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REGULAR ARTICLE

Multi-potent progenitors in freshly isolated and cultured human mesenchymal stem cells: a comparison between adipose and dermal tissue

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Abstract Mesenchymal stem cells (MSCs) from human adult adipose tissue (A-MSCs) have a better differentiative ability than MSCs derived from the derma (D-MSCs). To test whether this difference is associated with differences in the content of multi-potent progenitors in A-MSCs, the number and the differentiative properties of multi-potent progenitors have been analyzed in various preparations of A-MSCs and D-MSCs. Adipogenic and osteogenic differentiation performed on colony-forming units have revealed that adipogenic and osteogenic progenitors are similar in the two populations, with only a slighty better performance of A-MSCs over D-MSCs from passages p0 to p15. An analysis of the presence of tri-, bi-, uni- and nulli-potent progenitors isolated immediately after isolation from tissues (p0) has shown comparable numbers of tri-potent and bi-potent progenitors in MSCs from the two tissues, whereas a higher content in uni-potent cells committed to adipocytes and a lower content in nulli-potent cells has been observed in A-MSCs. Furthermore, we have characterized the progenitors present in A-MSCs after six passages in vitro to verify the way in which in vitro culture can affect content in progenitor cells. We have observed that the percentage of tri-potent cells in A-MSCs at p6 remains similar to that observed at p0, although bi-potent and uni-potent progenitors committed to osteogenic differentiation increase at p6, whereas nulli-potent cells decrease at p6. These data indicate that the greater differentiative ability of A-MSC populations does not correlate directly with the number of multi-potent progenitors, suggesting that other factors influence the differentiation of bulk populations of A-MSCs.

Keywords Mesenchymal stem cells · Adipose tissue · Differentiation · Progenitor cells · Human

Introduction

Mesenchymal stem cells (MSCs) represent a population of adult mesenchymal progenitor cells with the potential to differentiate not only into a variety of mesodermal cell types, such as pre-adipocytes, osteocytes and chondrocytes but also, with minor efficiency, into skeletal, cardiac and smooth muscle cells (Castro-Malaspina et al. 1980; Caplan 1991; Galmiche et al. 1993; Prockop 1997; Pittenger et al. 1999; Reyes et al. 2001; LaBarge and Blau 2002). Furthermore, MSCs can acquire morphologic and phenotypic characteristics of cells of non-mesodermal origin, such as neuronal cells, insulin-secreting cells and hepatic cells (Petersen et al. 1999; Theise et al. 2000; Deng et al. 2001; Sanchez-Ramos 2002; Woodbury et al. 2000; Jiang et al. 2002; D'Ippolito et al. 2004; Gallo et al. 2007). Originally, MSCs were

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isolated from human bone marrow (BM-MSCs; Friedenstein et al. 1976; Prockop 1997; Pittenger et al. 1999) within which they represented a small percentage of the total stromal cell population that contributes to the organization of the microenvironment supporting the differentiation of hematopoietic cells (Muller-Sieburg and Deryugina 1995; Koller et al. 1997; Strobel et al. 1986; Tavassoli and Friedenstein 1983; Sacchetti et al. 2007). In more recent years, the existence of MSCs with properties similar but not identical to BM-MSCs has been demonstrated in the stromal fraction of the connective tissue from several organs, including adipose tissue, trabecular bone, derma, liver and muscle (Zuk et al. 2001, 2002; Noth et al. 2002; Sottile et al. 2002; Tuli et al. 2003; Toma et al. 2001; Lee et al. 2004; Beltrami et al. 2007; da Silva Meirelles et al. 2006).

MSCs are a heterogeneous cell population of adherent spindle-shaped cells that can be expanded in vitro for up to 20 passages. When plated at low density, MSCs form small colonies, called colony-forming units of fibroblasts (CFU-f), which have been proposed to correspond to the progenitors that can differentiate into one of the mesenchymal lineages (Friedenstein et al. 1976; Prockop 1997; Castro-Malaspina et al. 1980; Kuznetsov et al. 1997; Pittenger et al. 1999; Colter et al. 2001). Alternatively, cells can be expanded starting from a CFU-f and the differentiative properties of these clonal populations can be tested for their ability to differentiate into three, two, one, or none of the three most relevant connective tissue lineages (e.g., adipocytes, chondrocytes and osteocytes). Studies of human bone marrow have revealed that about one-third of the MSC clones are able to acquire phenotypes of pre-adipocytes, osteocytes and chondrocytes (Pittenger et al. 1999). This has been confirmed in an independent study in which 30% of the clones from bone marrow have been found to exhibit a tri-lineage differentiation potential, whereas the remainder display a bi-lineage (osteo-chondro) or uni-lineage (osteo) potential (Muraglia et al. 2000). Moreover, MSC populations derived from adipose tissue and derma present a heterogeneous differentiation potential; indeed, only 1.4% of single cells derived from adipose-derived adult stem cell (ADAS) populations were tri-potent, the others being bi-potent or uni-potent (Zuk et al. 2002). The analysis of MSCs isolated from the derma has revealed that about 6% of clones are able to differentiate into pre-adipocytes, osteoblasts and chondroblasts, whereas 19% are bi-potent (adipo-osteo or osteo-chondro), 10% were uni-potent and 64% were nulli-potent (Chen et al. 2007). Together, these results indicate that MSC populations are heterogeneous and consist in cells with variable differentiation potentials (tri-, bi-, uni-, nulli-potent; Baksh et al. 2004). In this context, since MSCs from human adult tissues represent a promising source of cells for a wide range of cellular therapies (Caplan 2009) and considering that their usage requires in vitro expansion, a verification of the way that extended culture conditions can affect the properties and the number of MSC progenitors is of great interest.

In this study, we have analyzed the differentiation potential of populations of MSCs isolated from adipose tissue (A-MSCs) in comparison with that of MSCs isolated from the derma (D-MSCs). In particular, we have addressed the questions as to whether a variation in the differentiation capability is correlated with either the number of progenitors or with the differentiation ability of these progenitors and as to the way in which the number and the differentiation properties of multi-lineage progenitors are maintained following in vitro culture. To this aim, we have evaluated the number of CFU-f and their ability to differentiate into pre-adipocytes or osteoblasts immediately after cell isolation (p0) and following serial passages in vitro up to passage 15 (p15). We have also expanded clonal cell populations of A-MSCs and D-MSCs to determine the presence of tri-, bi-, uni- and nulli-potent progenitors immediately after isolation from tissues (p0). Lastly, we have isolated clonal cell populations from A-MSCs after six passages in vitro and compared their differentiative capabilities with cells at p0. We have found that the number of tri-potent progenitors is not changed after the in vitro culture of A-MSCs, whereas the number and properties of bi-potent and uni-potent and the content in nulli-potent cells are moderately changed.

Materials and methods

Isolation and culture of MSCs

Human BM-MSCs Human BM-MSCs were isolated as previously described (Pittenger et al. 1999) from ~2 ml bone marrow aspirate from three healthy donors. Samples were washed with phosphate-buffered saline (PBS; Sigma, St. Louis, Mo., USA) containing 0.2% bovine serum albumin (BSA) and digested in α-minimum essential medium (α-MEM; Cambrex, BioSciences Verviers, Belgium), 0.02% collagenase II (Sigma) and 100 U/ml DNase, for 45 min at room temperature with gentle agitation. After digestion, samples were centrifuged, resuspended in PBS and filtered through a 70-μm nylon mesh (BD, Biosciences, Mississauga, ON, Canada). Cells were then stratified on Ficoll-Paque centrifugation gradient, washed twice in PBS and resuspended in a expansion medium consisting in α-MEM supplemented with 100 IU/ml pencillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS; all from Sigma). To evaluate cell viability, cells were counted by means of the Trypan Blue exclusion test (Sigma).

Human A-MSCs Human adipose tissue was obtained from elective liposuction undertaken by seven donors. The raw lipoaspirate was processed according to Zuk et al. 2001, with some modifications. To isolate the stromal vascular

fraction (SVF), lipoaspirates (~10 ml) were washed with PBS and the extracellular matrix was digested with 0.075% collagenase type IA (Sigma), at 37°C for 1 h. Enzyme activity was neutralized with an equal volume of expansion medium and centrifuged at 600 *g* for 10 min to obtain a high-density SVF pellet. The pellet was resuspended in expansion medium, filtered through a 100-μm nylon mesh (BD, Biosciences, Mississauga) to remove cellular debris and centrifuged again. The final pellet was resuspended in expansion medium and filtered through 70-μm nylon mesh. Cells were counted by means of the Trypan Blue exclusion test to evaluate cell viability.

Human D-MSCs Fresh human foreskin samples were obtained from five donors. Cells were isolated according to Chen et al. 2007. The specimens were washed with PBS and the subcutaneous tissues were removed carefully. The tissue was cut into small pieces (1-3 mm^3) and then digested by incubation in 0.1% dispase (Whortington Biochemical, Lakewood, N.J., USA) at 4°C, overnight. The following day, the epidermal layers were removed and the remaining dermal parts were further digested with 0.1% collagenase I (Whortington) at 37°C for 4 h. The digested cells were passed through a 70-μm nylon mesh, centrifuged and resuspended in expansion medium. Isolated cells were counted by means of the Trypan Blue exclusion test. For proliferation, all MSCs were seeded at 4000 cells/cm^2 and maintained in expansion medium until 60%-70% confluent. Cells were harvested by using 0.25% trypsin/2mM EDTA (Sigma) and population doublings (PD) were calculated by using the formula: $PD=\ln_2/(\ln C2)/(\ln C1)$, where C1 represents the number of cells seeded at a given passage and C2 the number of cells harvested at the next passage. Cumulative PD was determined in cultures maintained until p15. The mean cumulative PD was obtained from three samples for each tissue.

Flow cytometry

Mesenchymal cells maintained in culture until p5-p6 were trypsinized and resuspended in expansion medium. Their viability was analyzed by using Trypan Blue. Cells were washed in ice-cold PBS, 0.5% BSA (fluorescence-activated cell-sorting [FACS] buffer) and resuspended in the same buffer. Samples containing 2×10^5 cells were incubated for 30 min at 4°C with the following monoclonal antibodies (mAbs): phycoerythrin (PE)-conjugated CD13 (1:10, clone WM47; DAKO), fluorescein-isothiocyanate (FITC)-conjugated CD14 (1:10, clone M5E2; BD, Biosciences, Franklin Lakes, N.J., USA), PE-conjugated CD29 (1:10, clone MAR4, BD), PE-conjugated CD31 (1:10, clone 10G9; Santa Cruz), allophycocyanin (APC)-conjugated CD34 (1:10, clone 581, BD), FITC-conjugated CD44 (1:10, clone DF1485, DAKO), FITC-conjugated CD45 (1:10, clone 35-Z6; Santa Cruz), FITC-conjugated CD49b (1:10, clone AK-7; BD), PE-conjugated CD49e (1:10, clone IIA1; BD), PE-conjugated CD54 (1:10, clone HA58; BD), purified CD73 (1:100, clone MOPC-21; BD), PE-Cy5-conjugated CD90 (1:10, clone 5E10; BD), PE-conjugated CD105 (1:10, clone N1-3A1; Ancell, Bayport, Minn., USA), FITC-conjugated CD106 (1:10, clone 51-10c9; BD), PE-conjugated CD117 (1:10, clone YB5. B8; BD), PE-conjugated CD133/1 (1:10, clone AC133; Miltenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated CD146 (1:10, clone P1H12; BD), APC-conjugated histocompatability antigen (HLA)-ABC (1:10, clone G46-2.6; BD), PE-Cy5-conjugated HLA-DR (1:10, clone G46-6; BD), purified Stro-1 (clone stro-1; R&D Systems, Minneapolis, Minn., USA). Identical IgG isotypes (BD, Biosciences) and secondary antibody alone were used as negative controls. Threshold was adjusted in forward and side scatter dot plots to exclude cellular debris. A total of 10,000 events were counted on a FACS Calibur flow cytometer and the data were analyzed on CellQuest software (BD, Biosciences).

Mesenchymal differentiation assays

Non-clonal MSC populations and clones derived from adipose tissue and derma were analyzed for their capacity to acquire adipogenic, osteogenic, or chondrogenic phenotypes. For the adipogenic and osteogenic differentiation assays, cells were seeded at 20,000 cells/cm^2 and cultured in expansion medium as a control. Each experiment was repeated three times for non-clonal MSCs and once for each clone.

Adipogenesis The day after plating, cells were induced by adipogenic medium consisting in α-MEM supplemented with 100 IU/ml pencillin, 100 μg/ml streptomycin, 2 mM L-glutammine, 10% FBS, 0.5 mM isobutyl-metylxanthine (IBMX), 1 μM dexamethasone, 10 μM insulin, 200 μM indometacin (all from Sigma) for 2 weeks. Oil Red O staining was performed to verify intracellular lipid accumulation. Prior to staining, cells were fixed for 15 min at room temperature in 4% paraformaldehyde and then washed with double-distilled H_2O followed by 70% ethanol. The cells were incubated in 2% (wt/vol) Oil Red O solution (Sigma) at room temperature for 10 min; excess stain was removed by washing with 70% ethanol followed by distilled water. The content of Oil Red O uptaked in samples was quantified by extaction with 100% ethanol and spectrophotometric measurements at 540/620 nm.

Osteogenesis Osteogenic differentiation was induced by culturing cells for 4 weeks in osteogenic medium consisting

in α-MEM supplemented with 100 IU/ml pencillin, 100 μg/ml streptomycin, 2 mM L-glutammine, 10% FBS, 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate (all from Sigma). Cultures were examined for calcified extracellular matrix deposition by Alizarin Red S (ARS) staining. Cells were fixed with 10% (v/v) formaldehyde for 15 min at room temperature, rinsed with distilled water and incubated for 20 min in 40 mM ARS, pH 4.1. ARS solution was removed and the cells were washed with distilled water and stored at -20°C until analysis. Quantification of the stain deposited was evaluated by extraction with 10% acetic acid for 30 min at room temperature and the dilution of each sample with 10% ammonium hydroxide; the spectral absorbance was measured at 450/620 nm.

Chondrogenesis Chondrogenic differentiation of BM-MSCs, A-MSCs and D-MSCs was induced by using the micromass culture technique (Zuk et al. 2001). Briefly, 10 μl concentrated cell suspension (8×10^6 cells/ml) was plated into the center of a 35-mm plate and the cells were allowed to attach at 37°C for 2 h. Chondrogenic medium viz., serum-free expansion medium containing 0.1 μM dexamethasone, 50 μg/ml L-ascorbate-2-phosphate, 40 μg/ml L-proline, 100 μg/ml sodium pyruvate, ITS-Premix (BD Biosciences, Bedford, Mass., USA) and 10 ng/ml TGF-β3 (R&D Systems) was gently overlaid onto the culture medium and the cultures were maintained for 21 days prior to analysis. Chondrogenesis was confirmed by Alcian Blue histological staining. Cell nodules were fixed with 4% paraformaldehyde for 15 min at room temperature, washed by PBS and stained with 1% Alcian Blue (wt/vol) in 0.1 N HCl for 30 min at room temperature; excess stain was removed by washes for 5 min in 0.1 N HCl. The chondrogenic potential of clones was tested by using the micromass technique with modifications. Samples containing 250,000 cells were resuspended in expansion medium and pelleted by sedimentation at 400 *g* for 10 min at 4°C. Cell pellets were resuspended in chondrogenic medium for the induction of chondrogenesis or in expansion medium for controls. The cells were centrifuged again and then cultured for 3 weeks. Nodules were fixed in 4% paraformaldehyde, washed with PBS, embedded in Tissue-Tek O.C.T (Sakura Finetek USA, Torrance, Calif., USA) and frozen in liquid nitrogen for 5 min. Cells that had been cultured in pellets were cryosectioned at a thickness of 7 μm and stained with 1% Alcian Blue (wt/vol) as described above. For immunohistochemistry, sections were fixed with 4% paraformaldehyde for 15 min at room temperature, pre-digested with 300 U/ml hyaluronidase (Sigma-Aldrich) and rinsed in PBS. Endogenous peroxidase activity was quenched by a 10-min incubation with 1% hydrogen peroxide in methanol and a wash in PBS; non-specific sites were blocked by incubation with 1.5% blocking serum (ABC Staining System, Santa Cruz). The expression of collagen II was revealed by using an anti-collagen II mAb (clone 2B1.5; Zymed Laboratories) overnight at 4°C. Immunostaining was revealed by using the ABC Staining System (Santa Cruz).

DNA extraction

To quantify DNA content in the differentiated MSCs, we used Hoechst 33258 (Sigma Aldrich). Briefly cells were digested with 1 mg/ml pronase, 0.2% SDS, in a nuclear lysis buffer (10 mM TRIS, 1 mM EDTA pH 8.0, 5 M NaCl in H_2O) overnight at 37°C; 100 μl each sample was added to 0.2 μg/ml Hoechst 33258 in a TE buffer (10 mM TRIS, 1 mM EDTA). Fluorescence was measured following excitation at 355 nm and by registering the emission at 460 nm. A standard curve was prepared with salmon sperm DNA. To measure the differentiation potential of MSCs, the amounts of Oil Red O and Alizarin Red S extracted were divided by the DNA content (reported as mOD/min per microgram).

Analysis by reverse transcription and quantitative polymerase chain reaction

BM-MSCs, A-MSCs and D-MSCs were induced toward chondrogenic lineages for 21 days. Total cellular RNA was extracted from induced and control cells by using the RNeasy kit (Qiagen, Italy) following the manufacturer's instructions: 100 ng total RNA from each sample was reverse-transcribed by using the High Capacity cDNA reverse transcription kit (Applied Biosystem). To evaluate chondrogenic differentiation, the relative expression of the human decorin gene was measured in control and induced samples with the following primers: forward 5′-CGCCTCATCT GAGGGAGCTT-3′; reverse 5′-TACTGGACCGGGTTGCT GAA-3′. The real-time polymerase chain reaction (PCR) was performed according to the manufacturer's instructions by using the Fast SYBR Green Master Mix (Applied Biosystem) and a Step One Plus Thermal Cycler (Applied Biosystem) and 0.2 μl of the cDNA solution prepared above. At the end of the runs, a melting curve was generated and analyzed. The relative abundance of each transcript was normalized by using the housekeeping gene β-actin (forward primer: 5′-GATGA GATTGGCATGGCTTT-3′; reverse primer: 5′CACCTTC ACCGTTCCAGTTT-3′). The ratio of expression of the gene of interest was calculated by means of the Pfaffl quantification method (Pfaffl 2001).

Analysis of differentiation potential in CFU-f cultures

After isolation, A-MSC and D-MSC cell populations were plated at 20 cell/cm^2 density and grown in expansion

medium. CFU-f were cultured at 37°C and 5% CO_2, with replacement of the media every 3 days, until individual colonies were visible under microscopic examination. After 10-15 days, plates with colonies consisting in ~100 cells were exposed to adipogenic and osteogenic differentiation media. Three plates were used for each differentiation assay. Cells were stained with Oil Red O and Alizarin Red S and the positive colonies in each plate were counted with respect to the number of total colonies revealed by Giemsa staining. This procedure was repeated for every passage until p15. The results obtained represented the mean of the relative frequencies of each A-MSCs and D-MSCs populations. Adipogenic and osteogenic CFU-f were observed under an inverted microscope (Zeiss Axiovert 40C) and images were captured by using a Canon Power Shot G6 digital camera.

Giemsa staining

Cells were washed with PBS, fixed with 100% methanol for 3 min at room temperature and stained with Giemsa-May-Grunwald (Sigma) for 10 min. Cells were washed twice with PBS and visible colonies were counted.

Cell cloning

The cells in at least six 100-mm plates, seeded as described above, were maintained in expansion medium to isolate clones. On the day prior to ring cloning, individual colonies, namely at passage 0, were selected for cloning. Colonies consisting in 100 cells were chosen if they were separated by at least one microscope field from other cell clusters. Single CFU-Fs were circumscribed with a cloning ring, washed with PBS and incubated with 0.25% trypsin/2 mM EDTA. Cells were harvested in expansion medium and plated in 24-well tissue plates. Clones were expanded until p4 and assayed for their differentiation capability.

Results

Isolation and characterization of MSC cell populations

Human MSCs were isolated from adult adipose tissue (A-MSCs), bone marrow (BM-MSCs) and derma (D-MSCs) and cultured in expansion medium. Long-term proliferation was analyzed by evaluating cumulative PD with respect to passage number. As showed in Fig. 1a, all MSC populations were cultured in vitro for at least fifteen passages (p15) (corresponding to 140 days for BM-MSCs and D-MSCs and 154 days for A-MSCs). A-MSCs and D-MSCs showed no obvious changes in their growth rate, reaching 25 and 34 cumulative PD, respectively, while BM-MSCs appeared to have a lower proliferative potential, reaching 13 PD.

The differentiative potentials of MSC populations were examined by culturing the cells under specific conditions for differentiation into adipogenic, osteogenic and chondrogenic lineages, as detailed in Materials and Methods. Adipogenic differentiation was evaluated by the measurement of Oil Red O accumulation after 2 weeks of treatment (Fig. 1b-e). Deposition of calcified extracellular matrix, positively stained with Alizarin Red, was used as a marker of osteogenic differentiation after 4 weeks of treatment (Fig. 1f-i). Following induction of adipogenic or osteogenic differentiation, a significant increase in the amount of Oil Red O or Alizarin Red was observed in A-MSCs and BM-MSCs compared with that of non-induced cells ($P<0.05$). In contrast, in D-MSCs, no significant level of either adipogenic or osteogenic differentiation was observed. Chondrogenic differentiation was induced by using the micromass culture technique (Zuk et al. 2001) and evaluated after 21 days of treatment by means of Alcian Blue staining to detect the deposition of sulfated proteoglycans within the extracellular matrix. As shown in Fig. 1l-n, all cell populations were able to form cell nodules that stained posititively with Alcian Blue. Analysis by quantitative RT-PCR revealed an increase in decorin m-RNA expression following chondrogenic differentiation in all MSC populations, although with some experimental variability (Fig. 1o).

In parallel, the characteristic cell surface antigen profile of MSCs was analyzed by flow cytometry (Pittenger et al. 1999). As shown in Supplemental Table 1, after 5 passages in culture, a high percentage of cells in A-MSC, BM-MSC and D-MSC populations expressed CD13, CD29, CD73, CD90 and CD105, whereas CD45, CD14, CD34, CD106, STRO-1 and CD133 markers were expressed in less than 5% of the cells. Interestingly, the percentage of cells positively stained for CD146 was higher in D-MSCs and BM-MSCs than in A-MSCs. On the contrary, the number of cells expressing CD54 was higher in D-MSCs than in A-MSCs and BM-MSCs.

Analysis of frequency and of adipogenic and osteogenic potential of CFU-f following extended in vitro culture

Since A-MSC cell populations showed significantly higher adipogenic and osteogenic differentiative potentials than D-MSCs, we wished to verify whether MSCs from these two tissues contained a different number of mesenchymal progenitors and whether their number changed following long-term expansion in vitro. The number of CFU-f derived from a single cell has been proposed to identify the number of progenitors in a MSC population (Castro-Malaspina et al. 1980; Kuznetsov et al. 1997). With this aim, we tested

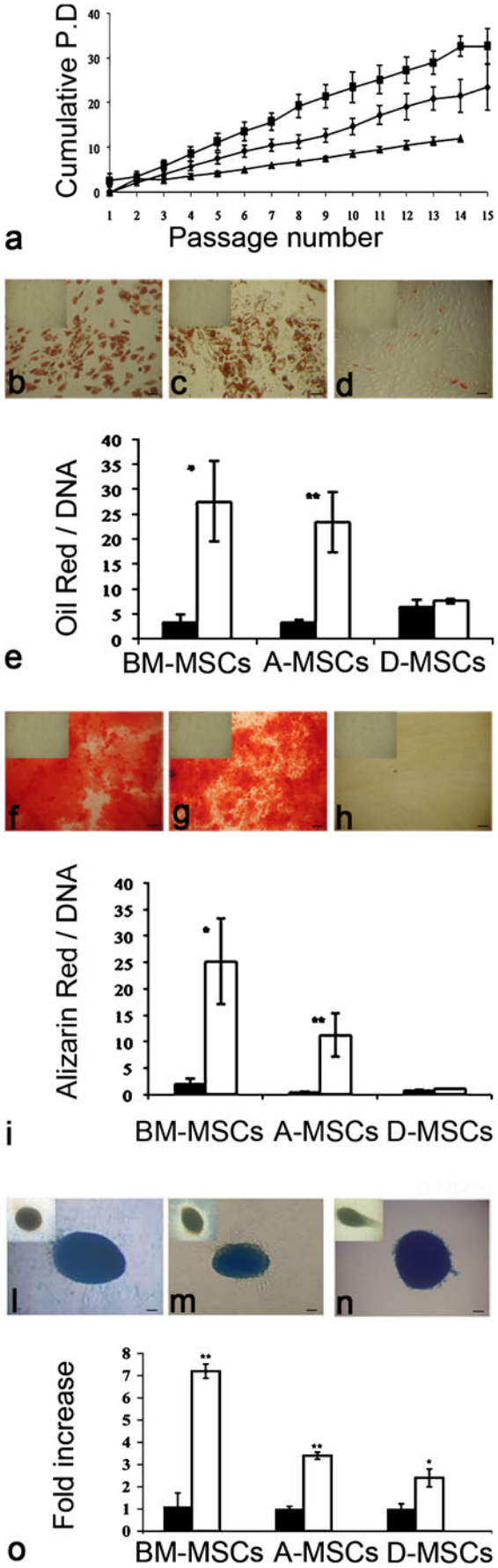

Fig. 1 **a** Growth kinetics of MSC populations. Cell viability was evaluated at every passage by the Trypan Blue exclusion test. Cumulative population doubling (*P.D*) was evaluated by cell counting after each passage. Data represent mean values of at least three different samples for each tissue: *triangles* bone marrow MSCs (*BM-MSCs*), *n*=3; *diamonds* adipose tissue MSCs (*A-MSCs*), *n*=7; *squares* derma MSCs (*D-MSCs*), *n*=5. **b–n** Mesenchymal lineage differentiation. Cytochemical staining of BM-MSCs (**b**, **f**, **l**), A-MSCs (**c**, **g**, **m**) and D-MSCs (**d**, **h**, **n**) at passage 5 induced to differentiate toward the adipogenic (Oil Red O, **b-d**), osteogenic (Alizarin Red, **f-h**) and chondrogenic (Alcian Blue, **l-n**) lineages. *Insets* Images of non-induced cells. **e**, **i** Quantitative analysis for adipogenic and osteogenic differentiation was performed by relating spectrophotometric measurements of extracted Oil Red O (**e**) and Alizarin Red (**i**) dyes from differentiated cells normalized to the DNA content (mean±SEM of three different MSC populations for each tissue; *black columns* control, *white columns* induced cell populations). **o** Expression of the chondrogenic marker decorin was evaluated in control and induced cells by quantitative RT-PCR (data are expressed as the fold increase±SD of decorin expression level in induced vs control cells for each MSC population; *black columns* control, *white columns* induced cell populations). Expression of β-actin was assessed as a housekeeping gene. Data were elaborated by using Microsoft Excel and statistical analysis was performed by using Instat Software (GraphPad, USA). **P*<0.05, ***P*<0.01. *Bars* 100 μm

the colony-forming efficiency of seven and five independent populations of A-MSCs and D-MSCs, respectively, at each passage from p0 to p15. As shown in Fig. 2a, the frequency of CFU-f formation was similar (about 10%) in both A-MSCs and D-MSCs and remained unchanged following in vitro culture up to p15. To test whether the number of progenitors from A-MSC and D-MSC populations changed following in vitro culture, adipogenic and osteogenic assays were performed on CFU-f at each passage from p0 to p15 (Fig. 2d, g). Chondrogenic differentiation is not reported, because, under our experimental conditions, it proved to be inefficient and poorly reproducible, as also observed by Gao et al. 2010. All CFU-f that appeared positive for Oil Red O and Alizarin Red staining by macroscopic observation were considered positive (Fig. 2b, c, e, f). The percentage of CFU-f able to acquire adipogenic and/or osteogenic phenotype was expressed as the average of positive colonies related to the number of total colonies revealed by Giemsa staining. As shown in Fig. 2d, the percentage of colonies positive for adipogenic differentiation in A-MSCs remained constant from p0 to p13. In contrast, in D-MSCs, the percentage of adipogenic CFU-f remained constant from p0 to p7 and started to decrease from p9 ($P<0.05$; Fig. 2d). The percentage of CFU-f positive for osteogenic differentiation started to decrease significantly in A-MSCs from p11, whereas in D-MSCs, the percentage of CFU-f positive for osteogenic differentiation started to decrease from p9 (Fig. 2g). Overall, these experiments indicated that the percentage of adipogenic progenitors in A-MSCs and D-MSCs was similar until p7 but, in D-MSCs, decreased in

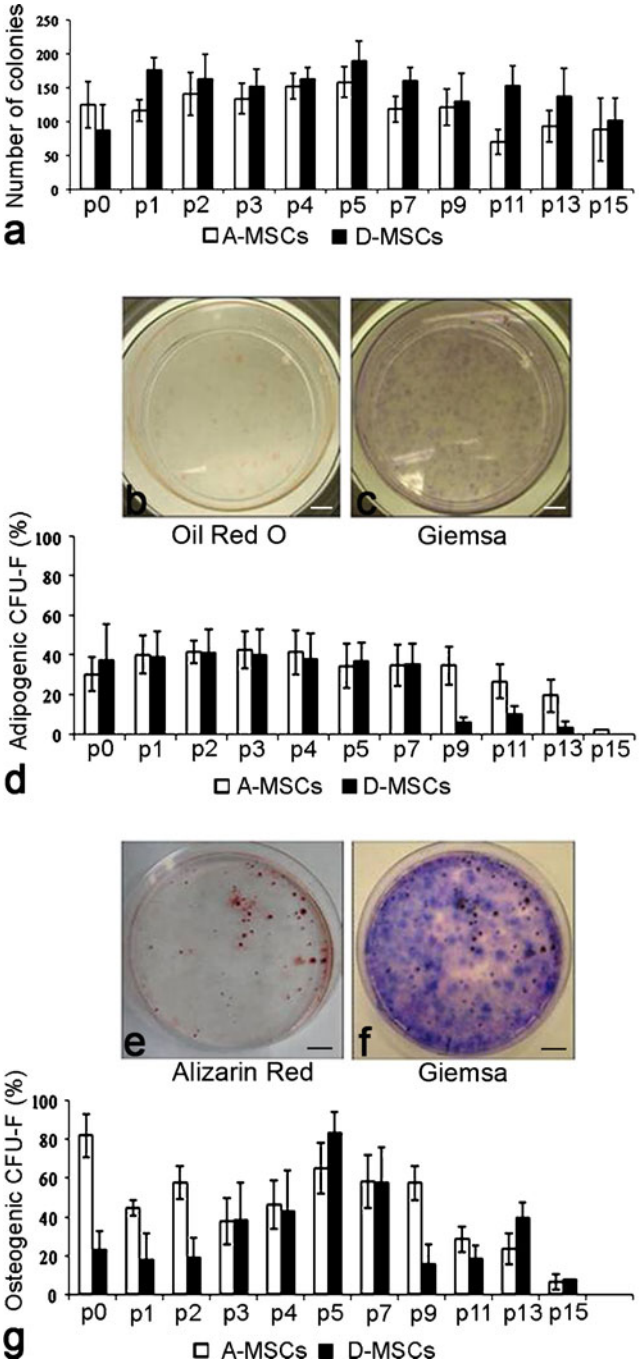

Fig. 2 Measurement of CFU-f. **a–c** A-MSC and D-MSC populations were plated at clonal density in 100-mm plates, at every passage from p0 to p15. When a single CFU-f consisted in ~100 cells, total colonies were counted in each plate. Data represent the means±SEM calculated for seven A-MSC and five D-MSC cell populations. **d-g** Analysis of adipogenic and osteogenic potential on CFU-f. A-MSC and D-MSC populations were plated at clonal density in 100-mm plates, from p0 to p15. When single CFU-f consisted in ~100 cells, growth medium was changed to adipogenic- or osteogenic-specific media. Adipogenic and osteogenic plates were counterstained by Giemsa to reveal the total number of CFU-f. Images were captured by using a Canon Power Shot G6 digital camera. Original magnification: ×20. **b** Oil Red O staining was performed at 2 weeks following adipogenic induction. **c** Gimsa staining of total CFU-f. **e** Alizarin Red staining was used to reveal the deposition of a calcified extracellular matrix at 4 weeks following osteogenic induction. **f** Gimsa staining of total CFU-f. **d**, **g** Comparison of frequencies of adipogenic and osteogenic CFU-f in A-MSCs and D-MSCs. Histograms represent the mean of relative frequencies of adipogenic (**d**) and osteogenic CFU-f (**g**) in A-MSC or D-MSC CFU-f measured in triplicate. Data were elaborated by using Microsoft Excel. Statistical analysis was performed by using Instat Software (GraphPad, USA). **P*<0.05. *Bars* 10 mm

the subsequent passages (p9-p15), although the total number of CFU-f was unchanged. In contrast, the frequency of osteogenic progenitors was higher in A-MSCs compared with D-MSCs only in the first three passages but was no different in the following passages.

Analysis of multipotency of MSC clonal populations

Although the analysis of differentiation performed by means of CFU-f provides a *bona fide* estimate of the number of progenitors able to differentiate into a specific mesodermal lineage, these experiments did not provide any information about the multipotency of individual CFU-f present in the two MSC populations. To evaluate the relative number of progenitors able to differentiate into either three, two, or one lineage (tri-potent, bi-potent and uni-potent progenitors), single-cell-derived clones were isolated and expanded from A-MSC and D-MSC cell populations at passage p0. Accordingly, 76 clones were isolated from five distinct dermal tissue specimens (10, 4, 19, 25 and 18 clones, respectively) and 49 clones were isolated from two distinct adipose tissue specimens (22 and 27 clones, respectively). Clones were expanded to reach 1-3 million cells and then assessed for their differentiative abilities toward adipogenic, chondrogenic and osteogenic lineages (Fig. 3).

As shown in Table 1, the analysis of clones isolated from passage 0 D-MSCs (column 2) revealed that 1/76 (1.3%) were tri-potent (adipogenic, osteogenic and chondrogenic), 16/76 (21%) were bi-potent, with 19.7% having the adipogenic/osteogenic phenotype and 1.3% with adipogenic/chondrogenic phenotypes. Of the remaining clones, 23/76 (30.2%) exhibited only a one-lineage potential, with 15.7%, 6.5% and 7.8% of the clones presenting adipogenic, osteogenic and chondrogenic phenotype, respectively. In addition, 36/76 clones (47.3%) from D-MSCs were nulli-potent. The parallel analysis of clones isolated from passage 0 A-MSCs (column 3) revealed that 1/49 (2%) of A-MSCs clones were tri-potent (adipogenic, osteogenic and chondrogenic), 11/49 (22.4%) were bi-potent, with clones showing adipogenic/osteogenic and adipogenic/chondrogenic phenotypes in 7/49 (14.2%) and 4/49 (8.1%), respectively, whereas no clones exhibited the osteogenic/chondrogenic phenotype. Uni-potent clones were found in 22/49 (44.8%) of cases, with clones expressing adipogenic, osteogenic, or chondrogenic phenotypes representing 32.6%, 10.2% and 2% of the total clones,

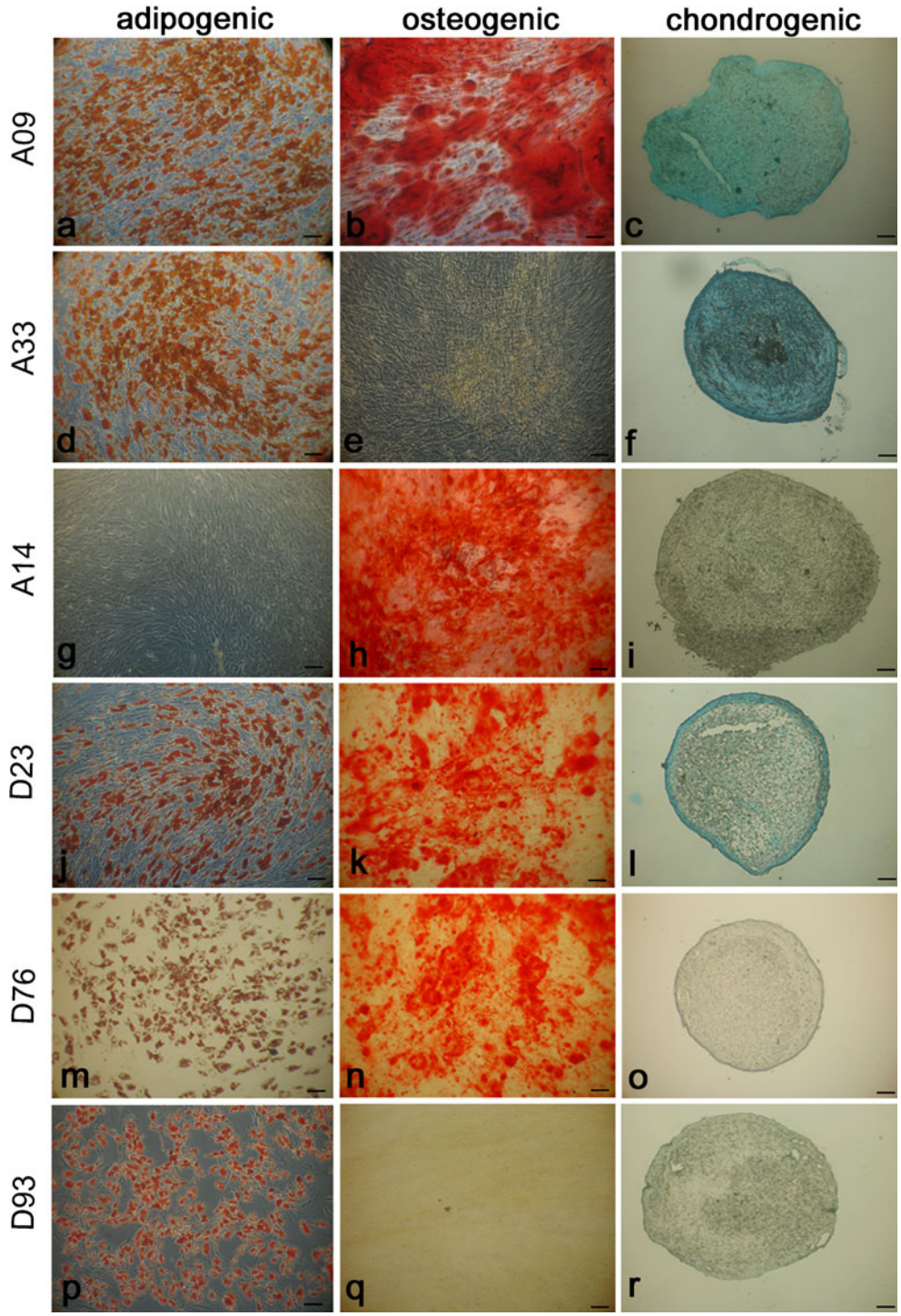

Fig. 3 Lineage differentiation potential of A-MSCs (**a-i**) and D-MSCs (**j-r**) clonal cell populations. Phenotypes of six representative clones are shown: *A09* and *D23* exhibited a three-lineage potential; *A33* and *D76* were bi-potent clones, viz., adipogenic/chondrogenic and adipogenic/osteogenic, respectively; *A14* and *D93* were uni-potent clones (osteogenic and adipogenic, respectively). **a**, **d**, **g**, **j**, **m**, **p** Oil Red O staining. **b**, **e**, **h**, **k**, **n**, **q** Alizarin Red staining. **c**, **f**, **i**, **l**, **o**, **r** Alcian Blue staining of sections of pellet frozen in OCT gel. *Bars* 100 μm

respectively. In addition, 15/49 clones (30.6%) showed no differentiation potential.

Analysis of multipotency of MSC clonal populations isolated from A-MSCs following expansion in vitro

We next wished to verify whether the number of multi-potent progenitors in A-MSCs changed following in vitro culture. With this aim, the same two A-MSCs preparations from which we had isolated 49 clones at p0 were cultured in vitro for six passages, after which 55 single-cell-derived clones were isolated. As shown in Table 1, clones isolated after the in vitro expansion of A-MSCs up to p6 (column 4), contained 1/55 (1.8%) tri-potent clones, comparable to those clones isolated at p0. Of note, the number of bi-potent clones increased from 11/49 (22.4%) detected in clones at

Table 1 Frequency and differentiation potential of MSC clonal populations isolated at passage 0 (*p0*) from human adult adipose tissue (*A-MSC*) and from derma (*D-MSC*) and of A-MSC clonal populations isolated at passage 6 (*p6*); 76 and 49 clones were isolated from D-MSCs and A-MSCs, respectively, at passage p0 and 55 clones were isolated from A-MSCs at passage p6. The frequency and differentiation potential of individual clones were assessed as indicated in Materials and Methods. The number and percentages of clones expressing specific phenotypes are listed (*A* adipogenic, *O* osteogenic, *C* chondrogenic)

Phenotype A/O/C	D-MSCs p0	A-MSCs p0	A-MSCs p6
	Total number of clones (% total clones)	Total number of clones (% of total clones)	Total number of clones (% of total clones)
Tri-potent (A O C)	1 (1.3)	1 (2.0)	1 (1.8)
Bi-potent	16 (21.0)	11 (22.4)	23 (41.8)
AO	15 (19.7)	7 (14.2)	21 (38.0)
AC	1 (1.3)	4 (8.1)	2 (3.6)
OC	0 (0)	0 (0)	0 (0)
Uni-potent	23 (30.2)	22 (44.8)	25 (45.4)
A	12 (15.7)	16 (32.6)	7 (12.7)
O	5 (6.5)	5 (10.2)	18 (32.7)
C	6 (7.8)	1 (2.0)	0 (0)
Nulli-potent	36 (47.3)	15 (30.6)	6 (10.9)

p0 to 23/55 (41.8%) in clones at p6. This increase was more evident for bi-potent cells expressing the adipogenic/osteogenic phenotype (21/55, 38% at p6 against 7/49, 14.2% at p0). The percentage of uni-potent cells was not greatly different, whereas the number of nulli-potent clones at p6 (6/55, 10.9%) was significantly lower than at p0 (15/49, 30.6%). On adding together the results from all clones isolated from A-MSCs at p0 and p6, the ability to differentiate into the osteogenic phenotype appears to be better represented in clones isolated at p6 than in those isolated at p0 (32.7% at p6 against 10.2 % at p0). On the other hand, we noted that, in clones isolated at p6, a decrease occurred in clones able to acquire the adipogenic phenotype (32.6% at p0 against 12.7% at p6). Overall, these experiments indicated that the in vitro passages of A-MSCs cultures did not significantly reduce the number of tri-potent clones, whereas changes were observed in the frequency and potency of the bi-potent cells and in the nulli-potent cells.

Discussion

Most of the available knowledge concerning MSCs has been obtained in experiments performed on non-clonal cell populations consisting in a heterogeneous population of cells with diverse self-renewal and differentiation properties (Friedenstein et al. 1976; Castro-Malaspina et al. 1980; Caplan 1991; Prockop 1997; Reyes et al. 2001; Jiang et al. 2002; Beltrami et al. 2007; Wagner et al. 2005; Baksh et al. 2007). As a consequence, variations in differentiation potentials are possible among MSC populations derived from distinct tissue origins. We have found that MSCs isolated from adipose tissues (A-MSC) show a higher differentiation potential than derma-derived MSCs (D-MSC). FACS analysis has revealed that A-MSC and D-MSC populations only differ with respect to the expression of CD54, a cell adhesion molecule expressed in several cell lineages (Springer 1995; Kvale and Holme 1996) and of CD146, a well-known marker of multi-potent progenitors in bone-marrow-derived MSCs (Sacchetti et al. 2007). However, whereas the role of CD54 in MSCs is still to be defined, the expression of CD146 appears not always to be associated with multi-potent properties in MSCs (Pierantozzi et al. 2010), suggesting that other mechanisms might explain the variations in the differentiation potential among MSCs. An alternative explanation is provided by the differences in either the number of progenitors or in the differentiation ability of the progenitors present in the distinct MSC populations. Indeed, experiments on adipogenic and osteogenic differentiation performed in cultures of CFU-f from A-MSCs and D-MSCs have shown that the pool of adipogenic progenitors present in the two populations is similar in early passages in culture but decreases after seven passages in cultures in D-MSCs but not in A-MSCs. The same analysis performed with regard to osteogenic differentiation has revealed a higher number of osteogenic progenitors in CFU-f in the early passages of A-MSCs compared with D-MSCs. Analysis of clonal cell populations derived at p0 from A-MSCs and D-MSCs has demonstrated a comparable number of tri-potent and bi-potent clones in the two tissues, whereas uni-potent clones are slightly more numerous and nulli-potent clones slightly less numerous in A-MSCs compared to D-MSCs. Adding together the potential of all clones (tri-, bi- and uni-potent) to differentiate into one

specific lineage, we find that 56.9%, 26.6% and 12.1 % of total clones from A-MSCs are able to differentiate into adipogenic, osteogenic and chondrogenic cells compared with 38%, 27.5% and 10.4% of clones isolated from D-MSCs, respectively. Nulli-potent clones represent 30.6% and 47.3% in A-MSCs and D-MSCs, respectively. These results indicate, therefore, that freshly isolated A-MSCs have a slightly higher number of clones able to differentiate into adipocytes, most of which are represented by uni-potent clones. These data are in agreement with those reported by Zuk et al. (2002) and Guilak et al. (2006) for A-MSCs and by Chen et al. (2007) for D-MSCs. However, considering the limitations attributable to the number of clones analyzed and to the experimental variation intrinsic to such experiments, the observed differences in the progenitor content of A-MSCs and D-MSCs might not justify the larger extent of differentiation properties observed in bulk MSC populations. Consequently, the differentiative properties of A-MSCs and D-MSC are likely to be affected by other mechanisms. One possibility is represented by positive and negative feedback among heterogeneous cells; these might control the ability of some progenitors to express fully their differentiative potentials. In addition, we cannot exclude the possibility that progenitors of distinct MSC cell preparations have intrinsic differences in their extent of mesenchymal differentiation (i.e., osteogenic); this might be related to the specific tissue of origin. The availability of larger samples and the identification of the mechanisms underlying lineage-specific differentiation should help in the future to improve the definition of these aspects.

A comparison of the differentiation properties of clones isolated from A-MSCs at p0 and at p6 has revealed that in vitro culture does not significantly affect the number of tri-potent and uni-potent clones, whereas the number of bi-potent clones capable of differentiating into osteogenic and adipogenic lineages increases. Adding up the number of clones with adipogenic and osteogenic potentials, we have observed that, among the clones derived from A-MSCs at p0 and at p6, an increase occurs from 26.4% to 72.5%, respectively, in clones able to differentiate into the osteogenic phenotype, whereas clones with an adipogenic potential remain constant (56.9% and 56.1%, respectively). During the last few years, various models of MSC lineage commitment have been proposed but no consensus has been reached regarding the way that these cells retain their proliferative and differentiative potentials (Caplan 1991; Owen 1988; Muraglia et al. 2000; Dennis and Charbord 2002; Russell et al. 2010). An analysis of clonal populations from BM-MSCs by Muraglia et al. (2000) has indicated a predominance of bi-potent and uni-potent progenitors with osteogenic but not adipogenic differentiation potential. These authors have reported that tri-potent and bi-potent clones preferentially retain osteogenic and chondrogenic potentials, while losing the adipogenic differentiation potential, because of an intrinsic commitment; this can be explained by the existence of a linear hierarchical model. Accordingly, the increase in osteogenic progenitors observed in A-MSCs at p6 might resemble the hierarchical model for the progression of MSCs, with the retention of the osteogenic potential, as suggested by Muraglia et al. (2000).

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