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Dissimilar Differentiation of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, and Adipose Tissue

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Mesenchymal stem cells (MSCs) have been investigated as promising candidates for use in new cell-based therapeutic strategies such as mesenchyme-derived tissue repair. MSCs are easily isolated from adult tissues and are not ethically restricted. MSC-related literature, however, is conflicting in relation to MSC differentiation potential and molecular markers. Here we compared MSCs isolated from bone marrow (BM), umbilical cord blood (UCB), and adipose tissue (AT). The isolation efficiency for both BM and AT was 100%, but that from UCB was only 30%. MSCs from these tissues are morphologically and immunophenotypically similar although their differentiation diverges. Differentiation to osteoblasts and chondroblasts was similar among MSCs from all sources, as analyzed by cytochemistry. Adipogenic differentiation showed that UCB-derived MSCs produced few and small lipid vacuoles in contrast to those of BM-derived MSCs and AT-derived stem cells (ADSCs) (arbitrary differentiation values of 245.57 ± 943 and 243.89 ± 145.52 μm^2 per nucleus, respectively). The mean area occupied by individual lipid droplets was 7.37 μm^2 for BM-derived MSCs and 2.36 μm^2 for ADSCs, a finding indicating more mature adipocytes in BM-derived MSCs than in treated cultures of ADSCs. We analyzed FAPB4, ALP, and type II collagen gene expression by quantitative polymerase chain reaction to confirm adipogenic, osteogenic, and chondrogenic differentiation, respectively. Results showed that all three sources presented a similar capacity for chondrogenic and osteogenic differentiation and they differed in their adipogenic potential. Therefore, it may be crucial to predetermine the most appropriate MSC source for future clinical applications. Exp Biol Med 233:901–913, 2008

Key words: mesenchymal stem cells; bone marrow; umbilical cord blood; adipose tissue; differentiation

Introduction

Mesenchymal stem cells (MSCs) comprise a population of multipotent progenitors capable of supporting hematopoiesis and differentiating into many tissues (1). MSCs are not ethically restricted and have low immunogenicity (2). MSCs are thought to be promising candidates for novel cell-based therapeutic strategies such as the repair of mesenchyme-derived tissues. In fact, MSCs have already been clinically used to repair or regenerate somatic tissues (3–6), to promote engraftment, and to prevent or treat severe graft-versus-host disease in allogeneic stem cell transplantation (7–8). In the appropriate microenvironment, MSCs differentiate into various cell types, including adipocytes, osteoblasts, chondrocytes (9–11), cardiomyocytes (12–14), and also into nonmesodermal-derived cells, including hepatocytes and neurons (15). Selective differentiation is dependent on specific environmental effectors: usually a combination of growth factors and cytokines supplied *in vitro* (1, 16). MSCs were originally isolated from bone marrow (BM) by Friedenstein *et al.* (17); however, similar populations have been reported in other tissues, such as peripheral blood (18), cord blood (19), trabecular bone (20),

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adipose tissue (1), synovium (21), skin (22), muscle, and brain (23).

MSCs have been characterized by their fibroblast-like morphology, plastic-adhesive and self-renewal properties, and their ability to differentiate *in vitro* into at least three mesodermal-derived tissues: bone, cartilage, and fat (1). Immunophenotypically, MSCs have been defined as cells expressing CD29, CD44, CD90, and CD105 and lacking hematopoietic lineage markers and HLA-DR (9–11). However, recent studies have demonstrated that MSCs isolated from several sources are not a homogenous population and that their differentiation potential may vary depending on the source and the donor (11, 24, 25). Unfortunately, the factors affecting these differences are still unknown. BM has been considered the main MSC source because of their potential to both proliferate and differentiate (3, 7). However, other sources of similar cell populations are being investigated, because BM-derived MSC isolation requires a painful and invasive procedure, the frequency of MSCs is low (1), and their ability to proliferate and differentiate decline with age (26).

Human umbilical cord blood (UCB)-derived MSCs are being evaluated for use in cellular therapies because they are ontogenically primitive, are less exposed to immunologic challenges, are abundantly available, and can be harvested without risk to the donor (27). Various reports are conflicting in relation to the presence of MSCs in UCB (28–30); however, several groups have successfully isolated MSCs from UCB (11, 15, 24, 31–35). The frequency of mesenchymal progenitors in the UCB of full-term deliveries is extremely low (0.00003% of nucleated cells) (31); however, these progenitors have the highest expansion potential when compared with that of other sources (11, 25).

Adipose tissue has recently been identified as a convenient alternative source of MSC-like cells. Adipose tissue–derived stem cells (ADSCs) are available in quantities of hundreds of million cells per individual (9), have an extensive self-renewal capacity (36), are easily isolated by differential sedimentation, and can be cultured for several months *in vitro* with low levels of senescence (37). ADSCs also have the potential to differentiate into various cells, including adipocytes, osteoblasts, chondrocytes, neurons, and multinucleated myocytes in response to lineage-specific induction factors (10, 11, 37–41).

The starting population for most of the trans-differentiation experiments is different; therefore, comparing results between various groups is difficult and may also partly account for the lack of reproducibility in some of the initial reports (10). MSCs are poorly defined, and this has led separate groups to assign diverse names and phenotypes to this cell population (42). Thus, a precise characterization of MSC and its properties relating to molecular differentiation represents an absolute condition for future development and exploitation of stem cell research for clinical applications (10, 11).

In this study we characterized for the first time adult stem cells isolated from three sources (BM, UCB, and AT) by flow cytometry and compared their differentiation properties to adipocytes, osteoblasts, and chondrocytes by cellular (cytochemistry) and molecular (reverse transcriptase polymerase chain reaction [RT-PCR]/quantitative polymerase chain reaction [qPCR]) approaches.

Materials and Methods

Collection of BM, UCB, and AT. Human BM stromal cells were obtained from the iliac crest of 10 patients with dilated cardiomyopathy who were aged between 50 and 70 years (60.36 ± 9.86 years) and who had applied for a stem cell transplantation procedure. About 5 ml of BM aspirate were collected in a syringe containing 10,000 IU heparin to prevent coagulation.

UCB units from full-term deliveries ($n = 10$) were collected from unborn placenta by a standardized procedure using syringes that contained anticoagulant citrate dextrose, and were processed within 12 hrs after collection. Donors faced no complications throughout their pregnancy.

AT was obtained from 10 donors, aged between 26 and 50 years (38.0 ± 12.55 years), who were undergoing elective bariatric surgery and dermolipectomy procedures. Typically, 100 ml of AT was processed.

All samples of BM, UCB, and AT were collected after informed consent was obtained in accordance with the guidelines on the use of human subjects, as approved by the ethics committee of Pontifícia Universidade Católica of Paraná (approval number 597).

Isolation and Culture of Adherent Cells. Three sources of adherent cells were used in this work.

BM. The aspirate was diluted 1:3 with Iscove's modified Dulbecco's medium (IMDM) (Gibco Invitrogen, Grand Island, NY) and carefully loaded onto Histopaque (1.077 g/ml) (Sigma Chemical Co., St. Louis, MO) to isolate BM mononuclear cells (MNCs). MNCs were isolated by density gradient centrifugation (400 *g*, 30 mins, room temperature) and washed twice with IMDM (43). BM-derived MNCs were cultured at a density of 1×10^5 cells/cm^2 in T75 culture flasks (TPP, Trasadingen, Switzerland) at 37°C in a humidified atmosphere that contained 5% CO_2; IMDM supplemented with 15% fetal calf serum (FCS) (Gibco Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Gibco Invitrogen) were also used. The culture medium was changed to remove the remaining nonadherent cells 2 days after the initial plating. Thereafter, the culture medium was replaced twice each week.

UCB. UCB MNCs were isolated by using two methods. In the first, each UCB unit was diluted 1:3 and processed as described for BM. The second was performed by using a commercially available kit (RosetteSep, Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's instructions; Histopaque density separation as described (15) followed. UCB-derived MNCs were set in culture at a density of 6×10^5 cells/cm^2 in T75 culture flasks in the same culture medium described in the preceding section. The cultures were incubated for 4 days at 37°C in a humidified atmosphere containing 5% CO_2. Nonadherent cells were then removed, and fresh medium was added to the flasks. Culture medium was removed by complete exchange every 7 days.

AT. ADSCs were isolated by using enzymatic digestion. In brief, 100 ml AT was washed with sterile phosphate-buffered saline (PBS) (Gibco Invitrogen). A one-step digestion by 1 mg/ml collagenase type I (Invitrogen) was performed for 30 mins at 37°C during permanent shaking and was followed by filtration through first a 40- and then 100-μm mesh filter (BD FALCON, BD Biosciences Discovery Labware, Bedford, MA, USA). The cell suspension was centrifuged at 800 *g* for 10 mins, and contaminating erythrocytes were removed by erythrocyte lysis buffer, pH 7.3. The cells were washed and then cultivated at a density of 1×10^5 cells/cm^2 in T75 culture flasks in DMEM-F12 (Gibco Invitrogen) supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) (44). The medium was changed 2 days after the initial plating. The culture medium was then replaced twice each week.

BM-derived and UCB-derived MSCs and ADSCs were subcultured after the cultures had reached 80% to 90% confluence; MSCs were detached by treatment with 0.25% trypsin/EDTA (Invitrogen) and were replated as passage-1 cells (the process was then continued as previously described).

Determination of the Cell-Surface Antigen Profile. BM-derived and UCB-derived MSCs and ADSCs, between the third and fifth passages (P_3 through P_5), were labeled with antibodies against several human proteins to analyze cell-surface expression of typical marker proteins: nonconjugated CD105; CD90, CD44, and CD31, each of which was conjugated with fluorescein isothiocyanate (FITC); CD73, CD166, and CD34, each of which was conjugated with phycoerythrin (PE); CD29, CD117, and CD14, each of which was conjugated with allophycocyanin (APC; BD Pharmingen, CA, USA); and CD45 conjugated with peridinin chlorophyll protein (PerCP; BD Pharmingen, San Diego, CA). Cells were detached by treatment with 0.25% trypsin/EDTA, washed with PBS, and incubated in the dark for 30 mins at room temperature with the respective antibody. Cells were then washed with wash flow buffer and resuspended in 500 μl of 1% formaldehyde solution. For the detection of CD105, cells were further washed and incubated for 15 mins with a secondary antibody Goat F(ab′)2 anti-human IgG (gamma) (Caltag Laboratories, Burlingame, CA) (45). Mouse isotype IgG1 antibodies were employed as controls (BD Pharmingen). Approximately 20,000 labeled cells were passed through a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and were analyzed by FlowJo software (Flowjo, Ashland, Oregon, USA).

Differentiation Procedures. BM-derived and UCB-derived MSCs and ADSCs were assessed for their potency by inducing their differentiation into adipocytes, osteoblasts, and chondrocytes. Cells between P_3 and P_5 from each source were incubated with three differentiation media.

Adipogenic Differentiation. Subconfluent (80%) MSCs were seeded on glass coverslips (Sarstedt, Newton, NC, USA) in 24-well plates (TPP) and were treated with three types of media: medium 1 consisted of 0.05 μ*M* dexamethasone (Sigma Chemical Co.), 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 60 μ*M* indomethacin (Sigma-Aldrich) in DMEM-HG (Gibco Invitrogen) with 15% FCS (46); medium 2 consisted of 1 μ*M* dexamethasone, 5 μg/ml insulin, 60 μ*M* indomethacin in IMDM with 15% FCS (46); and medium 3 consisted of Poietics Differentiation Basal Medium Adipogenic (Cambrex BioScience, Walkersville, MD) supplemented with hMSC Adipogenic SingleQuots (Cambrex BioScience). Adipogenic differentiation was induced by cyclic changes; the maintenance medium that contained the adipogenic inducer was changed every 3 days during 3 weeks. Oil Red O (Sigma-Aldrich) was used to visualize lipid-rich vacuoles. Briefly, cells were treated with Bouin's fixative (Biotec, Labmaster, Paraná, Brazil) for 10 mins at room temperature, washed twice with 70% ethanol and once with Milliq water, and stained with a solution of 0.5% Oil Red O (Sigma-Aldrich) for 1 hr. Hematoxylin-eosin (HE) (Biotec) was used for nuclear staining. Control cells were kept in IMDM medium with 15% FCS. To quantitatively analyze adipogenic differentiation, 70 fields in three biological replicates from each source of MSCs were counted by using Image-Pro Plus version 4.5. We also performed RT-PCR and qPCR to estimate the level of adipocyte-specific FABP4 mRNA in induced (medium 2) and noninduced (negative control) cultures.

Osteogenic Differentiation. Cells were seeded and cultured on slides placed in 24-chamber plates (TPP) to induce osteogenic differentiation. Subconfluent (80%) cultures were subjected to three types of osteogenic medium: medium 4 consisted of 0.1 μ*M* dexamethasone, 10 m*M* β-glycerolphosphate (Sigma-Aldrich), and 50 μ*M* ascorbate in DMEM-HG with 15% FCS (16); medium 5 consisted of 0.1 μ*M* dexamethasone, 10 m*M* β-glycerolphosphate, 100 μ*M* ascorbate, and IMDM with 15% FCS (16); and medium 6 consisted of Poietics Differentiation Basal Medium Osteogenic (Cambrex BioScience) supplemented with hMSC Osteogenic SingleQuots (Cambrex BioScience). Media were replaced every 3 days over a 3-week period. Induced monolayers were fixed for 10 mins in Bouin's fixative (Biotec) and washed (twice with 70% ethanol and once with Milliq water). Monolayers were then incubated for 15 mins with Alizarin Red S at pH 7.0 and pH 4.2 (Fluka Chemie, Buchs, UK) at room temperature to evaluate calcium accumulation. Light green (Sigma-Aldrich) was used to counterstain. Control cells were kept in IMDM with 15% FCS over the same period. In addition, RT-PCR and qPCR were performed to estimate the level of

Table 1. Primer Sets Used for RT-PCR and qPCR

Gene		Sequence (5′-3′)	Accession no.	Amplicon (bp)
GAPDH	Forward:	GGCGATGCTGGCGCTGAGTAC	2597	150
	Reverse:	TGGTTCACACCCATGACGA		
FABP4	Forward:	ATGGGATGGAAAATCAACCA	2167	97
	Reverse:	GTGGAAGTGACGCCTTTCAT		
Osteonectin	Forward:	ACATCGGGCCTTGCAAATACATCC	6678	437
	Reverse:	GAAGCAGCCGGCCCACTCATC		
ALP	Forward:	TACAAGGTGGTGGGCGGTGAACGA	249	92
	Reverse:	TGGCGCAGGGGCACAGCAGAC		
Collagen type II, α1	Forward:	CCGGGCAGAGGGCAATAGCAGGTT	1280	128
	Reverse:	CAATGATGGGGAGGCGTGAG		

the osteoblast-specific osteonectin and alkaline phosphatase (ALP) mRNA in MSCs cultured in induction medium (medium 5) and in noninduction or control medium.

Chondrogenic Differentiation. Cells were grown in micromass culture to promote chondrogenic differentiation (47). Briefly, 2×10^5 cells in 0.5 ml of medium were centrifuged at 300 *g* for 10 mins in a 15-ml polypropylene tube to form a pellet. Without disturbing the pellet, cells were cultured for 21 days in three different chondrogenic media: medium 7 consisted of DMEM-HG supplemented with 15% FCS and 0.01 μ*M* dexamethasone, 397 μg/ml ascorbic acid-2-phosphate (Sigma-Aldrich), 1 m*M* sodium pyruvate (Gibco Invitrogen), 10 ng/ml TGF-β1 (Sigma-Aldrich), and 1% insulin-transferrin-selenium-X (Gibco Invitrogen) (27); medium 8 consisted of DMEM-HG supplemented with 1% FCS and 10 ng/ml TGFβ1, 0.5 μg/ml of insulin, 50 μ*M* ascorbic acid (27); and medium 9 consisted of Differentiation Basal Medium Chondrogenic supplemented with hMSC Chondrogenic SingleQuots. Media was changed every 3 days. On day 21, cell aggregates were fixed in 10% formaldehyde for 1 hr at room temperature, dehydrated in serial ethanol dilutions, and embedded in paraffin blocks. Paraffin sections (4-μm thick) were stained for histologic analysis with HE, Mallory (Biotec), or Toluidine Blue solution (Sigma-Aldrich) to demonstrate the presence of intracellular matrix mucopolysaccharides. Chondrogenic differentiation was further confirmed by RT-PCR analysis of the chondrocyte-specific protein collagen type II mRNA in induced (medium 8) and noninduced cultures.

Total RNA Extraction and RT-PCR. Total RNA was obtained with the RNeasy kit (QIAGEN, Austin, TX) and treated in column with DNAse I (QIAGEN). Concentrations were determined by spectrophotometry (GeneQuant, Amersham Biosciences, Sunnyvale, CA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using 1 μl of 10 μ*M* oligo-dT primer (USB Corporation, Cleveland, OH) and 1 μl of reverse transcriptase (IMPROM II, Promega, Fitchburg, WI) according to the manufacturers' instructions. PCR was carried out with 20 ng of cDNA as template, 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 5 pmol of primers (10 pmol for the *FABP4* gene; Table 1), 2.5 m*M* $MgCl_2$, 0.0625 m*M* dNTPs, and 1 unit *Taq* polymerase (Invitrogen). The oligonucleotide primer sets used for PCR and the amplicon size are depicted in Table 1. PCR included heating at 94°C for 2 mins, and the heating was followed by 30 cycles of 94°C for 15 secs, 55°C for 30 secs, 72°C for 40 secs, and a final extension of 72°C for 3 mins by using a Bio-Cycler II thermocycler (Peltier Thermal Cycler; Bio-Rad, Hercules, CA). Ten microliters of RT-PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed under ultraviolet illumination (UV White Darkroom, UVP Bioimaging Systems, Upland, CA).

qPCR. Quantitative PCR was performed by using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Amplifications were carried out in a final reaction volume of 20 μl with the SYBR Green master mix (Applied Biosystems), 10 ng cDNA template, and 5 pmol primers (10 pmol for *FABP4*). PCR conditions were 50°C for 2 mins and 95°C for 10 mins, and this initial step was followed by 45 cycles of 95°C for 15 secs, 60°C for 30 secs, and 72°C for 40 secs. The melting curves were acquired after PCR to confirm the specificity of the amplified products. A standard curve based on cycle threshold values was used to evaluate gene expression. In brief, we used 1:5 dilutions of known concentrations of cDNA in triplicate to generate curves extending from 50 pg to 80 ng cDNA. We generated standard curves for each gene, including the control (housekeeping) gene. The relative amount of gene expression for each sample was normalized by dividing the value obtained for the analyzed gene by the value obtained for each control gene. Results were analyzed as gene expression relative to the housekeeping gene expression. Differences in expression were observed by comparing cells induced to differentiate with control samples that had not been induced (48).

Statistical Analysis. Continuous variables were presented as means ± standard deviations, and categorical variables were presented as frequencies and percentages. Comparisons between BM-derived and UCB-derived MSCs and ADSCs were performed by using the nonparametric

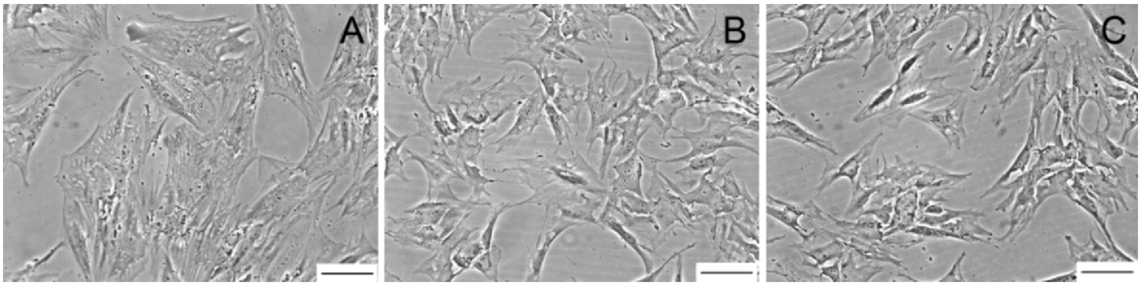


Figure 1. Microscopic appearance of MSCs. (A) BM-derived MSCs. (B) UCB-derived MSCs. (C) ADSCs. Magnification: ×400. The bar indicates 20 μm.

Kruskal-Wallis exact test, and values of $P < 0.05$ were considered statistically significant. Analysis was performed with the SPSS V.14 software package.

Results

Isolation, Expansion, and Morphology of BM-Derived and UCB-Derived MSCs and ADSCs. The success rate for isolating BM-derived MSCs and ADCSs was 100% (10/10). By contrast, the success rate for UCB was only 30% (3/10). UCBs were processed no longer than 12 hrs after umbilical cord collection. A net volume of 74.4 ± 28.7 ml and $88.1 \times 10^6 \pm 48.4 \times 10^6$ MNCs were obtained. No correlation was detected between volume, number of MNCs in the UCB after gradient separation, and success in obtaining MSCs. Although evidence for the isolation of fibroblastoid cells with MSC characteristics from UCB is still under debate (11, 15, 24, 28–30, 33–35, 44), we observed that MSC-like cells can be isolated from UCB units of full-term newborns.

The commercial kit (RosetteSep) did not significantly improve the isolation of UCB-derived MSCs. Therefore, the density gradient method (Histopaque) was used because it was less expensive and faster. Only a few cells attached to the plastic culture flasks and formed spindle-shaped adherent cells within 3 to 4 weeks after the plating of UCB-derived MNCs. By contrast, BM-derived MNCs and ADSCs formed clusters of elongated, spindle-shaped (fibroblast-like) MSCs within 3 days and reached cell confluence after 1 week.

Confluent cells were treated with trypsin and were subcultured (1:2 split). Cells from BM-derived and UCB-derived MSCs and ADSCs after two passages were homogeneous in size ($P = 0.159$) and granulosity ($P = 0.165$), having a fibroblastic shape (Fig. 1).

MSC Cell-Surface Antigen Profile. Cell-surface antigen expression was evaluated by flow cytometry in at least 3 samples each from BM-derived and UCB-derived MSCs and ADSCs between P_3 and P_5 (Fig. 2). With few exceptions, all three sources displayed similar immunophenotypes for the markers analyzed (Fig. 2 and Table 2). Cells were uniformly positive for the endoglin receptor CD105, the extracellular matrix protein CD90, the surface enzyme ecto-5′-nucleotidase CD73, the activated leukocyte cell adhesion molecule CD166, the β_1-integrin CD29, and the hyaluronate receptor CD44. No detectable contamination of hematopoietic cells was observed, as flow cytometry analysis was negative for markers of hematopoietic lineage, including the lipopolysaccharide receptor CD14, the leukocyte common antigen CD45, and the endothelial cell marker CD31. The percentages of expression of CD34, a hematopoietic progenitor cell marker, in MSCs isolated from BM, UCB, and ADSCs were 2.16% ± 2.48%, 10.52% ± 10.58%, and 10.37% ± 8.37%, respectively (Table 2). Statistical analysis comparing the MSCs sources regarding CD34 showed a significant difference only between BM and ADSCs ($P = 0.02$). Flow cytometry experiments for CD117 (c-kit) were independently analyzed by three experts. The independent analyses showed that CD117 is a complex marker to evaluate. Whereas ADSCs were clearly positive (98.11 ± 3.06), BM-derived and UCB-derived MSCs showed dimly positive-to-negative staining for CD117. This pattern became evident when the mean values and standard deviations of BM-derived and UCB-derived MSCs positive for CD117 were evaluated (52.7 ± 46.46 and 38.84 ± 40.80, respectively; Table 2).

Differentiation Assays. After careful visual examination, the following differentiation media were considered the most efficient in inducing adipogenic (medium 2), osteogenic (medium 5), and chondrogenic (medium 8) differentiation. Using these media, MSCs from the three sources between passages P_3 and P_5 were compared for their multilineage differentiation plasticity by *in vitro* assays. Differentiation to adipocytes, osteoblasts, and chondrocytes was qualitatively assessed on the basis of cell morphology and cytochemistry.

We used the presence of lipid-rich vacuoles stained with Oil Red O to analyze adipogenic induction. BM-derived MSCs and ADSCs presented large, round cells with cytoplasmic lipid-rich vacuoles (Fig. 3); however, UCB-derived MSCs displayed few and very small intracellular lipid droplets (Fig. 4). Seventy fields in three biological replicates from each source of MSCs were analyzed to estimate the differentiation value (DV), which was calculated by dividing the lipid droplet area by the number of nuclei so that possible differences in field cell confluences were considered. No differences in the adipogenic potential

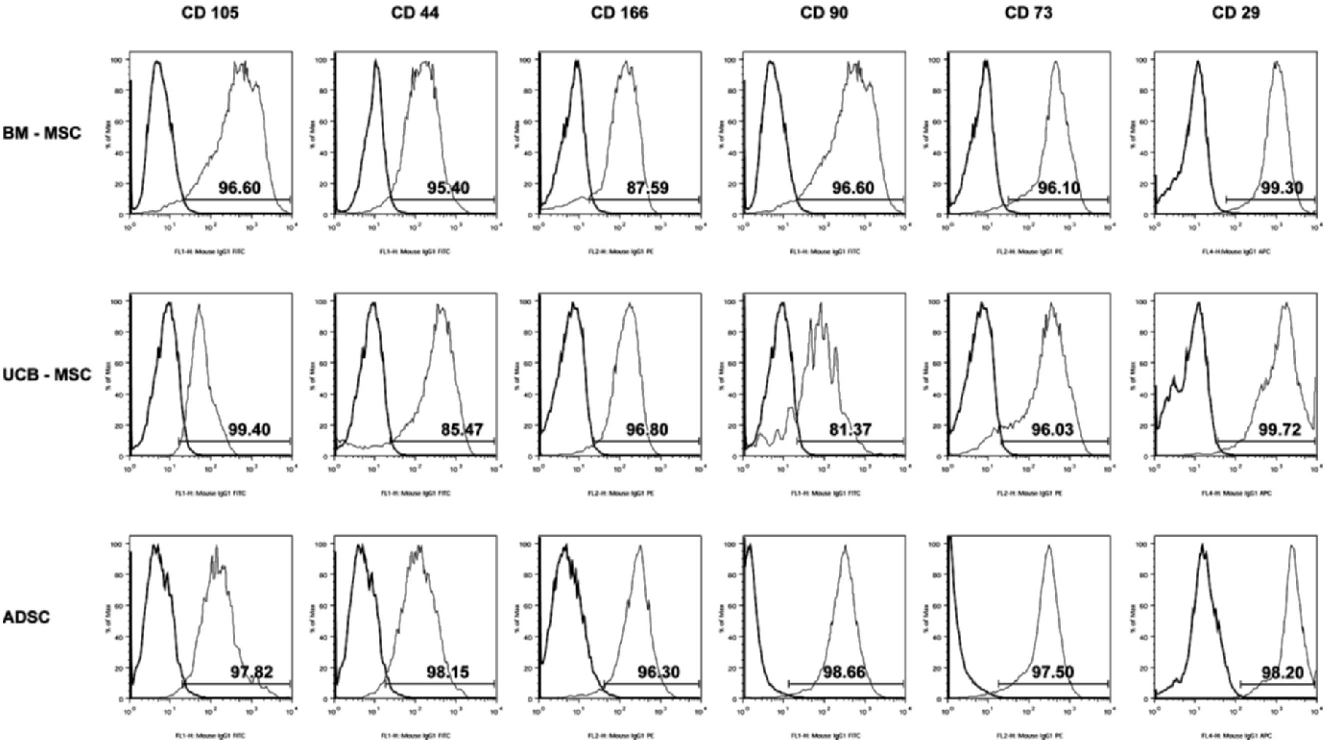


Figure 2. Immunophenotype assessed by flow cytometry. BM-derived MSCs, UCB-derived MSCs, and ADSCs were labeled with antibodies against the indicated antigens and analyzed by flow cytometry. Representative histograms are displayed. On the y axis is the % Max (the cell count in each bin divided by the cell count in the bin that contained the largest number of cells), and the x axis is the fluorescence intensity in a log (10^0–10^4) scale. The isotype control is shown as a thick black-line histogram.

were found between BM-derived MSCs (DV = 245.57 ± 943 μm^2 per nucleus) and ADSCs (DV = 243.89 ± 145.52 μm^2 per nucleus). The impressively high variations observed in BM-derived MSCs DV may be a consequence of the heterogeneous cell population present at the moment analyzed. However, the mean area occupied by individual lipid droplets was 7.37 μm^2 in BM-derived MSCs and 2.36 μm^2 in ADSCs, indicating that adipocytes in BM-derived MSC cultures are more mature than in treated ADSC cultures.

Osteogenic differentiation was assessed by the mineralization of the extracellular matrix, visualized by Alizarin Red S staining at pH 4.2. We detected calcium carbonate and phosphate in cells from all sources after 21 days of differentiation induction (Fig. 3). No differences in the osteogenic differentiation capacity were detected among BM-derived and UCB-derived MSC and ADSC samples.

In chondrogenic differentiation assays, MSCs formed aggregates that dislodged and floated freely in the suspension culture. High-density micromass MSC cultures generated cellular nodules, which produced large amounts of cartilage-related extracellular matrix molecules such as collagen. Paraffin sections of the aggregates stained with HE, Mallory, or Toluidine Blue showed a condensed structure with cuboidal cells and chondrocyte-like lacunae. The cells stained positively for Toluidine Blue; this dye is specific for the highly sulfated proteoglycans of cartilage matrices. All samples tested, irrespective of their origin, demonstrated a cartilage-like phenotype with chondrocyte-like lacunae (Fig. 3).

Untreated control cultures, which were grown in regular medium without adipogenic, osteogenic, or chondrogenic differentiation stimuli, did not exhibit spontaneous adipocyte, osteoblasts, or chondrocyte formation after 14 and 21 days of cultivation (Fig. 3).

Expression Profile of Differentiation Markers by RT-PCR and q-PCR Analysis. The mRNA levels of various marker genes were analyzed by RT-PCR and qPCR of total RNA isolated from induced and noninduced cultures. GAPDH mRNA was used as an internal control.

Levels of mRNA for FAPB4 were analyzed as a marker of adipogenic differentiation. RT-PCR easily detected FAPB4 expression in induced BM-derived MSCs and ADSCs in comparison with FAPB4 expression in the noninduced control cells; importantly, control cells were cultured for the same period as treated cells were. The overall RT-PCR profile was very similar for replicates from the same MSC source. However, results of qPCR detected significant variability in expression among independent biological samples (Fig. 5A). No expression or low levels of expression of FAPB4 were detected in induced and noninduced UCB-derived MSCs; this low expression is in contrast to that observed in BM-derived MSCs and ADSCs (Fig. 5A). Therefore, poor adipogenic potential detected in

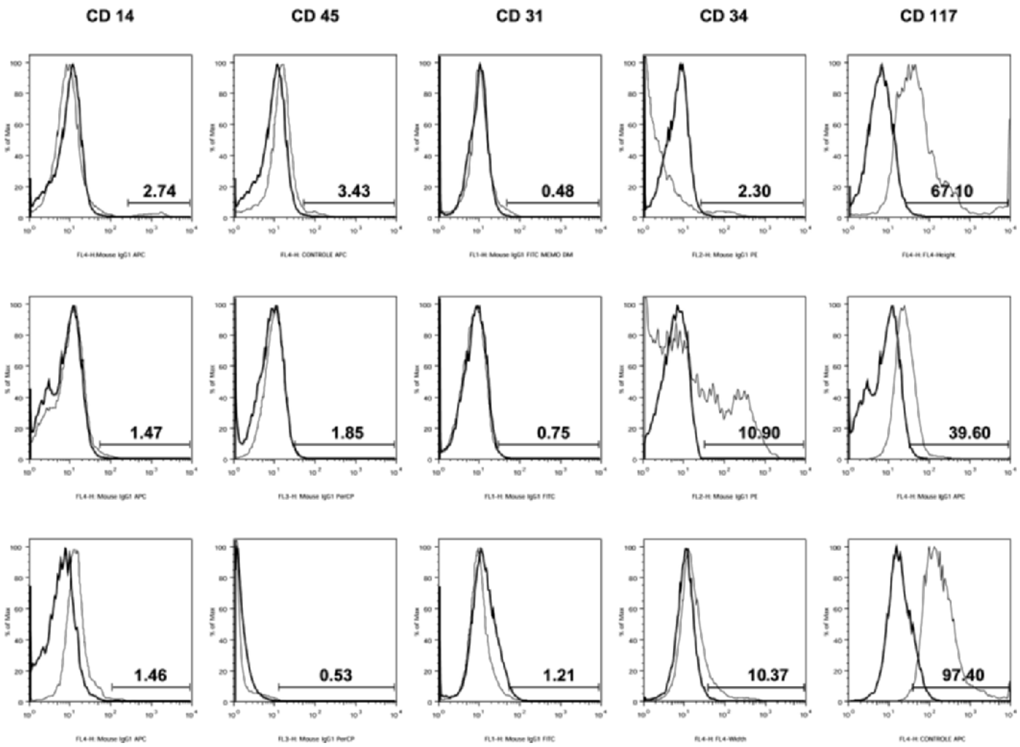


Figure 2. Continued.

UCB-derived MSCs by microscopic analysis was consistent with the results observed in FAPB4 expression analyses.

We analyzed osteonectin and ALP expression to evaluate osteogenic induction. Osteonectin is a glycoprotein that has been used as a differentiation marker for bone cells (49). RT-PCR showed no difference in osteonectin expression between induced and noninduced cells(data not shown). By qPCR, we observed discordant osteonectin expression profiles among biological samples from all three MSC sources. Whereas osteonectin expression in the induced culture in one BM sample was considerably greater than that in the noninduced culture, no difference was observed in the remaining samples. Therefore, we concluded osteonectin did not appear to be a suitable marker for osteogenic differentiation, at least in the culture conditions used in this study. Therefore, ALP mRNA levels were analyzed. We detected higher ALP mRNA levels in the induced cells than in noninduced cells from all sources after performing qPCR (Fig. 5B). In all the induced UBC-derived MSCs replicates analyzed, ALP mRNA levels were higher than those in the induced samples from the other sources (Fig. 5B).

Chondrogenesis was further studied by analyzing the mRNA level of a well-known marker, the cartilage-specific type II collagen gene. Similar to osteonectin expression, a

Table 2. Comparison of the Expression of Surface Proteins of Mesenchymal Stem Cells Derived from at Least 3 Samples of BM-Derived MSCs, UCB-Derived MSCs, and ADSCs Analyzed by Flow Cytometry[a]

Antibody	BM	UCB	AT
CD105	95.75 ± 5.52	96.96 ± 4.33	98.83 ± 1.01
CD90	93.16 ± 4.61	87.16 ± 5.79	96.78 ± 1.88
CD73	97.61 ± 2.83	96.84 ± 0.81	96.42 ± 2.82
CD166	91.69 ± 4.10	80.71 ± 25.31	93.79 ± 6.78
CD44	95.43 ± 4.27	92.48 ± 7.01	98.77 ± 0.62
CD29	98.72 ± 2.28	99.78 ± 0.06	97.45 ± 4.18
CD14	4.06 ± 4.35	4.32 ± 3.57	2.13 ± 1.79
CD45	1.97 ± 1.46	0.97 ± 0.88	0.45 ± 0.58
CD31	0.28 ± 0.20	0.41 ± 0.43	0.94 ± 1.54
CD34	2.16 ± 2.48	10.52 ± 10.58	10.37 ± 7.98
CD117	52.70 ± 46.46	38.84 ± 40.80	98.11 ± 3.06

[a] Each value is the mean percentage of at least three experiments ± standard deviation.

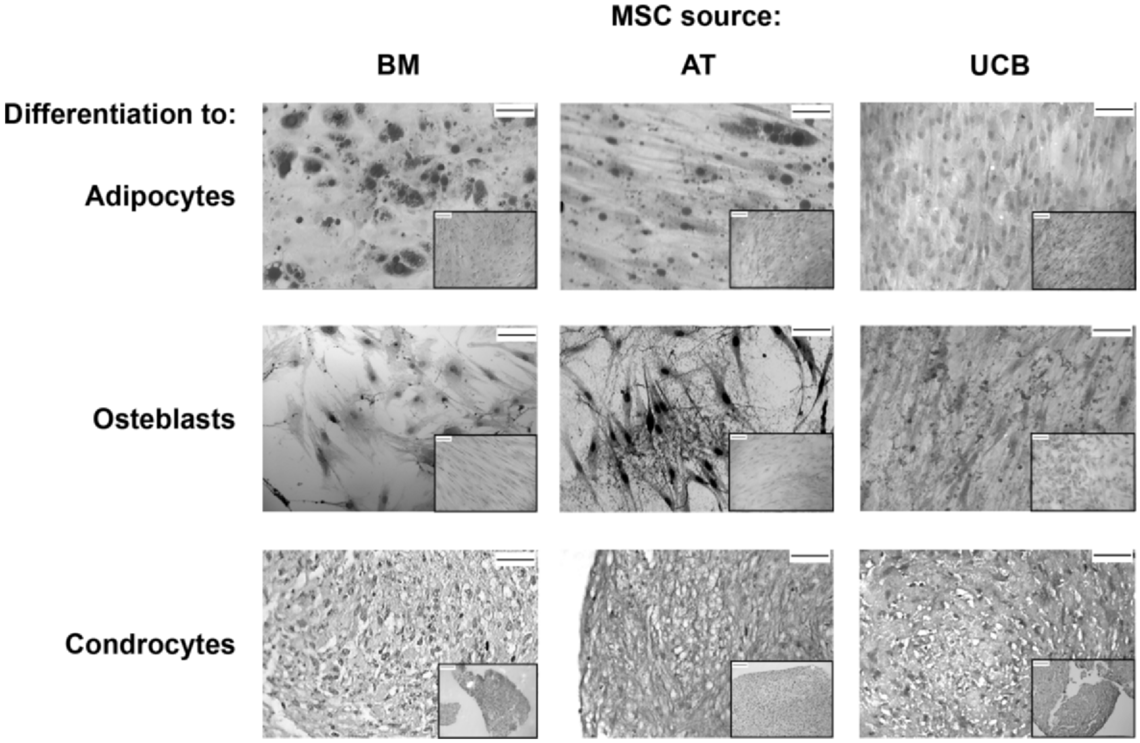


Figure 3. Differentiation of BM-derived MSCs, UCB-derived MSCs, and ADSCs. Cells between P_3 and P_5 from each source were incubated for 21 days in the presence of specific differentiation agents for adipocytes (medium 2), osteoblasts (medium 5), and chondrocytes (medium 8). Differentiation into the adipocyte lineage was demonstrated by staining with Oil Red O. Alizarin Red S staining shows mineralization of the extracellular matrix. Toluidine Blue shows the deposition of proteoglycans and lacunae Untreated control cultures without adipogenic, osteogenic, or chondrogenic differentiation stimuli are shown on the bottom right corner of each photograph. Magnification: ×200. The bar indicates 20 μm.

strong band was detected in all induced and noninduced MSCs under the RT-PCR conditions used in this study. However, we detected higher type II collagen expression in induced cells than in noninduced cells after qPCR; this increase in expression was evident for most induced cells although individual expression levels varied (Fig. 5C). In a few cases, no significant differences between induced and noninduced cells were seen (2 of 4 ADSCs).

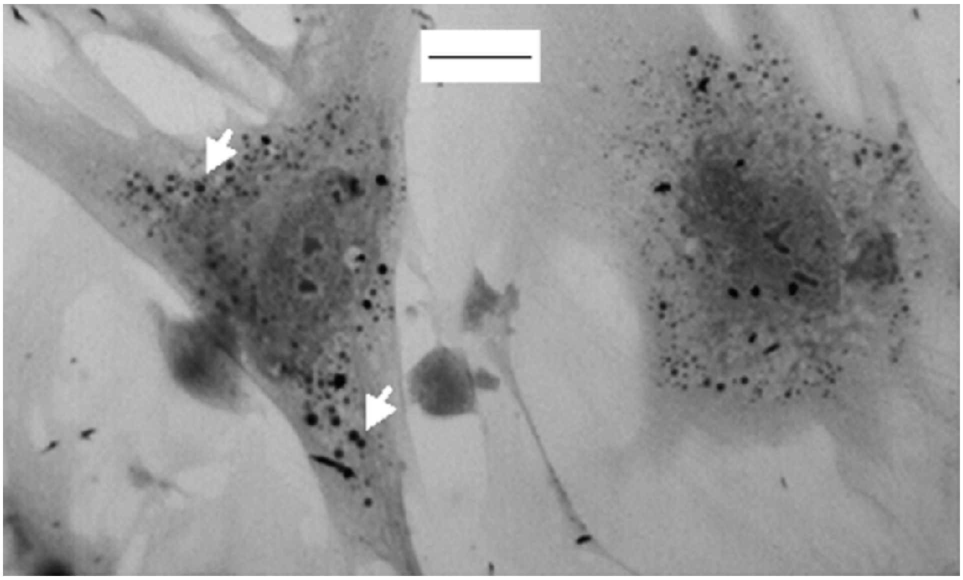


Figure 4. Tiny intracytoplasmic lipid droplets (arrowheads) present in UCB-derived MSCs under standard differentiation conditions. Magnification: ×1000. The bar indicates 20 μm.

Discussion

The expected plasticity of human mesenchymal progenitors is paramount for upcoming therapeutic strategies for cellular therapy and tissue engineering. Functional assays are required to establish the presence of MSCs in a tissue because there are no specific and universal molecular markers of adult MSCs. Here we compared the biological properties and differentiation potential of MSCs isolated from presently the most important sources: BM, UCB, and AT.

MSC isolation differed depending on the source. Whereas BM-derived MSCs and ADSCs isolation efficiency was 100%, that for UCB-derived MSCs was only 30%. Other groups have also reported low levels of efficiency in the isolation and establishment of UCB-derived MSCs (10, 11, 24). Sharing UCB-derived MSCs with the fetus (50) and cross-contamination with monocytes and osteoclast-like cells during culture establishment (24) are some of the hypotheses to explain the low yields of MSCs from this source. Also, successes in obtaining UCB-derived MSCs are related to the time between collection and isolation, and the UCB unit volume (24). In this study, the storage time was less than 12 hrs, and the mean volume was 74.4 ± 28.7 ml; however, the low number of MNCs (88.11 $\times 10^6 \pm 48.37$) might account for the extremely low frequency of UCB-derived MSCs obtained in comparison with the frequencies of BM-derived MSCs or ADSCs. The period for establishing BM-derived MSCs or an ADSC monolayer was shorter than that for UCB-derived MSCs. Growth of the latter was slower than that of BM-derived MSC and ADSC cultures, but once cultures were established, growth was maintained over multiple passages. This result probably reflects the low precursor frequency of MSCs in UCB (32).

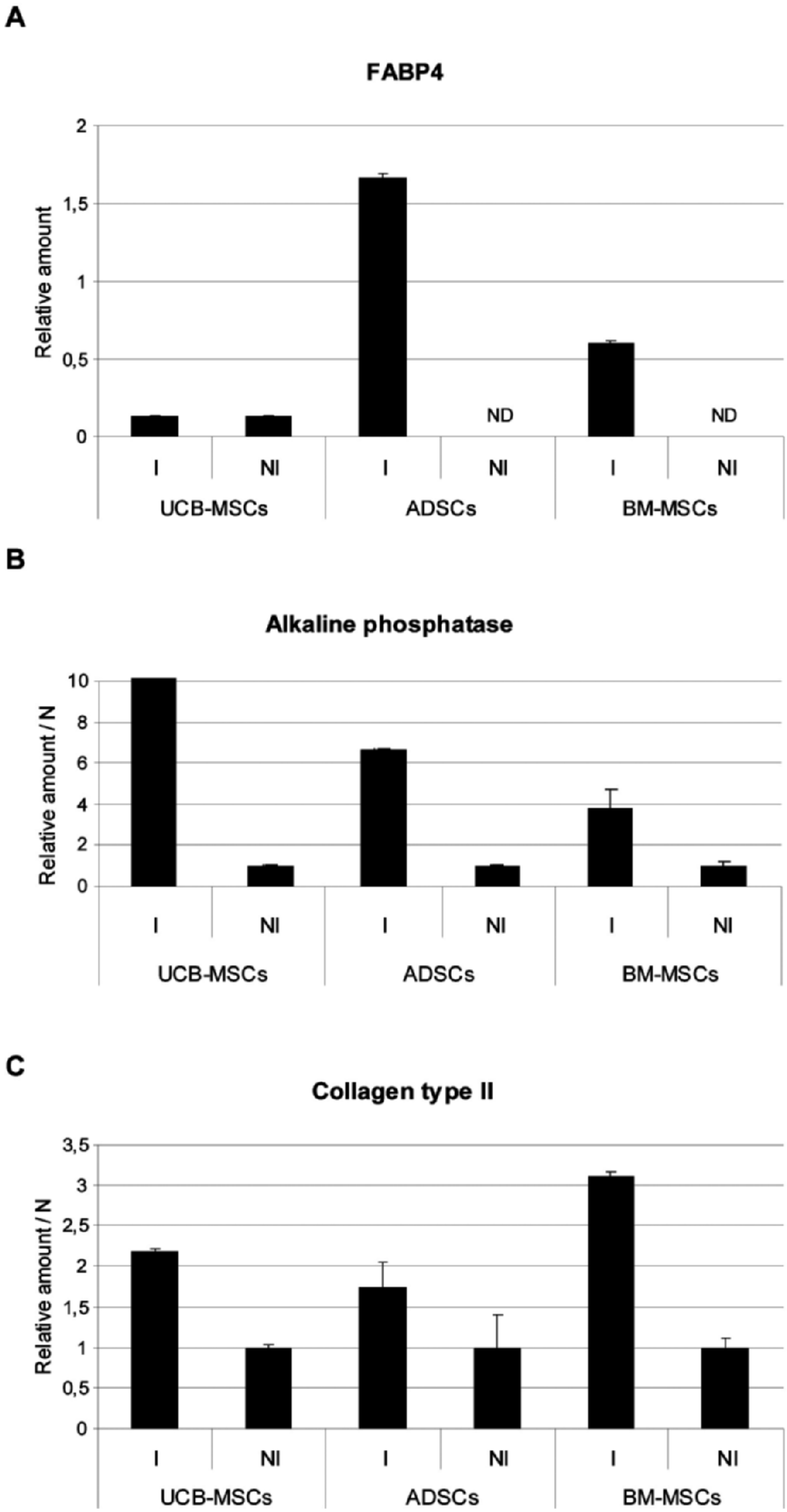
No morphologic differences were observed between BM-derived and UCB-derived MSCs and ADSCs, as has been previously reported (10, 11, 51). Also, flow cytometry measurements showed no significant differences concerning cell size and complexity in all MSC populations (data not shown). The homogeneity of MSC cultures at specific passages was apparent after assessment of the cell-surface antigen profile. The direct comparison reported here showed that BM-derived and UCB-derived MSCs and ADSCs share classic MSC marker proteins (52). As expected, these cells lacked the hematopoietic marker CD14, CD45, and the endothelial marker CD31. However, CD34 gene expression was 2% in BM-derived MSCs and about 10% in UCB-derived MSCs and ADSCs. This observation was not unusual as freshly isolated or primary cultures of BM-derived and UCB-derived MSCs and ADSCs have been reported to be dimly to significantly positive for CD34 (15, 53–56).

CD117 was present in ADSCs and dim in BM-derived and UCB-derived MSCs. Expression of this protein by MSCs is controversial. It has been previously reported that MSCs do not express CD117 (10, 57–59), whereas other reports have shown that embryonic stem cells, hematopoietic stem cells, and MSCs are dimly or strongly positive for this marker (60–63); our results are consistent with the latter. Together, these data strongly suggest that BM-derived MSCs, UCB-derived MSCs, and ADSCs are highly similar morphologically but are not so immunophenotypically (54, 57, 62–65).

In this study, we used qualitative assays to demonstrate the *in vitro* multilineage developmental potential of BM-derived and UCB-derived MSCs and ADSCs after exposure to specific culture conditions. BM-derived MSCs and ADSCs demonstrated a high *in vitro* potential to differentiate into adipocytes, osteoblasts, and chondrocytes, whereas UCB-derived MSCs presented a more restricted, or at least delayed, adipocyte differentiation capacity. Immaturity of these neonatal cells cannot account for their low adipocyte differentiation potential because differentiation to osteoblasts and chondrocytes was similar to that of BM-derived MSCs and ADSCs.

BM-derived MSCs and ADSCs cultures had a greater propensity to differentiate into adipocytes than did UCB-derived MSCs under similar culture conditions. Induced BM-derived MSCs presented more mature adipocytes (unilocular lipid vacuoles) by morphometric assessment than did induced ADSCs. Karahuseyinoglu *et al*. (66) reported that some MSCs in the BM stroma may already be committed to form mature adipocytes *in situ*. Previous studies had reported conflicting data regarding the adipogenic differentiation potential of UCB-derived MSCs (10, 11, 15, 24, 25, 32, 50). UCB-derived MSCs rarely differentiated toward adipocytes under our standard differentiation protocols. Only tiny lipid vacuoles were observed in a few UCB-derived MSCs after 21 days of induction, and FABP4 expression was poor or even absent; FABP4 is a fatty acid-binding protein characteristically present in adipocytes. These tiny lipid vacuoles suggest that differentiation is at its initial stages, and it is highly probable that a longer culture period is necessary for UCB-derived MSC adipogenic differentiation. In fact, human umblical cord stromal cells achieved adipogenic differentiation only after 40 days of induced culture (66); this represents a relatively longer period than with BM-derived MSCs and ADSCs. Also, Bieback *et al*. (24) showed that adipogenic differentiation could solely be induced in MSC-like cells cultured continuously in adipogenic induction medium for at least 5 weeks.

In this study, human BM-derived and UCB-derived MSCs and ADSCs were able to proliferate and subsequently differentiate into osteoblasts. Incubation with differentiation medium induced cell aggregation and matrix production, which positively stained with the calcium-specific marker Alizarin Red S. The mRNA profiles for osteonectin were not satisfactory for the detection of osteoblast differentiation, at least under our conditions. Compared with induced MSCs, untreated MSCs from all three sources


A
FABP4
Relative amount
2
1,5
1
0,5
0
ND
ND
I
NI
I
NI
I
NI
UCB-MSCs
ADSCs
BM-MSCs
B
Alkaline phosphatase
Relative amount / N
10
8
6
4
2
0
I
NI
I
NI
I
NI
UCB-MSCs
ADSCs
BM-MSCs
C
Collagen type II
Relative amount / N
3,5
3
2,5
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1,5
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0,5
0
I
NI
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NI
I
NI
UCB-MSCs
ADSCs
BM-MSCs

mostly showed no differences in osteonectin mRNA levels. Data deposited at the Gene Expression Omnibus profile at National Center for Biotechnology Information (accession number GDS1288 record | GPL96 212667) show that osteonectin mRNA levels in BM-derived MSCs are quite high, and it has also been shown by Serial Analysis of Gene Expression analyses that noninduced BM-derived and UCB-derived MSCs significantly expressed this glycoprotein (67, 68); these data are consistent with our observations. Therefore, we suggest that osteonectin is not an appropriate hallmark gene for cultures induced to differentiate into osteoblasts during 21 days. Matrix mineralization is the latest stage of osteoblast differentiation process, and osteonectin may be considered a marker for terminal differentiation (69); terminal differentiation was not achieved in the 21 days of our induced cultures. Accordingly, Plant and Tobias (70) studied osteoblast differentiation and observed that osteocalcin, osteopontin, and osteonectin expression showed modest increases only at later times, such as 20 and 24 days after induction. Conversely, ALP appeared to be a good osteogenic marker under conditions used in this study. In addition, identifying genes associated with osteoblast differentiation is a very complex task in MSCs induced to become an osteoblast lineage (71–74).

BM-derived and UCB-derived MSCs and ADSCs cultured with TGF-β developed typical morphologic features of chondrocytes and produced mucopolysaccharide, an indicator of chondrogenic differentiation. Although the extracellular matrix protein collagen type II is expressed by chondrocytes and MSCs, q-PCR assays clearly showed that its mRNA levels were higher in induced MSCs than in noninduced MSCs. A common observation for all the molecular markers analyzed was the considerable variability seen among all biological samples. The overall profiles were similar among samples that had undergone similar treatment, but the relative mRNA levels differed enormously. It is highly probable that the variation observed was mainly due to the age, the health condition, and the genetic background of the patients and donors rather than due to technical variations (75, 76).

Here we presented comparative data from human BM-derived and UCB-derived MSCs and ADSCs. It is reasonable to conclude that MSCs can be found in these three various tissues, and although MSCs from the 3 sources analyzed here may be considered morphologically and immunophenotypically similar with the usual markers available, they clearly diverge in their differentiation capacity and/or differentiation kinetics. Presently, stem cell–based therapies are being extensively studied *in vivo*. Whereas BM-derived MSCs and ADCS can produce a variety of tissues of mesodermal and nonmesodermal origins (77–82), the *in vivo* adipocyte differentiation potential of UCB-derived adherent cells seems to be reduced (27), as it was observed in our *in vitro* assays. Therefore, further basic research is still necessary to understand the biology of MSCs obtained from different tissues and to delineate their extent and significance on clinical applications.

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Figure 5. Expression profile of differentiation markers. BM-derived MSCs, UCB-derived MSCs, and ADSCs were maintained in induced or control medium for 21 days and assayed for the expression of adipogenic, osteogenic, and chondrogenic specific mRNA levels. (A) The adipogenic differentiation marker FABP4, (B) the osteogenic differentiation marker ALP, and (C) the chondrogenic differentiation marker collagen type II were analyzed by q-PCR. Abbreviations: BM, bone marrow; UCB, umbilical cord blood; MSCs, mesenchymal stem cell; ADSC, adipose tissue derived stem cells; I, induced cells; NI, noninduced cell (negative control); ND, not detected. Representative results of three independent experiments are shown. Results of q-PCR are expressed as mean and standard deviation of the technical triplicate. GAPDH was used as an internal control. When possible, the relative amount values were normalized with the noninduced values (relative amount/N); thus, the value for the noninduced sample was 1.

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