These flow cytometric profiles are in accord with the immunophenotype of BM- and UCB-derived hMSCs revealed by other investigators [3,6,9–15], leading us to conclude that the adherent cells prepared in this study represent hMSCs. This suggests that upon a serial passage of adherent cells, a homogeneous population of hMSCs can be obtained from a MNC fraction of human UCB.

UCB-derived hMSCs exhibit in vitro osteogenic and chondrogenic potential

Incubation of UCB-derived hMSCs under the osteogenic condition for 2 weeks resulted in a dramatic increase in alkaline phosphatase (ALP) activity and accumulation of calcium deposit, as assessed by ALP staining and alizarin red S staining, respectively (Fig. 3A). This osteogenic potential was further confirmed by the RT-PCR analysis where expressions of two osteoblast-specific genes, osteopontin and ALP, were shown to increase gradually upon exposure to osteogenic medium (Fig. 3B).

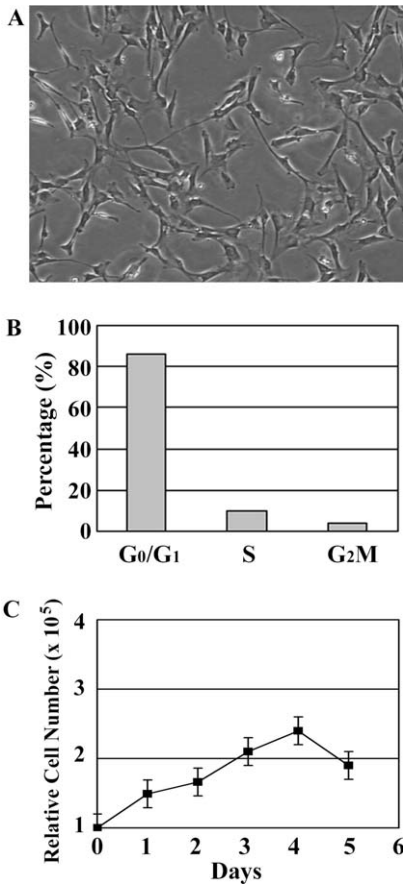

Fig. 1. Morphology and proliferative characteristics of adherent cells isolated from UCB. (A) Morphology of a homogeneous cell population in the fourth passage that was isolated from human UCB. Magnification 40×. (B) Cell cycle analysis by flow cytometry. Percentages of cells in G_0/G_1, S, and G_2/M phases were indicated. (C) Growth curve of UCB-derived cells. The cell numbers of three different cell cultures ($n=3$) were measured by a trypan blue exclusion assay and expressed as means ± SD of three experiments. An average population doubling time was estimated to be around 30 h.

Incubation of pelleted hMSCs in chondrogenic medium for 5 weeks yielded a multi-layered matrix-rich morphology and accumulation of the sulfated proteoglycans as evidenced by staining with safranin red O (Fig. 3C). This chondrogenic differentiation was further confirmed by the RT-PCR analysis of Runx2 and SOX9, two transcription factors involved in chondrocyte differentiation, as well as aggrecan, a chondrocyte-specific gene product. Expression of both Runx2 and aggrecan was observed in the first week of induction while mRNA of SOX9 appeared after 2 weeks, and then expression of all of these molecules gradually increased for up to 4 weeks.

UCB-derived hMSCs exhibit in vitro adipogenic and myogenic potential

Incubation of UCB-derived hMSCs with adipogenic agents for 2 weeks resulted in notable morphological changes of cells including the formation of lipid droplets around nuclei (Fig. 3E), and strong positive staining of oil red O (Fig. 3E). The RT-PCR analysis showed that expression of PPARγ, a transcription factor important for adipogenesis, increased significantly after 2 weeks (Fig. 3F).

Incubation of UCB-derived hMSCs in myogenic medium for 6 weeks produced cells that were highly immunoreactive to a monoclonal antibody against MyHC (Fig. 3G). The flow cytometry analysis also showed that this protein was expressed in more than a half of hMSCs at the 6th week (Fig. 3G). Skeletal myogenic differentiation was also demonstrated by the RT-PCR analysis of expression of MyoD and myogenin, two important transcription factors in myogenic differentiation. Their mRNAs were visible at the 1st week of induction but disappeared from the 2nd week (Fig. 3H), which is consistent with skeletal muscle development where these proteins are involved in early but not late myogenesis.

Discussion

By definition, hMSCs are self-renewable and have the potential to differentiate along a mesengenic lineage. Adherent fibroblast-like cells isolated from human UCB in this study appeared to be hMSCs, as judged by morphological and proliferative characteristics, as well as immunophenotype analysis. In order to further verify the identity of these UCB-derived cells, the mesenchyme-related multipotency was investigated.

Osteogenesis of the cells was proven by the detection of increased ALP activity and calcium deposits, and also elevated gene expression of ALP and osteopontin. Chondrogenic differentiation was confirmed by observing a multi-layered matrix-rich morphology and accumulated sulfated proteoglycan in pellet culture, and also increased expression of Runx2, SOX9, and aggrecan. Adipogenesis was manifested by appearance of lipid droplets and strong PPARγ expression. And finally, skeletal myogenesis was evaluated by morphological changes and increased expression of myosin, MyoD, and myogenin. Taken together, these results indicated that the cells, under appropriate conditions, could differentiate into cells of osteogenic, chondrogenic, adipogenic, and skeletal myogenic lineages.

Since these four lineages cover the most, if not all, of the mesengenic process, the observed multipotency demonstrates that the adherent cells prepared in this study are truly hMSCs, and this in turn corroborates the view that neonatal UCB does contain hMSCs. The existence of hMSCs in full-term UCB has been a source of dispute for many years among several groups of investigators. It has been reported that homogeneous cell populations of hMSCs could be successfully isolated from full-term UCB samples by Erices et al. [10], Goodwin et al. [11], and Lee et al. [12], but a series of studies by Wexler

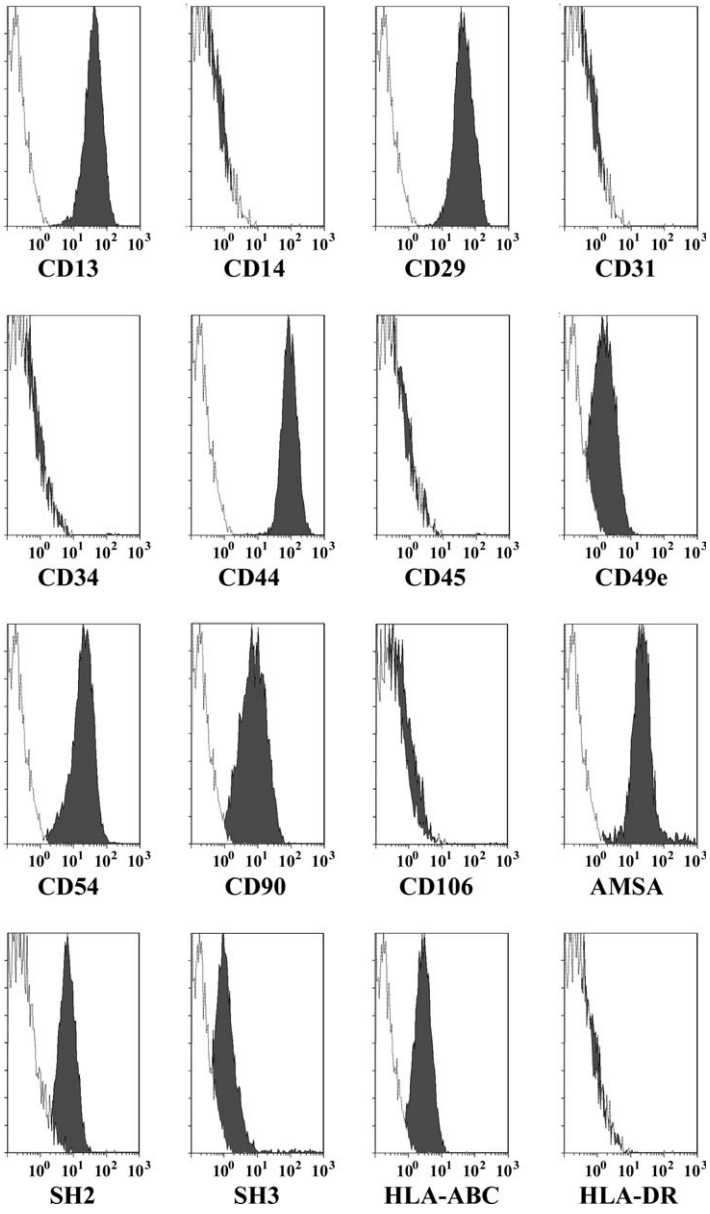

Fig. 2. Immunophenotypes of adherent cells derived from UCB. Flow cytometric analysis of hematopoietic (CD14, CD34, and CD45), endothelial (CD31 and CD106), and mesenchymal (CD44, CD49e, CD54, CD90, and ASMA) lineage markers as well as human leukocyte antigens (HLA-ABC and HLA-DR) on UCB-derived adherent cells. An open profile represents an isotype control for background fluorescence and a shaded one shows a positive signal.

et al. [6], Mareschi et al. [8], and Yu et al. [9] failed to obtain hMSCs from newborn UCB. It remains unknown what led these contradictory results, but it is most likely that frequency of hMSCs in the neonatal circulating blood system is so low that their survival/death is largely affected by minute differences in culture conditions between different laboratories.

Among a wide variety of human tissues, BM has been regarded as a primary source for hMSCs. Functional and differentiation properties of BM-derived hMSCs have been extensively studied over the past 3 decades [2,17], and also their clinical applications have been recently launched with the aim to cure diseases such as osteogenesis imperfecta [18,19], myocardial infarction [20,21], metachromatic leukodystrophy, and Hurler syndrome [22]. However, the wider use of BM in clinical studies has been impeded by a number of disadvantages, such as donor-invasive procurement, tight HLA restriction, and high risk of transmitting viral diseases. Our results here showed that UCB-derived hMSCs were highly similar to BM-derived hMSCs, with respect to cell characteristics and multilineage differentiation potential, suggesting that UCB can be an alternative source of hMSCs to BM. Therefore, given that a methodology was developed to derive hMSCs from most, if not all, UCB, UCB-derived hMSCs may be the most practical

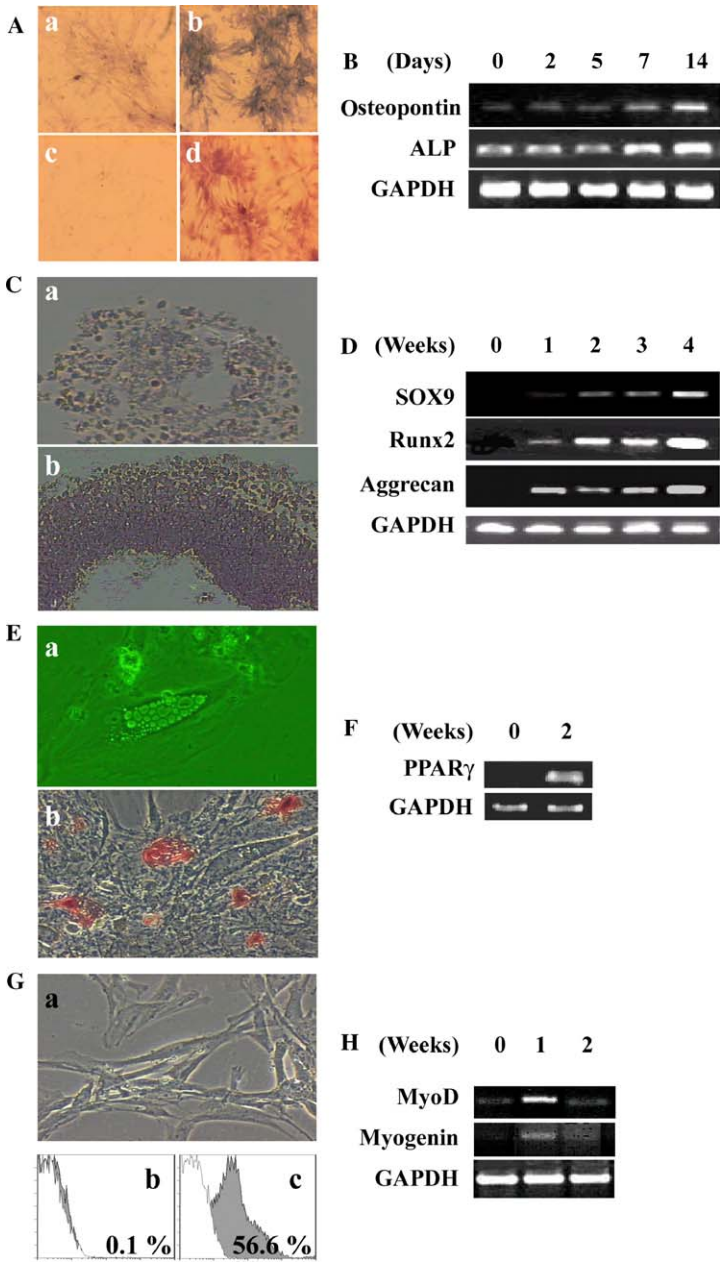

Fig. 3. Mesengenic differentiation of UCB-derived hMSCs. (A) Cytochemical analysis of osteogenic differentiation. For detection of ALP activity, the cells incubated for 2 weeks in regular culture medium (a, control) and osteogenic medium (b) were investigated by ALP staining. For visualizing calcium deposits, the cells cultured for 2 weeks in culture medium (c, control) and osteogenic medium (d) were stained by alizarin red S. Magnification 40×. (B) RT-PCR analysis of gene expression of ALP and osteopontin in the cells incubated in osteogenic medium during the first one week. (C) Histochemical analysis of chondrogenic differentiation in pellet culture. The pelleted cells were incubated for 5 weeks in regular culture medium (a, control) and chondrogenic medium (b), and stained with safranin red O to visualize sulfated proteoglycan. Magnification 40×. (D) RT-PCR analysis of gene expression of SOX9, Runx2, and aggrecan in the cells incubated in chondrogenic medium during four weeks. (E) Phase contrast microscopic images of the cells cultured for 2 weeks in adipogenic medium (a) and their positive staining with oil-red O (b). Magnification 100×. (F) RT-PCR analysis of gene expression of PPARγ. (G) Phase contrast microscopic image (a) and flow cytometric analysis of expression of MyHC in cells incubated for 6 weeks in regular culture medium (b, control) or myogenic medium (c). Magnification 100× (H) RT-PCR analysis of MyoD and myogenin in cells incubated for 2 weeks in myogenic medium.

tool for treatment of diseases and damages in a variety of connective tissues.

In addition to mesenchyme-related multipotency reported in this study, it was found that the UCB-derived cells were able to rapidly differentiate along a neuroectodermal lineage, as previously reported [23]. This finding is in line with a recent report demonstrating that UCB-derived hMSCs can differentiate into other germ layer cells such as hepatic cells of endodermal origin and neuroglial cells of neuroectodermal origin [12], as well as a

substantial body of evidence that the neural cells can be derived in vitro and in vivo from BM-derived hMSCs (for review see [24]). All of these suggest that cell fates of BM- and UCB-derived hMSCs are not confined to a mesoderm layer, but can traverse embryonic germ layer boundaries. This plasticity of hMSCs and the molecular mechanisms underlying it should be more extensively studied in the future.

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