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Comparative investigation of the differentiation capability of bone-marrow- and adipose-derived mesenchymal stem cells by qualitative and quantitative analysis

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Abstract Mesenchymal stem cells (MSCs) hold promise for cell-based therapy in regenerative medicine. To date, MSCs have been obtained from conventional bone marrow via a highly invasive procedure. Therefore, MSCs are now also isolated from sources such as adipose tissue, cord blood and cord stroma, a subject of growing interest. As the characterization and differentiation potential of adipose-derived MSCs (AD-MSCs) and bone-marrow-derived MSCs (BM-MSCs) have not been documented, we have evaluated and compared the characteristics of both MSC types by qualitative and quantitative analyses. Both cell types show similar morphology and surface protein expression, being positive for stromal-associated markers and negative for hematopoietic and endothelial markers. The colony-forming potential of AD-MSCs is distinctly higher than that of BM-MSCs. Nonetheless, similar adipogenic and osteogenic differentiation is observed in both groups of MSCs. Cytochemical qualitative analysis and calcium mineralization demonstrate higher levels toward osteogenic differentiation in BM-MSCs than in AD-MSCs. On the contrary, the percentage of Nile red oil staining for differentiated adipocytes is higher in AD-MSCs than in BM-MSCs. Quantitative real-time polymerase chain reaction shows similar patterns of osteogenic- and adipogenic-associated gene expression in both cell types. Each of the MSCs respond in functional analysis by exhibiting unique properties at the differentiation level according to their micro-environmental niche. Thus, quantitative analysis might be a valuable means of describing stem cell multipotency, in addition to qualitative investigation.

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Introduction

Human mesenchymal stem cells (MSCs) are characterized by their plastic adherent growth, specific surface antigen expression and multipotent differentiation potential (Dominici et al. 2006; Yoo et al. 2009). These cells readily proliferate under in vitro conditions. When cultured within a specific environment, they hold the capability to differentiate into multiple tissues, including bone, adipose, tendon, cartilage, skeletal muscle, marrow stroma, cardiac, neural cells and connective tissue (Yoo et al. 2009; Nekanti et al. 2010; Pittenger et al. 1999; Zhu et al. 2008). MSCs have been isolated from almost all the organs of the body including conventional bone marrow, umbilical cord, umbilical cord blood, adipose tissue, dental pulp, periosteum, tendon, skin, synovial membrane,

amniotic fluid, limbal tissue and menstrual blood (Nekanti et al. 2010; Zhu et al. 2008). The origin of MSC is highly important in determining their biological activity; clinical applications might thus differ according to the respective stem cell niche. Furthermore, scientists continue to show interest in the search for novel and potent sources that might be suitable for specific regenerative applications. Significant differences have been identified in the properties of MSCs that are derived from different sources (Sakaguchi et al. 2005). Previously, human fibroblasts have been used as a negative control for MSC comparative studies as these cells do not possess in vitro differentiation capacity (Pittenger et al. 1999; Wagner et al. 2005). Recently, MSC-like cells have been derived from human foreskin with boundless multipotent differentiation capabilities, such as for osteocytes, adipocytes, neural cells, smooth muscle cells, Schwann-like cells and hepatocyte-like cells (Huang et al. 2010). To address the difficulties in this fast-growing research scenario, concomitant quantitative and qualitative analyses should therefore be encouraged to identify and define MSCs intended for human use.

In this study, we have isolated MSCs from bone marrow and adipose tissue and compared their morphology, colony-forming potential, surface antigen expression and multi-lineage differentiation. Our aim has been to analyze the potency of these cells by universal qualitative and novel quantitative experiments. This might serve as a valuable tool for the definition of MSCs.

Materials and methods

Cell isolation and culture

MSCs from bone marrow

MSCs from bone marrow were isolated from various donors and processed under the same conditions. Bone marrow aspirates were obtained from the knee joints of five donors of 40–50 years of age who had undergone knee replacement surgery; all the samples were received from the Department of Orthopedics, King Khalid University Hospital (Riyadh, Kingdom of Saudi Arabia [KSA]). With approval from the Ethics Committee of King Saud University Medical College and Hospital, the patients were informed about and gave written consent for the collection of bone marrow samples. The mononuclear cell fraction was isolated by lymphoprep density gradient centrifugation (density 1.077 ± 0.001 g/ml; Axis-Shield Poc, Norway). BM-MSCs were cultivated as previously described by Pountos et al. (2007). In brief, mononuclear cells were plated in an expansion medium at a density of 10^5 cells/cm^2 in tissue culture flasks (BD, USA). The expansion medium consisted in Dulbecco's modified Eagle's medium (DMEM; supplemented with D-glucose 4500 mg/l, 4 mM L-glutamine and 110 mg/l sodium pyruvate), 10%

fetal bovine serum (FBS), $1 \times$ penicillin-streptomycin (Penstrep) and non-essential amino acids (all purchased from Gibco-Invitrogen, USA). On reaching 80% confluency, the cells were trypsinized with 0.25% trypsin/1 mM EDTA (Invitrogen, Germany) and seeded at about 9000 cells/cm^2. Cells were expanded for two to four passages.

MSCs from adipose tissue

Human adipose tissue (lipoaspirate) of five healthy donors of 40–50 years of age was obtained from elective liposuction procedures under anesthesia after informed and written consent was received as set down in the guidelines approved by the Ethics Committee on the Use of Human Subjects (King Saud University Medical College and Hospital, KSA). MSCs were isolated as previously described (Zhu et al. 2008). In brief, adipose tissue was washed in phosphate-buffered saline (PBS) and digested with 0.1% collagenase type I in DMEM for 20 min at 37°C with constant agitation. Mature adipocytes, debris and connective tissue were separated from pellets by centrifugation (800g, 10 min). Pellets were resuspended in red blood cell lysis buffer (Sigma, Germany) and incubated for 5 min at room temperature. The processed suspension was passed through a 100-mm nylon mesh filter (Millipore, USA). After being washed, filtered cells were cultivated in the same expansion medium as described above for BM-MSCs.

Colony-forming unit—fibroblast assay

Both types of MSC were expanded in culture to 70%–80% confluence, harvested with trypsin-EDTA (GIBCO, Invitrogen, Germany) and counted. Cells were diluted in routine culture media in triplicate dishes of 6 cm at a cell seeding density of 1×10^3. After incubation for 14 days at 37°C in 5% humidified CO_2, the cells were washed with PBS and stained with 0.5% Crystal Violet (Sigma-Aldrich, Germany) for 15 min at room temperature. Cells were washed twice with PBS and visible colonies were counted from 3 dishes (triplicate) at passage 1. The minimum number of input cells required to produce a single colony determined the colony-forming unit—fibroblast (CFU-F) frequency. This was repeated at passage 4.

Immunofluorescence

Adherent cells were fixed with 4% paraformaldehyde (Sigma, Germany) for 15 min and permeabilized with 0.1% Triton X-100 (Sigma) for 10 min. After being washed with PBS, the cells were blocked with 3% bovine serum albumin (BSA; Sigma) for 30 min, followed by incubation with primary antibody (purified mouse anti-vimentin; BD Pharminogen, USA) diluted in PBS (1:100) at 4°C

overnight. After removal of primary antibodies, cells were washed three times with PBS and incubated with the secondary antibody (goat polyclonal anti-mouse IgG; Abcam, USA) conjugated to fluorescein isothiocyanate (FITC; 1:4000) for 1 h at room temperature. Cells were washed three times with PBS and mounted with a medium containing DAPI (4'-6-diamidino-2-phenylindole) to detect nuclei (VectaShield; Vector Labs, Burlingame, Calif., USA).

Flow-cytometry

Trypsinized cells were washed twice in PBS supplemented with 2% FBS and resuspended to a concentration of about 1×10^5 cells/antibody test. Thus, 10 μl PE-conjugated mouse anti-human CD146, CD73, CD29, and HLA-DR, FITC-conjugated mouse anti-human CD34, CD90, CD45, CD13, and CD31, and APC-conjugated mouse anti-human CD105, CD14, and CD44 antibodies (BD Biosciences, USA; except monoclonal antibody against human CD105, which was from R&D Systems, USA) were used. Negative control staining was performed by using a FITC/PE/APC-conjugated mouse IgG1 isotype antibody. To analyze the cell viability, 10 μl 7-AAD dye (7-aminoactinomycin D; BD Biosciences) was added. After storage for 30 min at 4°C in the dark, cells were washed with PBS, resuspended in 500 μl PBS and analyzed in a BD FACS Calibur flow cytometer (BD Biosciences, USA). The ratios of fluorescence signals versus scatter signals were calculated and histograms were generated by using the software Cell Quest Pro Software Version 3.3 (BD Biosciences, USA).

In vitro osteoblast differentiation

Cells were seeded at a density of 0.05×10^6 cells/ml in 6-well plates (for cytochemical staining and RNA isolation) and grown in standard DMEM growth medium. At 70%-80% confluence, the medium was replaced in test wells by osteogenic induction medium supplemented with DMEM containing 10% FBS, 1% Pen-strep, 50 μg/ml L-ascorbic acid (Wako Chemicals, Neuss, Germany), 10 mM β-glycerophosphate (Sigma) and 10 nM calcitriol (1α,25-dihydroxy vitamin D3; Sigma), and 10 nM dexamethasone (Sigma). The ostogenic medium was changed every 3 days and the experiments were terminated at day 10. Cells cultured in regular culture medium were considered as an experiment control.

In vitro adipocyte differentiation

Cells were seeded at a density of 0.05×10^6 cells/ml in 6-well plates (for cytochemical staining and RNA isolation) and grown in standard DMEM growth medium. At 90%-100% confluence, the medium was replaced in all test wells with adipogenic induction medium supplemented with 10% FBS and with adipogenic-induction mixture (AIM) containing 10% horse serum (Sigma), 1% Pen-strep, 100 nM dexamethasone, 0.45 mM isobutyl methyl xanthine (IBMX; Sigma), 3 μg/ml insulin (Sigma) and 1 μM Rosiglitazone (BRL49653; Novo Nordisk, Bagsvaerd, Denmark). The adipogenic medium was replaced every 3 days; experiments were terminated at day 10. Cells cultured in the regular culture medium were considered as an experiment control.

Cytochemical staining

Alkaline phosphatase staining for osteoblasts

Cells induced toward osteoblast differentiation for 10 days as described above were washed in PBS twice, fixed in 10 mM acetone/citrate buffer (1.5:1) at pH 4.2 for 5 min at room temperature and incubated with alkaline phosphatase (ALP) substrate solution (naphthol AS-TR phosphate prepared 1:5 in water plus 10 mg Fast red TR, in 24 ml 0.1 M TRIS buffer pH 9.0; Sigma) for 1 h at room temperature. Cells were rinsed with water, stored in PBS and photographed by using Carl Zeiss—Axio observer.1 equipped with a digital camera (Axiocam MRc5, Göttingen, Germany).

Alizarin red S and von Kossa staining for osteoblasts

Mineralization of the cell layer was examined by the use of alizarin red S or von Kossa staining. The cell layers were washed twice with PBS, fixed with 10% formalin solution for 30 min and then washed with pure water twice. For alizarin red S staining, the cells were placed in 2% alizarin red S (pH 4.1; Sigma) solution for 10 min, washed with pure water three times and then air-dried. For von Kossa staining, the cell layers were placed in a 5% silver nitrate (Sigma) solution for 10 min under UV light and immersed in 2.5% sodium thiosulfate for 5 min. After being washed with pure water, the cell layers were then photographed.

Oil red-O staining for adipocytes

Cells differentiated after 14 days into adipocytes were washed twice in PBS and staining for accumulated cytoplasmic lipid droplets was performed with Oil red-O stain. Cells were fixed in 4% formaldehyde for 10 min at room temperature, rinsed once in 3% isopropanol and stained for 1 h at room temperature with filtered Oil red-O staining solution (prepared by dissolving 0.5 g Oil red-O powder in 60% isopropanol). Cells were rinsed with water and then photographed by using Carl Zeiss—Axio observer.1 equipped with a digital camera (Axiocam MRc5, Göttingen, Germany).

Calcium concentration measurements

A Fluo-4 Direct Calcium Assay kit (Invitrogen, Germany) was used to measure Ca^{2+} concentration on a fluorometer (Tecan, Switzerland) as previously described by Xu et al. (2009) and the manufacturer's protocols. Briefly, both BM-MSCs and AD-MSCs were cultured and osteoblast-induced separately in 96-well plates. After 10 days of induction, cells were treated as recommended by the manufacturer with Fluo-4 dye solution containing probenecid to prevent extrusion of the dye out of cells. The plates were maintained at 37°C for 30 min and then at room temperature for an additional 30 min. The assay was carried out at 494 nm (excitation) and 516 nm (emission).

Adipocyte enumeration by flow cytometry

Nile Red Staining was performed as previously described by Schaedlich et al. (2010). In brief, following trypsination, the undifferentiated and differentiated MSCs were once washed with PBS. The dye was then added at a final concentration of l00 ng/ml. The cells were then incubated on ice for 5 min, centrifuged and washed once with PBS. They were subsquently resuspended in an appropriate volume of PBS and kept on ice prior to flow cytometric analysis.
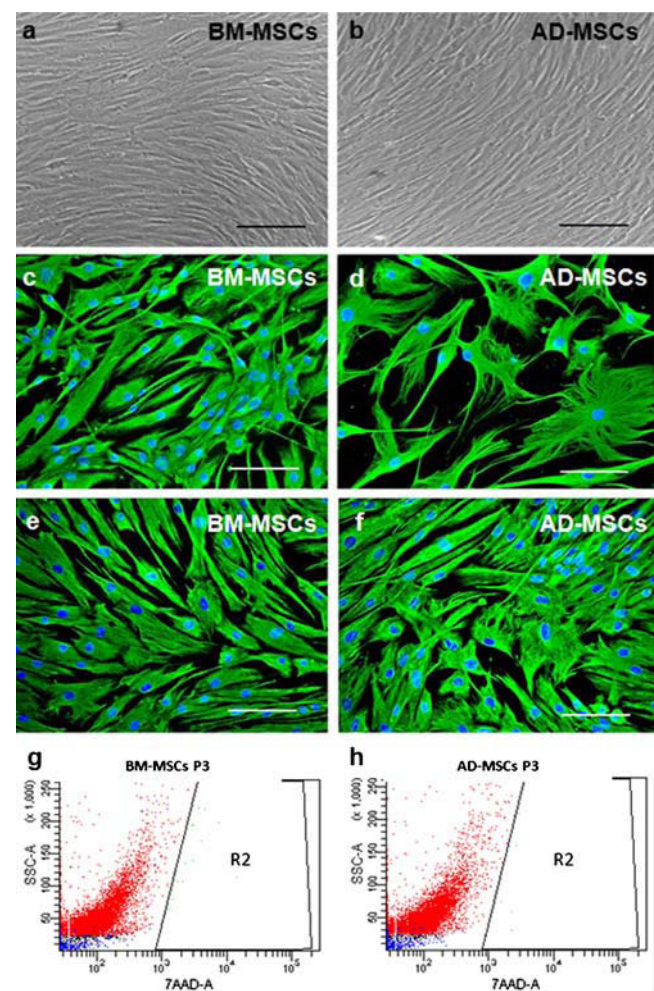
Reverse transcriptase with real-time quantitative polymerase chain reaction

Following differentiation of the human MSCs, the specific genes related to each differentiation event were analyzed at

the mRNA level. Total RNA from differentiated (test) and undifferentiated (control) cells on day 10 was harvested and washed in PBS. Total RNA was extracted by using the MagNA pure compact RNA isolation kit (Roche Applied Science, Germany) in an automated MagNA pure compact system (Roche, Germany) according to the protocol for cultured cells as recommended by the manufacturer. The primer sequences (all from Invitrogen, UK) and the sizes of each product, as listed in Table 1, were used to quantify mRNA by real-time reverse transcription (RT) with normalization to β-actin with the delta Ct method. The relative abundance of target mRNA was quantified relative to the control β-actin gene expression from the same reaction. Samples for cDNA synthesis and the polymerase chain reaction (PCR) were prepared by using a iScript One-step

Table 1 Primer sequences for polymerase chain reaction analysis and their respective product sizes (*PPAR* peroxisome proliferator activated receptor, *aP2* fatty-acid-binding protein 4, *ALP* alkaline phosphatase, *F* forward, *R* reverse)

Gene	Primer sequence (5' to 3')	Product size (bp)
β–Actin	F: TGTGCCCATCTACGAGGGGTATGC	448
	R: GGTACATGGTGGTGCCGCCAGACA	
PPAR-γ2	F: TTCTCCTAT TGACCCAGAAAGC	307
	R: CTCCACTTTGATTGCACTTTGG	
aP2	R: GCCAGGAATTTGACGAAGTC	107
	F: TGGTTGATTTTCCATCCCAT	
Adiponectin	R: TGTTGCTGGGAGCTGTTCTACTG	234
	F: ATGTCTCCCTTAGGACCAATAAG	
ALP	F: ACGTGGCTAAGAATGTCATC	475
	R: CTGGTAGGCGATGTCCTTA	
Osteocalcin	R: CATGAGAGCCCTCACA	310
	F: AGAGCGACACCCTAGAC	
Osteopontin	R: CCAAGTAAGTCCAACGAAAG	347
	R: GGTGATGTCCTCGTCTGTA	

Fig. 1 Morphology and viability analysis of mesenchymal stem cells derived from human bone marrow (hBM-MSCs) or human adipose tissue (hAD-MSCs). Cells were plastic-adherent with a fibroblast-like spindle-shaped morphology (**a**, **b**). Immunofluorescent staining of primary cultures (**c**, **d**) and cells at passage 2 (**e**, **f**) for vimentin; nuclei were visualized with DAPI (4'-6-diamidino-2-phenylindole). Viability was analyzed by 7-AAD DNA affinity dye flow cytometry of passage 3 (*P3*) cells, before the differentiation study (**g**, **h**). *Bars* 100 μm

RT-PCR Kit with SYBR Green (Bio-Rad, USA) and run on a Light Cycler (Roche, Germany) PCR machine. Relative quantification of PCR products was based on value differences between the target and β-actin control by using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data are presented as means±SD and are results from a minimum of three experiments. Student's *t*-test (paired) was performed to analyze results between the different groups. A $P<0.05$ was considered to be statistically significant. The SPSS software package version 17.0 (SPSS, USA) was used for the statistical tests.

Results

Isolation of MSC from different tissues

MSCs were isolated from human bone marrow and adipose tissue. BM-MSCs and AD-MSCs were isolated in the same culture medium with 10% serum content. Both isolated MSC populations displayed a spindle-shaped morphology (Fig. 1a, b). BM-MSCs showed a homogeneous morphology, whereas AD-MSCs were heterogeneous varying from elongated cells to flat round cells within a primary culture system (Fig. 1c, d). In subsequent passages, both cell types had a similar morphology. MSC viability was analyzed by fluorescence-based flow-cytometric 7-AAD nucleic acid dye assessment before the cells were stimulated to differentiate into lineages; both MSC types were >90% viable (Fig. 1g, h).

Immunophenotypes of MSC

A collection of surface markers were tested by flow cytometric analysis (Fig. 2a, b). Both types of MSC were negative for CD34, CD45, CD14, CD31 and HLA-DR, whereas they were positive for CD13, CD29, CD44, CD73, CD90 and CD105 in agreement with earlier reports (Dominici et al. 2006; Zhu et al. 2008; Baglioni et al. 2009). This expression of surface proteins indicated that they were of non-hematopoietic or endothelial origin. Flow cytometric analysis of marker CD146 showed that this distinct characteristic was exhibited by the
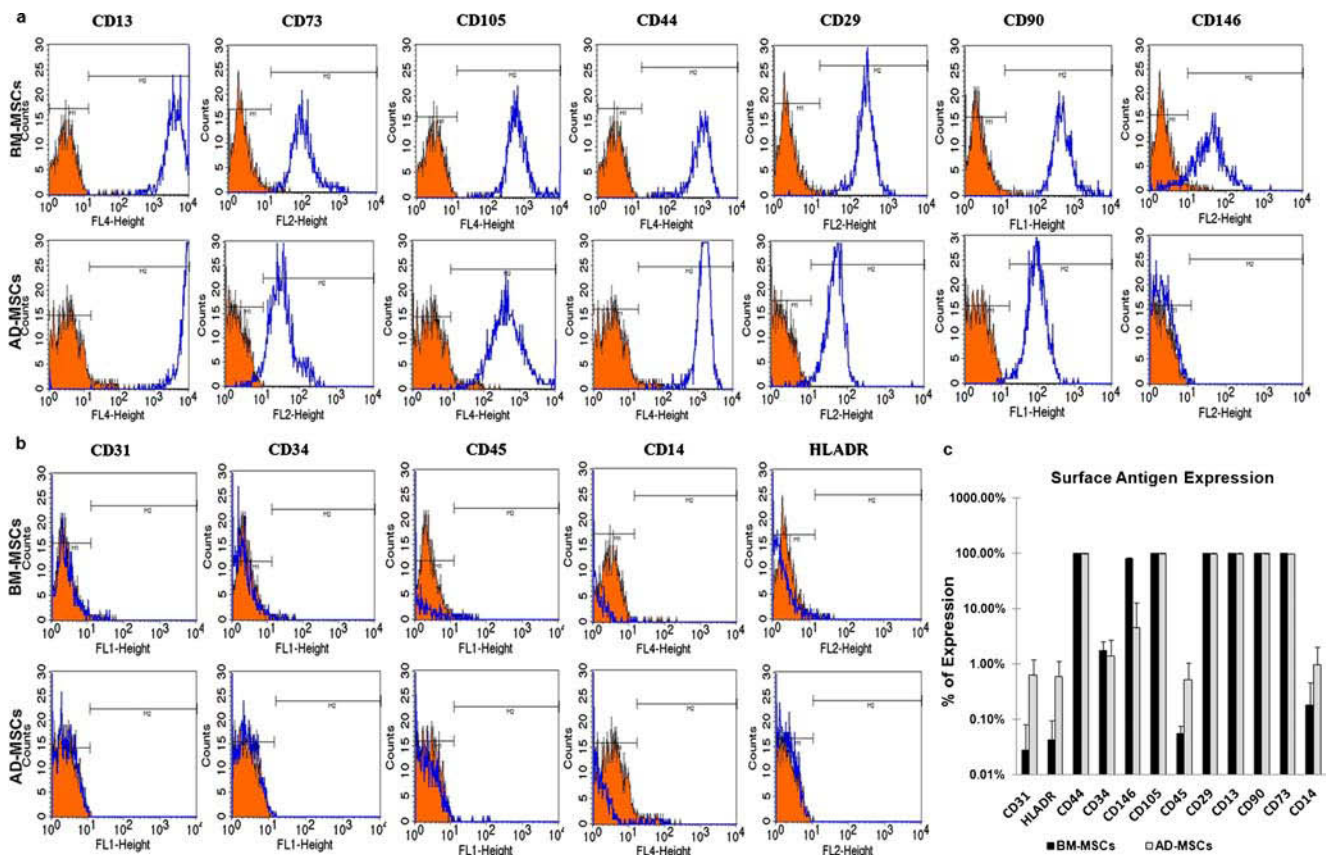
Fig. 2 Phenotype analyses of hBM-MSCs and hAD-MSCs by flow cytometry. Flow cytometry analysis of cell surface protein expression. **a** Stromal-cell-associated markers CD13, CD29, CD44, CD73, CD90, CD105 and CD146. **b** Hematopoietic and endothelial lineage markers CD31, CD34, CD45, CD14 and HLADR. *Filled histograms* in **a**, **b** represent cells stained by the corresponding isotype control antibody. Five thousand events were collected and counted. **c** Bar diagram representing percentages of cells showing CD marker expression (mean±SD)

80% of the MSC population derived from bone marrow but only by less than 3% of the MSC population derived from adipose tissue. In comparison, except for CD146, no obvious differences were observed in the expression of other surface antigens (Fig. 2c).

CFU-F assay

CFU-F numbers were compared at passage 1 and passage 4 of MSCs. Significant changes were observed in both groups of BM-MSCs and AD-MSCs (Fig. 3c). CFU-F numbers in bone marrow determined at passages 1 and 4 after primary culture were found to decline with the passage numbers. The CFU rate of recurrence in BM-MSC declined significantly by passage 4 (three-fold), with over a one-fold increase in prevalence of CFU in AD-MSC. Each colony had approximately 40 cells (Fig. 3a, b). Immunohistochemical analysis showed that these cells at passage 2 expressed vimentin suggesting their mesenchymal lineage property (Fig. 1e, f).

Multilineage capability of MSCs: qualitative and quantitative analysis

In all of our BM-MSC and AD-MSC populations, osteogenic differentiation could be induced, as studied qualitatively by staining for mineralization by alizarin red S and the
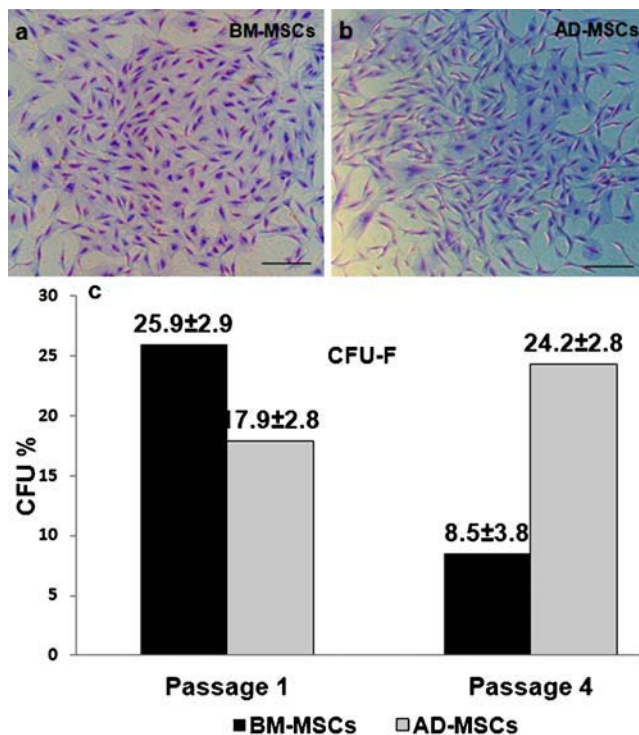

Fig. 3 CFU-F analysis of hBM-MSCs and hAD-MSCs. Colony-forming unit assay (CFU-F) on day 14 at passage 1 (**a**, **b**). *Bars* 100 μm. Comparative analysis of CFU-F percentages (**c**)

von Kossa method and by staining for ALP activity (Fig. 4a-h). Mineralization and ALP activity was low in AD-MSCs compared with BM-MSCs; moreover fluorescence-based calcium-level quantification confirmed that AD-MSCs (non-induced: 0.2 ± 0.04, induced: 0.4 ± 0.1) had significantly less relative fluorescence than BM-MSCs (non-induced: 0.4 ± 0.1, induced: 0.9 ± 0.1; paired t-test between the groups: at day 0, $P=0.031$; at day 10, $P=0.022$; Fig. 4i). The expression of osteogenic genes was assessed by real-time RT-PCR at day 0 and 10 after induction. The genes included were ALP, osteocalcin (a transcript; bone gamma-carboxyglutamic-acid-containing protein [BGLAP]) and osteopontin (bone sialoprotein I [BSP-1 or BNSP]), an extracellular matrix protein and a marker of mature osteoblasts. Although osteogenic genes were upregulated in both induced MSCs, AD-MSCs were less significantly affected compared with BM-MSCs. The paired t-test illustrated non-significance for ALP ($P=0.085$) and significance for osteocalcin ($P=0.007$) and osteopontin ($P=0.016$) between the groups (Fig. 4j).

With regard to adipogenic differentiation, lipid vacuoles were observed in both MSC populations (Fig. 5a-d) but this was seen in marginally fewer BM-MSCs (70%) compared with AD-MSCs (80%) by quantitative flow cytometry Nile red staining (Fig. 5f-h). The expression of genes for peroxisome proliferator activated receptorγ (PPARγ; a lipocyte-specific transcription factor), aP2 (fatty-acid-binding protein 4) and adiponectin (a transcript that is over-expressed in preadipocytes) was analyzed by real-time RT-PCR at day 0 and day 10 after induction. PPARγ, aP2 and adiponectin were upregulated during adipogenesis. The expression of these three genes was detected in both groups, with significantly lower expression in BM-MSCs compared with AD-MSCs. The paired t-test showed that aP2 ($P=0.118$) was non-significantly different between the groups but that both PPARγ ($P=0.016$) and adiponectin ($P=0.031$) were significantly different between the groups (Fig. 5e).

Discussion

Great expectations are connected with MSCs in the field of regenerative medicine. The unlimited differentiation of stem cells have made researchers isolate it from various tissues of the body. Protocols for the expansion, characterization and functional analysis of MSC are widely heterogeneous. To resolve this issue, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed minimal criteria to characterize human MSCs (Dominici et al. 2006). In addition to their plastic adherence and surface marker expression, MSCs should possess the ability to differentiate into osteoblasts, adipocytes and chondroblasts in vitro (Dominici et al. 2006). However, a plethora of publications of qualitative analyses exist to
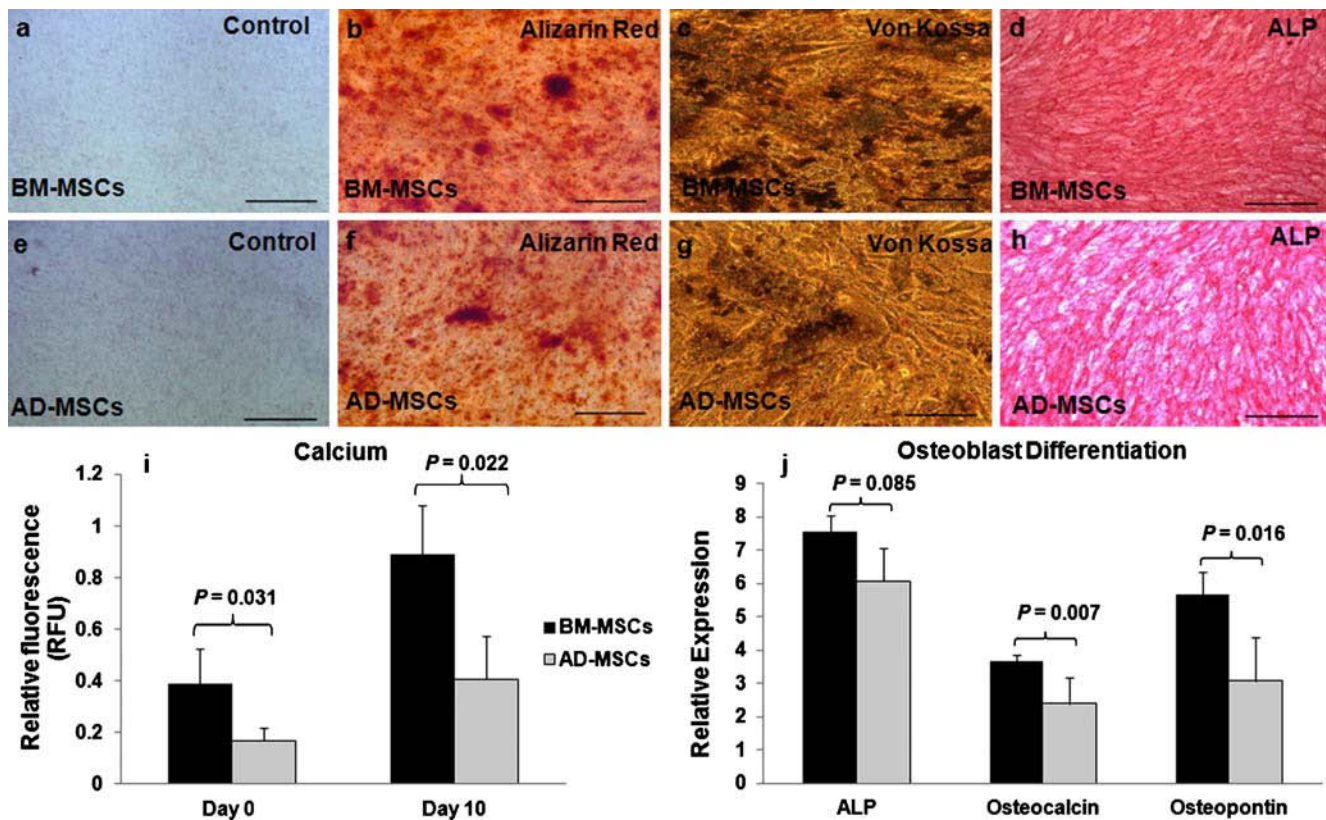
Fig. 4 Osteoblast differentiation potential of hBM-MSCs and hAD-MSCs. Qualitative and quantitative analyses in vitro. **a**, **e** Non-induced cells. **b**–**d**, **f**–**h** Induced cells were stained by Alizarin red S (**b**, **f**) and von Kossa (**c**, **g**) staining and for alkaline phosphatase activity (*ALP*; **d**, **h**) to evaluate osteogenesis. *Bars* 100 μm. **i** Calcium assay by fluorometry. Results are expressed in relative fluorescence units (*RFU*). **j**
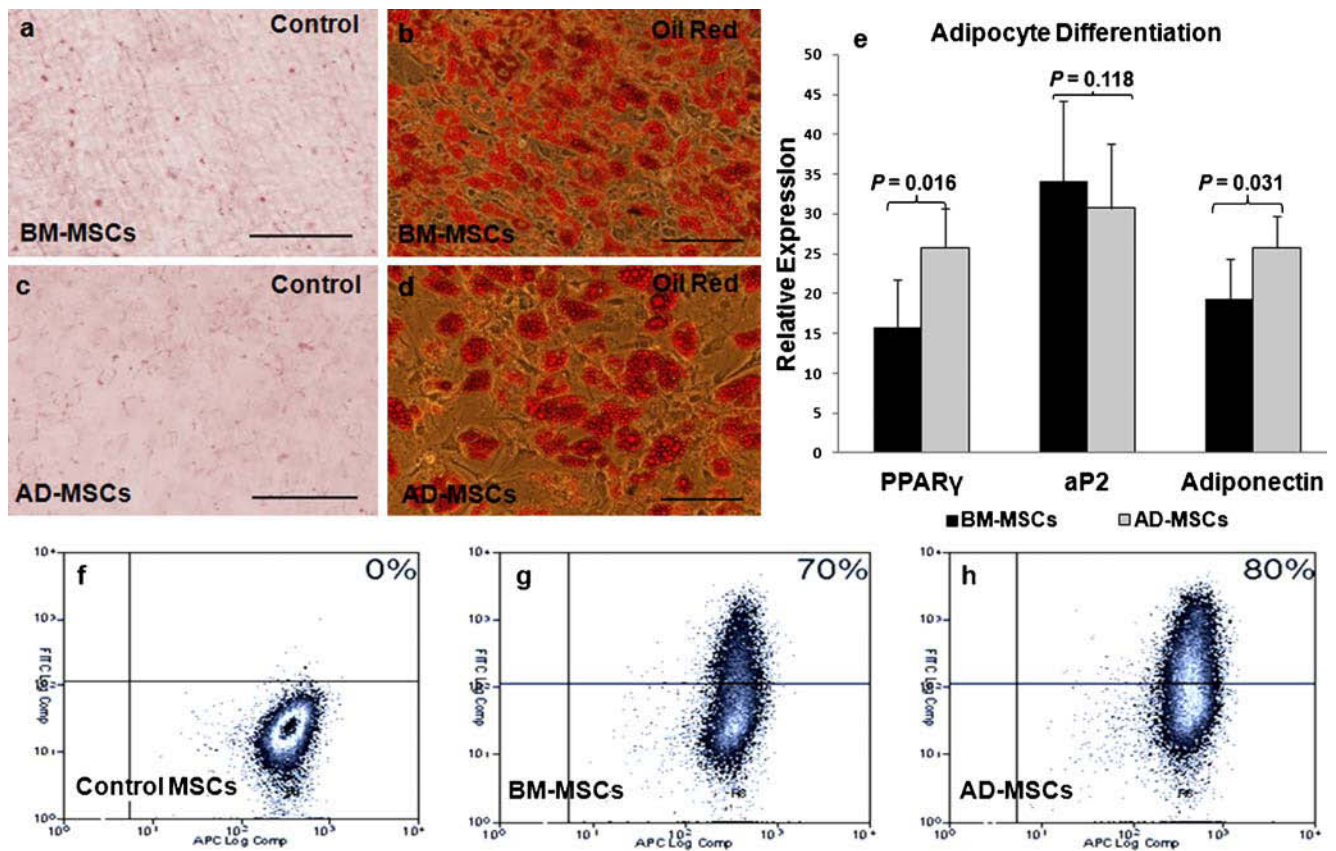
Analysis by quantitative reverse transcription with the polymerase chain reaction (qRT-PCR) of osteogenic-associated gene expression in induced and non-induced hBM-MSCs and hAD-MSCs. Data are shown as mean±SD (Student's paired *t*–test [2-tailed] between two groups, hBM-MSCs versus hAD-MSCs of five donor biological samples with at least three independent experiments)

demonstrate MSC multipotency from different sources (Yoo et al. 2009; Nekanti et al. 2010; Pittenger et al. 1999; Zhu et al. 2008; Wagner et al. 2005). The existence of differences needs to be addressed based on the comparative evaluation of MSCs from different sources. Thus, qualitative investigations are not sufficient to define MSC multipotency and need additional studies that offer a detailed understanding of MSCs.

Microscopic observation during expansion has shown that both BM-MSCs and AD-MSCs display vimentin positivity and similar morphology (Vidal et al. 2011). We have observed that the adherent cell percentage is comparable between bone marrow and adipose tissue (De Ugarte et al. 2003). Disparate statements have been observed regarding MSC colony numbers and age-related declines (Stolzing et al. 2008). In our report, we have analyzed the colony-forming efficiency of BM-MSCs and collagenase-digested AD-MSCs under the same conditions and passage numbers. We have found a passage-related decline in BM-MSCs, whereas considerable improvement occurs in AD-MSCs. Thus, in the later passages, the abundance of CFU-F in the adipose-derived population (24.2%) is significantly greater than in that of BM-MSCs (17.9%).

According to the phenotypic analysis described earlier (Dominici et al. 2006; Wagner et al. 2005; Yoshimura et al. 2006), both BM-MSCs and AD-MSCs are positive for known stromal-cell-associated markers, such as CD73, CD29 and CD90 and negative for the hematopoietic and endothelial markers CD45, CD14, CD34 and CD31. CD105, CD13 and CD44 are also expressed by both cell types, whereas HLA-DR (Class II MHC) is not detectable. Interestingly, CD146 (melanoma cell adhesion molecule; MCAM) is not expressed on AD-MSCs, whereas strong expression has been noticed in BM-MSCs. CD146 is one of the most commonly reported positive markers on bone marrow and MSCs derived from other sources (Mafi et al. 2011). A previous study has reported that a CD146-positive multipotential stem cell population exists within adult human adipose tissue and that this population is closely associated with perivascular cells surrounding the blood vessels (Zannettino et al. 2008). CD146-positive endothelial and pericyte progenitors from freshly isolated adipose-derived populations have been identified as CD146-negative in the major population after the plating of the AD-MSCs (Yoshimura et al. 2006). CD146 alone cannot determine

Fig. 5 Adipocyte differentiation potential of hBM-MSCs and hAD-MSCs. Qualitative and quantitative analysis in vitro. **a, c** Non-induced cells. **b, d** Induced cells were stained with Oil Red O to analyze neutral lipid vacuoles. *Bars* 100 μm. **e** qRT-PCR analyses of adipogenic-associated gene expression in induced and non-induced hBM-MSCs and hAD-MSCs. Data are shown as mean ± SD (Student's paired *t*–test [2-tailed] between two groups, hBM-MSCs versus hAD-MSCs of five donor biological samples with at least three independent experiments). **f–h** Nile red staining for adipocyte differentiated and undifferentiated cells by flow cytometry

the character of MSCs, since it is expressed on endotheilal (CD31$^+$, CD34$^+$, CD45$^-$, CD90$^+$, CD105^{low}, CD146$^+$) and pericyte/perivascular (CD31$^-$, CD34$^-$, CD45$^-$, CD90$^+$, CD105$^-$, CD146$^+$) cells (Zannettino et al. 2008; Yoshimura et al. 2006). Moreover, human fibroblasts show an indistinguishable phenotype, like MSCs (Wagner et al. 2005). Thus, this CD marker panel is not sufficient to define MSCs and requires modification as the ISCT has proposed in its minimal criteria for defining multipotent mesenchymal stromal cells (Dominici et al. 2006). Many studies have shown that adherent cells represent true MSCs as demonstrated by adipogenic and osteogenic activity (Barry and Murphy 2004; Lee et al. 2004). No substantial quantitative information exists to describe their lineage differentiation capability, which might provide the best alternative source for MSC-based regenerative application. As previously described, MSCs acquire osteoblastic morphology with deposition of a Ca^{2+}–rich mineralized extracellular matrix and the upregulation of ALP activity by osteogenic supplements. Similarly, adipocyte differentiation has been confirmed by lipid-filled vacuoles analysis (Barry and Murphy 2004).

Adipocyte differentiation in our cells has been demonstrated by the well-acknowledged Oil red O stain and has further been confirmed by flow cytometric analysis with the lipophilic fluorescent dye. Further, Nile red has been used to quantify the extent of lipid accumulation within the differentiated MSCs (Schaedlich et al. 2010; Gimble et al. 1995). We have observed a greater adipogenic potential in AD-MSCs (80%) than in BM-MSCs (70%), whereas both are indistinguishable and strongly positive for Oil red O stain. Furthermore, previous studies strongly recommend this technique for adipocyte cell sorting from the heterogeneous cell population (Schaedlich et al. 2010). Therefore, it might be a more efficient and reliable technique for adipocyte quantitative analysis.

Based on previous reports concerning the osteogenic potential of human MSCs derived from bone marrow and adipose tissue (Lee et al. 2004; Gimble and Guilak 2003), we have aimed to compare the osteogenic potential of both MSCs based on identical features. Because of the lack of definite and established molecular, CD, and gene markers to recognize adult stem cells, functional analyses are required

to identify these cells. In our study, BM-MSCs and AD-MSCs have been demonstrated to be able to differentiate in vitro toward the osteogenic lineage, as reported earlier (Lee et al. 2004; Gimble and Guilak 2003). Although both MSC types differentiate and are positive for ALP activity and mineralization as shown by Alizarin red S and von Kossa qualitative staining, they have considerable differences in calcium deposition. BM-MSCs are more potent than AD-MSCs in this respect and we could even observe these characteristics in a naive condition (Shafiee et al. 2011). Together, the linage differentiation potential have been analyzed and confirmed by appropriate transcription factors such as ALP, osteocalcin and osteopontin for osteogenic lineages and PPARγ, aP2 and adiponectin for adipogenic lineages, in addition to cytochemistry and quantitative analysis.

In conclusion, we confirm that both human BM-MSCs and AD-MSCs have an identical morphology and phenotype and that both undergo the process of adipogenic and osteogenic differentiation. Comparative analyses have illustrated the lowered colony-forming and adipogenic potential of BM-MSCs in comparison with AD-MSCs. However, both MSC types have responded to functional analyses by each of them having unique properties in differentiation levels according to their micro-environmental niche; this has been attested by quantitative analysis. Thus, quantitative analysis can be a useful parameter in order to define stem cell multipotency, in addition to qualitative investigations. Both the assessed MSCs might play a vital role in future regenerative medicine.

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