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Comparison of Alternative Mesenchymal Stem Cell Sources for Cell Banking and Musculoskeletal Advanced Therapies

Carola Cavallo,[1] Carmela Cuomo,[2,3] Sara Fantini,[3] Francesca Ricci,[4] Pier Luigi Tazzari,[4] Enrico Lucarelli,[5] Davide Donati,[5] Andrea Facchini,[1] Gina Lisignoli,[1] Pier Maria Fornasari,[3] Brunella Grigolo,[1] and Lorenzo Moroni[3*]

[1]*Immunorheumatology and Tissue Regeneration Laboratory/RAMSES, Rizzoli Orthopaedic Institute, Bologna, Italy*
[2]*Clinical Pathology Centralized Laboratory, Sant'Orsola-Malpighi Hospital, Bologna, Italy*
[3]*Muscoloskeletal Tissue Bank, Rizzoli Orthopaedic Institute, Bologna, Italy*
[4]*Service of Transfusion Medicine, S.Orsola-Malpighi Hospital, Bologna, Italy*
[5]*Bone Regeneration Laboratory and Clinic of Orthopaedics and Traumatology, Rizzoli Orthopedic Institute, Bologna, Italy*

ABSTRACT

With the continuous discovery of new alternative sources containing mesenchymal stem cells (MSCs), regenerative medicine therapies may find tailored applications in the clinics. Although these cells have been demonstrated to express specific mesenchymal markers and are able to differentiate into mesenchymal lineages in ad hoc culture conditions, it is still critical to determine the yield and differentiation potential of these cells in comparative studies under the same standardized culture environment. Moreover, the opportunity to use MSCs from bone marrow (BM) of multiorgan donors for cell banking is of relevant importance. In the attempt to establish the relative potential of alternative MSCs sources, we analyzed and compared the yield and differentiation potential of human MSCs from adipose and BM tissues of cadaveric origins, and from fetal annexes (placenta and umbilical cord) after delivery using standardized isolation and culture protocols. BM contained a significantly higher amount of mononuclear cells (MNCs) compared to the other tissue sources. Nonetheless, a higher cell seeding density was needed for these cells to successfully isolate MSCs. The MNCs populations were highly heterogeneous and expressed variable MSCs markers with a large variation from donor to donor. After MSCs selection through tissue culture plastic adhesion, cells displayed a comparable proliferation capacity with distinct colony morphologies and were positive for a pool of typical MSCs markers. In vitro differentiation assays showed a higher osteogenic differentiation capacity of adipose tissue and BM MSCs, and a higher chondrogenic differentiation capacity of BM MSCs. J. Cell. Biochem. 112: 1418–1430, 2011.

KEY WORDS: MESENCHYMAL STEM CELLS; ALTERNATIVE SOURCES; PLACENTA; ADIPOSE TISSUE; ALLOGENEIC THERAPIES; BONE; CARTILAGE; REGENERATIVE MEDICINE

Stem cells are well-known for their self-renewing potential, immunomodulatory properties, and ability to differentiate into specific lineages. These properties are very appealing for advanced therapies in tissue regeneration if harnessed reliably on large scales. Adult mesenchymal stem cells (MSCs) are particularly interesting from a clinical perspective, due to the maintained regenerative potential and the lack of controversial "side effects" such as teratoma formation [Ramalho-Santos et al., 2002; Mimeault and Batra, 2006]. MSCs are already used in clinical trials for graft versus host disease thanks to their immunomodulatory capacity [Le Blanc et al., 2008]. Due to their mesoderm origin, these cells can be easily differentiated into musculoskeletal tissue lineages and are studied to repair bone and cartilage showing functional tissue regeneration in animal models [Murphy et al., 2003; Alhadlaq et al., 2004; Au et al., 2008]. In addition, the discovery of MSCs in alternative tissues other than the BM makes these cells readily

[†]Authors share co-authorship
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*Correspondence to: Dr. Lorenzo Moroni, Tissue Regeneration Department, University of Twente Drienerlolaan 5, 7522 NB, Enschede. E-mail: l.moroni@tnw.utwente.nl
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available for cell banking, thus highlighting the possibility to create more easily personalized autogeneic or allogeneic cell therapies that can be soon translated into the clinics.

Bone marrow (BM) MSCs are currently considered the gold standard, by which newly discovered sources of MSCs are compared on the basis of renewal and multipotency. BM MSCs are typically isolated from the iliac crest, but they have also been found in other BM cavities like vertebrae bodies [Ahrens et al., 2004]. Alternatively, adipose tissue (AT) derived MSCs have also been isolated from various locations in the body. The easiest source for AT MSCs isolation is the abdomen after liposuction plastic surgeries, thanks also to the relative large quantities of removed and otherwise discarded fatty tissue. Additionally, AT MSCs have been isolated from the stromal vascular fraction of AT in the thigh region [Jurgens et al., 2008]. Another discarded tissue that has been demonstrated to contain MSCs is from the term placenta. In particular, MSCs can be found in the amniotic and chorionic membranes, and in the connective tissue of the umbilical cord (UC) named "Wharton's jelly" [Parolini et al., 2007; Troyer and Weiss, 2008]. More recently, MSCs have also been discovered in different tissues including the dental pulp and hair bulbs [Gronthos et al., 2002; Marchionni et al., 2009; Liu et al., 2010].

Although MSCs from different sources have shown to express similar surface markers, self-renewal capacity, and multipotent differentiation properties, comparative studies of these cells under standardized culture and differentiation conditions are limited [Im et al., 2005; Hoogduijn et al., 2006; Kern et al., 2006; Miao et al., 2006; Bernardo et al., 2007]. This is of particular importance if the translation of MSCs expansion from the laboratory to the clinic is to take place, especially with regard to good manufacturing practice rules (GMPs). In these settings, it would be ideal to expand and test MSCs multipotency with a standardized culture protocol for different cell sources. This would not only allow for less deviations from GMPs, but would also reduce operational costs. Furthermore, the hypothesis to use tissues from cadaveric donors as source of MSCs is of great interest. Since the transplanted allogenic cells may not give rise to an immunological response, the opportunity to have an MSCs bank would avoid the harvest of healthy tissues.

Here, we set to compare different tissue sources of MSCs in terms of isolation efficiency and differentiation capacity into the osteogenic and chondrogenic lineages with standardized culture media. MSCs were isolated from the iliac crest BM and thigh AT of cadaveric donors, and from the fetal annexes of human term placentas, specifically amniotic and chorionic membranes and Wharton's jelly. These tissues were selected because they are otherwise discarded and easily available; moreover, they have previously been evaluated for in vitro and pre-clinical studies for clinical translation.

MATERIALS AND METHODS

MSCs ISOLATION

Mesenchymal stem cells were isolated in conformity with the Italian national laws for tissue banking and with the local Ethical Committee for BM and AT derived from cadaveric sources. Only individuals <50 years old who died of causes not associated to congenital or chronic diseases were considered. Tissues were harvested within 24 h of death in conformity with the quality control and risk assessment procedures required by the national and European norms (2004/23/CE, 2006/17/CE, 2006/86/CE). Human term placenta was obtained after delivery pending a signed written consent of the parents. Term placentas from pregnant women between 20 and 40 years old were collected.

Bone marrow MSCs were isolated from the BM (~15 ml) retrieved from the iliac crest ($n = 5$) and diluted 1:2 with phosphate-buffered saline (PBS, Invitrogen, San Giuliano Milanese, Italy) containing 100 U/ml penicillin and 100 μl/ml streptomycin (Invitrogen). In case of a live donor (age < 43 years old), 2 ml of BM aspirates were collected. For all BM donors, the surgical procedure was performed as described in Lucarelli et al. [2003]. The aspirates were centrifuged at 1500 rpm for 10 min and washed 4 times with PBS. The cell suspension was counted with a nucleocounter (Chemometec, Allerød, Denmark) according to the procedure provided by the manufacturer, and then plated in MSC expansion medium at a variable density between 5×10^4 and 5×10^5 mononuclear cells (MNCs)/cm^2 in order to evaluate the minimum cell density at which isolation of adherent MSCs was achieved. The MSC expansion medium comprised high glucose Dulbecco Modified Eagle's Media (DMEM, Invitrogen) containing 10% v/v highly qualified fetal bovine serum (FBS, Invitrogen, certified USA), 1% v/v L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μl/ml streptomycin (Invitrogen).

AT MSCs were isolated from thigh AT ($n = 5$). The tissue was washed several times with PBS, minced in small pieces, and digested in 0.1% w/v collagenase type I (Invitrogen) for 60 min in a cell culture incubator at 37°C on a rolling bed. Collagenase activity was neutralized by adding DMEM containing 10% v/v FBS. The digested solution was filtered through Falcon nylon filters with a sieving net of 100 μm (BD Bioscience, Buccinasco, Italy). The filtered cell suspension was centrifuged at 1500 rpm for 10 min and washed 4 times with PBS. Cells were counted with a nucleocounter, and plated in MSC expansion medium at a variable cell density between 5×10^4 and 1×10^5 MNCs/cm^2.

Placentas ($n = 6$) were stored and transported in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml fungizone (Invitrogen), and 100 μg/ml antimycotic (Invitrogen). The amniotic and chorionic membranes were separated and treated differently than the UC. The amniotic membrane was minced and digested in a 0.15% w/v collagenase type I solution in PBS at 37°C for 90 min. The chorionic membrane was chopped and incubated in 0.5 U/ml trypsin/EDTA (Invitrogen) at 37°C for 15–20 min. The supernatant was collected and stored, while the chopped remaining membrane was digested in a 270 U/ml collagenase type II (Invitrogen) solution in PBS at 37°C for 120 min. In both cases, the digested tissue was filtered with Falcon nylon filters with a sieving net of 100 μm. The filtered cell suspension was centrifuged at 1500 rpm for 10 min and washed 4 times with PBS. Cells were counted with a nucleocounter, and plated in MSC expansion medium at a variable cell density between 5×10^4 and 1×10^5 MNCs/cm^2.

Umbilical cords with an average length of 70 cm were cut from the respective placentas and preserved in the same transporting

media as previously described. Umbilical arteries and veins were removed by pulling. The remaining connective tissue was cut in small pieces (3–5 cm) and mechanically disaggregated. The tissue was digested in a 0.1% w/v collagenase type I and hyaluronidase (VWR-International, Milan, Italy) solution in PBS at 37°C for 60 min, followed by a second digestion in 0.5 U/ml trypsin/EDTA for 30 min [Seshareddy et al., 2008]. The digested tissue was filtered and the derived cell suspension centrifuged and washed, while cells counted as previously described for the other sources. Finally, cells were plated in MSC expansion medium at a variable cell density between 5×10^4 and 2×10^5 MNCs/cm^2.

MSCs EXPANSION CULTURES

Mononuclear cells were cultured in MSCs expansion media at variable cell density to determine the minimum cell density at which isolation of adherent MSCs could be achieved. Adhered cells were cultured until reaching 80%–90% confluence, collected using 0.25% Trypsin/EDTA, counted, and plated at a density of 5×10^3 cells/cm^2 for four passages. At each passage, aliquoted cells were frozen in a medium containing 40% v/v DMEM, 50% v/v FBS, and 10% v/v dimethyl sulfoxide (Sigma-Aldrich, Bologna, Italy). At each passage, the cumulative population doubling was calculated following the equation [Kern et al., 2006]:

$$PD = \left[\frac{LN(C_h/(C_i)}{LN(2)}\right] \quad (1)$$

where PD is the population doubling, C_h the cell concentration at the harvesting time, and C_i is the cell concentration at seeding time. Expansion media was changed every 2–3 days. Cell area and width was also measured following Sekiya et al. [2002].

MSCs DIFFERENTIATION CULTURES

Mesenchymal stem cells were expanded until passages 2–4 and differentiated into osteogenic and chondrogenic lineages. The osteogenic media comprised DMEM, 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FBS, 10 nM dexamethasone (Sigma–Aldrich), 50 μg/ml L-ascorbic acid (Sigma–Aldrich), and 10 mM β-glycerolphosphate (Sigma–Aldrich). The chondrogenic medium contained DMEM, 100 nM dexamethasone, 40 μg/ml proline (Sigma–Aldrich), 50 μg/ml L-ascorbic acid-2-phosphate, 1% v/v sodium pyruvate (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 mg/ml ITS premix (BD Biosciences), and 10 ng/ml TGF-β1. Cells were plated in six well plates at a cell density of 1×10^5 cells/well. Cultures were conducted at Day 0, 10 and 28 days. Undifferentiated MSCs were plated at the same cell density and cultured for the same time period in expansion media as control. At the end of the culturing time the plates were treated for histological analysis. To analyze the gene expression of MSCs in chondrogenesis, cells were cultured in micromasses (200 000 cells/micromass) for the same culturing time as above mentioned.

FACS ANALYSIS

Mesenchymal stem cells immunophenotypical characterization was performed immediately after isolation or at passages 2 and 4 by fluorescent activated cell sorting (FACS, Becton Dickinson, Buccinasco, Italy) and analyzed with CellQuest (Becton Dickinson, Buccinasco, Italy). Surface fluorescent markers were obtained from Instrumentation Laboratory, Italy. CD90-PC5, CD105-PE, CD44-FITC, and CD166-PE were used as positive markers for MSCs characterization. CD45-FITC and CD117-PC5 were used as negative markers. In addition, the presence of major histocompatibility complex (HLA) surface markers was determined by measuring HLA-ABC-FITC and HLA-DR-PE levels. Correspondent IgG1 anti-FITC, IgG1-anti-PE, and IgG1 anti-PC5 mice antibodies were used as negative controls.

CELL STAINING

Alizarin red staining. To analyze osteogenic differentiation, the calcium deposition was evaluated using Alizarin red S staining. Cultured cells were washed with PBS and fixed in 10% neutral-buffered formalin for 1 h at room temperature (RT). After washes in double deionized water, cells were dehydrated in an alcohol graded series and stained with 1% Alizarin red S solution (Sigma-Aldrich, Bologna, Italy) for 2 min. **Stained cells were washed extensively with double deionized water to remove the nonspecific precipitation. Experiments were performed in duplicate wells.**

Alcian blue staining. To evaluate chondrogenic differentiation, Alcian blue staining was performed. Briefly, cultured cells were washed with PBS and fixed in 10% neutral buffered formalin for 30 min at RT. Then, cells were incubated for 3 min at RT with 3% acetic acid and stained with 1% Alcian blue (Sigma) for 30 min at RT. Stained cells were washed extensively in running tap water and rinsed in double deionized water. Experiments were performed in duplicate wells.

RT-PCR ANALYSIS

Cells were analyzed by Real-Time RT-PCR to investigate and compare temporal changes in gene expression of osteogenic and chondrogenic markers. In particular, gene expression profiles of alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OC) for osteogenesis, and collagen type II, aggrecan and sox-9 for chondrogenesis were evaluated. Cultured cells (passage 2–4) were directly lysed on wells by the addition of 0.5 ml of TRIzol reagent (Invitrogen, Life Technologies, San Giulian Milanese, Italy). RNA was recovered by precipitation with isopropyl alcohol and then treated with DNase I (DNAfree Kit, Ambion, Austin, TX). Total RNA was reverse transcribed using the Multiscribe reverse transcriptase (Applied Biosystems, Courtaboeuf, France), according to the manufacturer's protocol.

Complementary DNA was synthesized from 1 μg of total RNA per sample with 45 min incubation at 42°C, using MuLV reverse transcriptase (Applied Biosystems). PCR primers for aggrecan and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control were obtained from Martin et al. [2001] and Blanco et al. [1995]. PCR primers for type II collagen, ALP, BSP, and OC were designed using the PRIMER3 software (Steve Rozen, Helen J, Skaletsky 1998 Primer 3, http://frodo.wi.mit.edu/primer3/) while for Sox-9 using the LightCycler Probe design Software (Roche Molecular Biochemicals, Mannheim, Germany). All were chosen to span exon junctions. Specific primer pairs, PCR product length, annealing temperatures and references are reported in Table I. Real-Time PCR was run in a LightCycler

TABLE I. List of Primers Used for PCR Analysis of MSCs Differentiation into the Osteogenic and Chondrogenic Lineages

Collagen II	5′-GAC AAT CTG GCT CCC AAC
	3′-ACA GTC TTG CCC CAC TTA C
Osteocalcin	5′-GCA GCG AGG TAG TGA AGA
	3′-TCC TGA AAG CCG ATG TGG
ALP	5′-GGA AGA CAC CTC TGA CCG T
	3′-GCC CAT TGC CAT ACA GGA
BSP	5′-CAG TAG TGA CTC ATC CGA AG
	3′-CAT AGC CCA GTG TTG TAG CA
Aggrecan	5′-T CG AGG ACA GCG AGG CC
	3′-TCG AGG GTG TAG CGT GTA GAG A
Sox-9	5′-GAG CAG ACG CAC ATC TC
	3′-CCT GGG ATT GCC CCG A
GADPH	5′-TGG TAT CGT GGA AGG ACT CAT GAC
	3′-ATG CCA GTG AGC TTC CCG TTC AGC

Instrument (Roche) using the QuantiTect™ SYBR® Green PCR Kit (Qiagen, Milan, Italy) with the following protocol: initial activation of HotStarTaq™ DNA Polymerase at 94°C for 15 min, 45 cycles of 94°C for 15 s, 56/60°C for 20 s and 72° for 10 s. The increase in PCR product was monitored for each amplification cycle by measuring the increase in fluorescence caused by the binding of SYBR Green I dye to dsDNA. The threshold cycle (CT) values (i.e., the cycle number at which the detected fluorescence reaches a threshold value in the range of exponential amplification) were determined for each sample (run in triplicate). For each target gene, mRNA levels were normalized using the reference gene GAPDH.

STATISTICAL ANALYSIS

To determine whether there was any statistical significant difference among cells from different tissue sources, samples were analyzed with one-way ANOVA followed by a post hoc Bonferroni test. Statistical significance level was set for $P < 0.05$. Values are shown as mean ± standard deviation.

RESULTS

MSCs ISOLATION EFFICIENCY

BM resulted to be the tissue source with the richest fraction of isolated MNCs compared to all the other sources analyzed, when the same amount of tissue was processed (Fig. 1A). To successfully isolated MSCs through adherence selection to tissue culture plastics, a minimum cell density of 1×10^5 cells/cm^2 was necessary for MNCs derived from AT, amniotic, and chorionic membranes. The cell density had to be doubled to achieve MSCs colony formation for Wharton's jelly MNCs. Interestingly, the minimum cell density required to isolate MSCs from the BM was 5×10^5 cells/cm^2, thus implying a relative lower amount of MSCs present in BM and UC compared to AT, amniotic, and chorionic membranes. Yet, the first mature colony forming units were formed much faster for BM and AT derived MSCs (within one week after MNCs plating) compared to the fetal annexes derived MSCs (3–4 weeks after MNCs plating).

The population of isolated MNCs was found to be extremely heterogeneous, expressing a variable number of positive and negative markers characteristics of MSCs with a large spreading likely due to donor to donor variations (Table II). Although no significant difference was found for the cells isolated from the analyzed tissue sources, it seemed that MNCs from placental tissues may have a higher amount of cells that displayed positive markers for MSCs (CD44, CD90, CD105, and CD166). Furthermore, all MNCs showed a limited expression of HLA-DR suggesting a restricted rejection capacity already immediately after isolation if used for cell therapy strategies [Fritsch et al., 1999; Weiss et al., 2006]. MNCs were plated on tissue culture flasks to select adherent MSCs. After 2 passages, the adherent cells displayed distinctively positive MSCs markers, while they were negative for haematopoietic markers CD45 and CD117 (Fig. 2). Furthermore, the isolated MSCs were negative for HLA-DR marker confirming the earlier finding at P0 that these cells may have limited rejection capacity for allogeneic transplantation. Interestingly, MSCs derived from chorion, AT, and UC maintained a highly positive HLA-ABC expression while MSCs derived from amnion and BM were only slightly positive for the same marker (<25%). These MSCs may find optimal use for regenerative medicine applications (Fig. 3).

Mesenchymal stem cells maintained a comparable population doubling at early passages (Fig. 1B). From a morphological point of view, MSCs from fetal annexes had the tendency to form better defined colonies than MSCs from BM or AT. In particular, MSCs from the chorionic membrane and in part MSCs derived from the Wharton's jelly seemed to continuously grow in confined colonies, suggesting that these cells may have a more primitive phenotype and a higher potency likely due to aging differences (Fig. 4).

MSCs DIFFERENTIATION CAPACITY

For differentiation experiments, MSCs derived from the Wharton's jelly of the UCs were not used. Immediately after isolation, UC MSCs possessed a higher cell area and cell width compared to the other cells (Figure S1). With increasing doubling population, UC MSCs displayed a larger and more spread morphology. This prevented the collection of a sufficient amount of cells for differentiation experiments. In both osteogenic and chondrogenic differentiation protocols, an attempt to withdraw FBS from the culture media was made to remove potential xenogenic factors during culture. MSCs from BM and AT appeared to support a stronger staining for alizarin red, which implied a better osteogenic differentiation compared to the fetal annexes MSCs. Yet, when FBS was removed from the culturing media in the attempt to obtain serum free differentiation, no mineralization was observed (Fig. 5). When cultured in chondrogenic media, MSCs showed comparable differentiation potential, as detected by Alcian blue staining (Fig. 6). Conversely to osteogenic differentiation, the absence of FBS during chondrogenesis resulted in enhanced differentiation.

Quantitative PCR showed that MSCs from various sources differentiated into osteogenic and chondrogenic lineages to varying degrees and this process was influenced by the presence of FBS. ALP mRNA was expressed at very low levels only in AT MSCs in absence of FBS while in presence of serum this expression increased and ALP was also expressed by BM MSCs at similar levels (Fig. 7 A,B). BSP mRNA was slightly detectable from day 28 only in BM MSCs. An increase of this expression was evident in presence of FBS, while no expression was reported in the cells derived from other sources (Fig. 7C,D). OC was expressed in all the cell types evaluated, statistically unaffected by the absence or presence

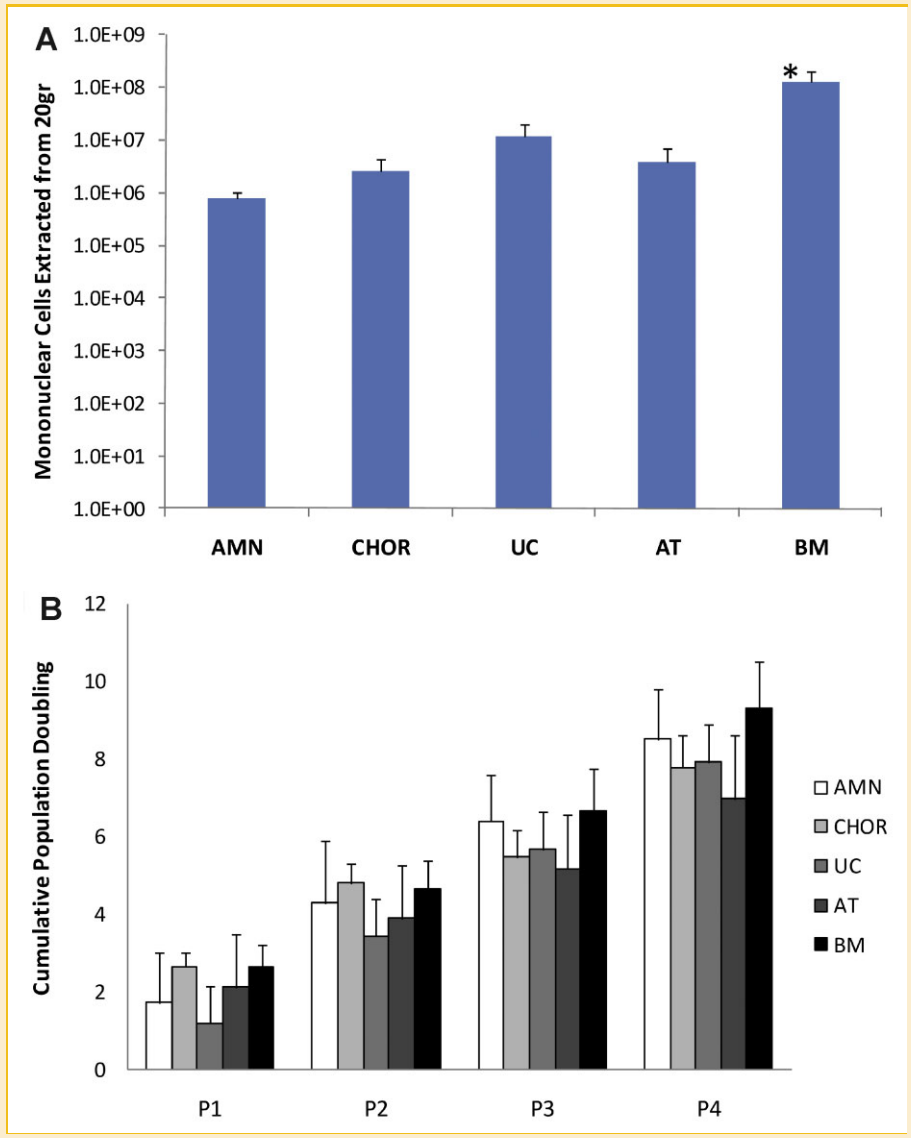

Fig. 1. (A) BM contains a significant higher amount of MNCs compared to all the other alternative sources ($P < 0.05$). (B) The isolated MSCs displayed a similar population doubling at early passages.

TABLE II. Amount of MNCs Positive for MSCs Typical Markers by FACS Analysis

	AMN	CHOR	UC	AT	BM	BM patient
CD44	33.7 ± 26.0	25.1 ± 27.2	13.6 ± 15.8	21.9 ± 17.1	61 ± 32.2	91.6
CD90	29.3 ± 8.8	22.9 ± 10.3	33.4 ± 28.3	28.1 ± 26.4	31.8 ± 40.4	2.5
CD105	60.6 ± 12.9	44.2 ± 3.6	54.0 ± 22.2	36.1 ± 41.3	58.2 ± 22.4	27.3
CD166	51.3 ± 6.6	29.4 ± 4.1	40.8 ± 26.7	26.5 ± 39.9	10.9 ± 12.4	90.5
CD45	27 ± 41.8	7.5 ± 8.1	18.2 ± 31.4	45.6 ± 10.1	83.5 ± 14.7	92.2
CD117	18 ± 1.3	2.7 ± 3.7	6.8 ± 6.2	5.6 ± 7.7	3.2 ± 4.4	8.9
HLA-ABC	53.6 ± 45.5	67.6 ± 5.9	74.6 ± 3.0	99.8 ± 0.2	84.2 ± 14.1	98.6
HLA-DR	14.8 ± 15.2	37.2 ± 5.6	18.9 ± 2.9	0.0 ± 0.0	6.6 ± 8.1	1.7

A comparison of the different tissue sources highlights the highly heterogeneous cell population right after isolation (primary cells). As a control, FACS analysis was performed on BM MNCs obtained from a biopsy of a patient undergoing hip prosthesis. For each tissue sources, 5 donors have been analyzed, while 1 donor was used for BM MNCs from a patient.

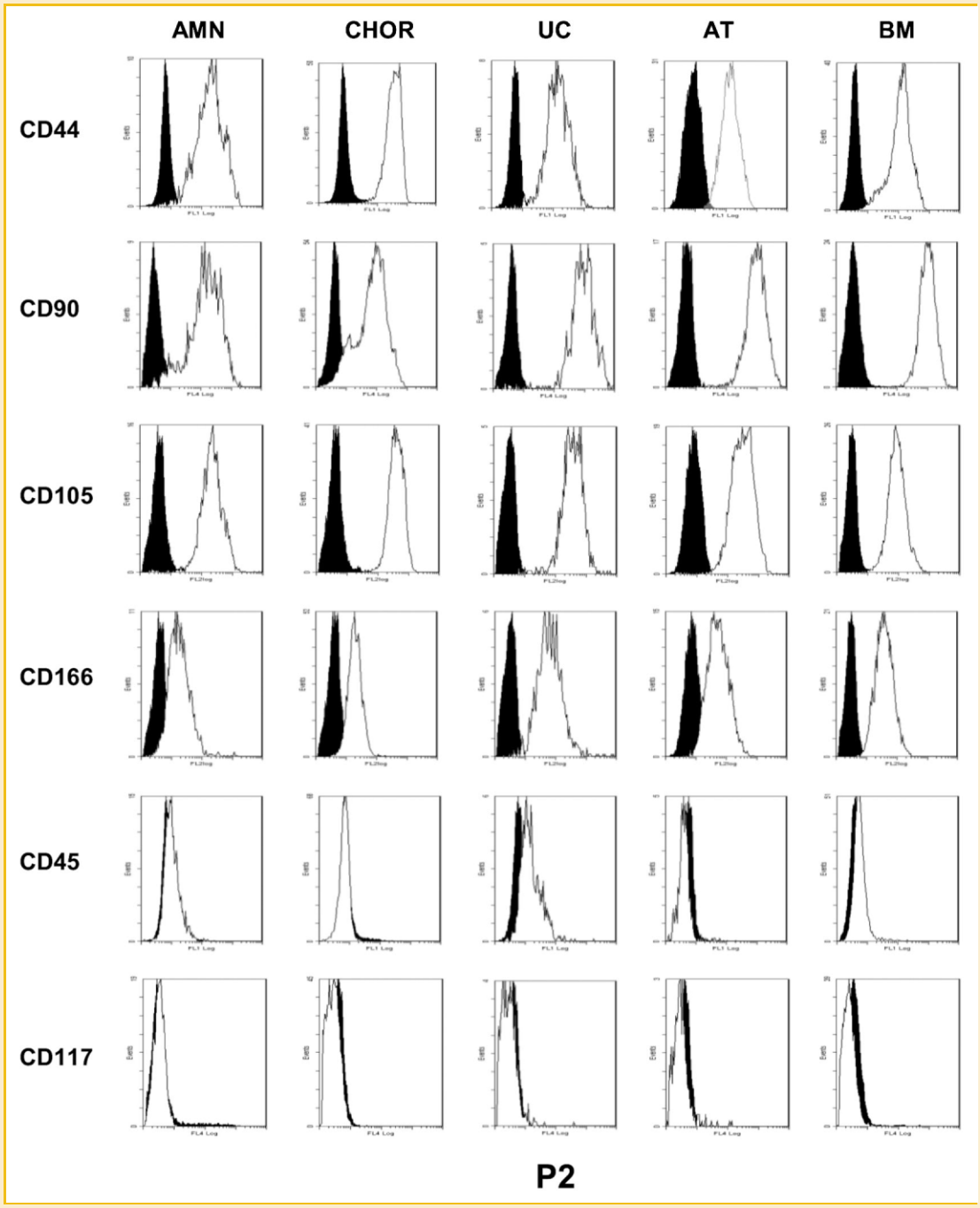

Fig. 2. FACS analysis shows that isolated hMSCs from different tissue sources were positive for MSCs typical markers (CD44, CD90, CD105, and CD166) after in vitro selection and expansion. Negative MSCs markers (CD45, CD117) were also founds negative for the analyzed cells.

of FBS. Higher expression of OC was visualized only in BM MSCs in presence of FBS at day 10 compared to the culture without FBS (Fig. 7E,F).

Collagen type II was mainly expressed in BM MSCs cultured without serum and this expression increased over time. At day 28, Chorion MSCs also expressed collagen type II, yet at a significantly lower level than BM MSCs. Cells cultured in presence of FBS did not express this marker, with the exception of amnion MSCs (Fig. 8A,B). A similar trend was observed for aggrecan mRNA with BM MSCs supporting the highest gene upregulation (Fig. 8C,D). Sox-9 mRNA expression levels were similar in Amnion, Chorion, and AT cells, while higher values were observed for BM MSCs in absence of serum. In presence of FBS, Sox-9 mRNA expression became similar in all the cell types evaluated, with a slight increase in AT MSC derived cells (Fig. 8E,F).

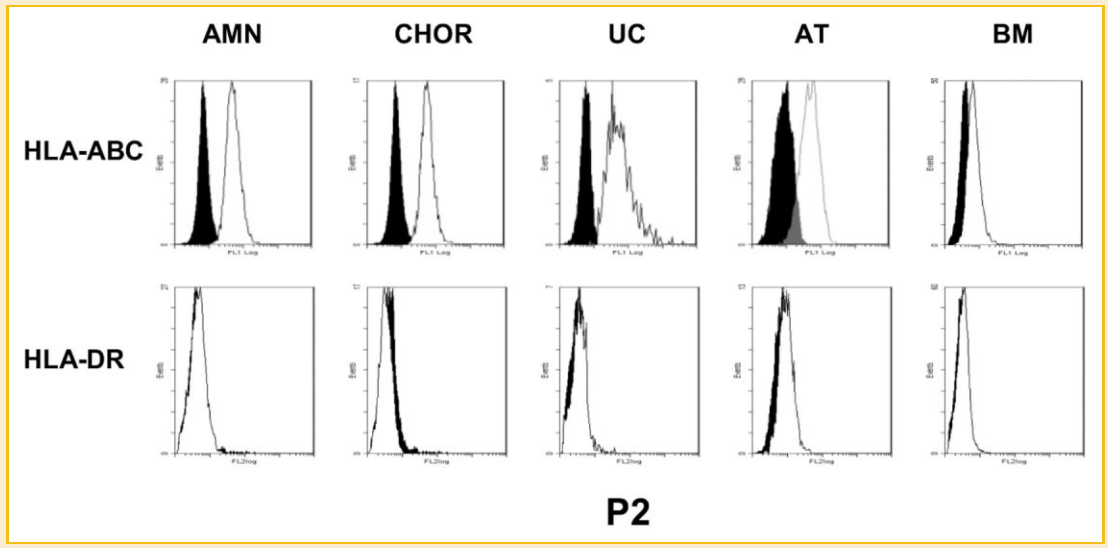

Fig. 3. FACS analysis shows that all isolated hMSCs are negative for HLA-DR, suggesting a limited rejection capacity for allogeneic transplantation. hMSCs from BM of cadaver origin were also negative for HLA-ABC.

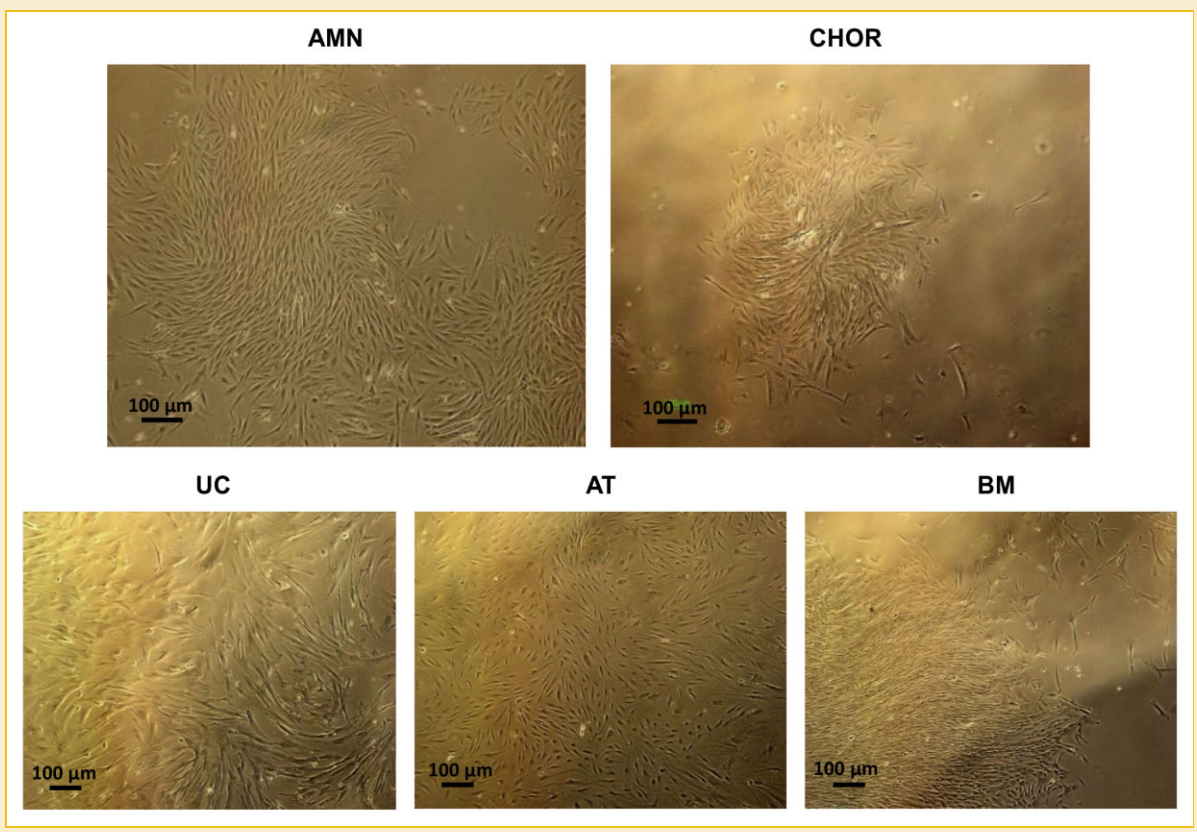

Fig. 4. Plated hMSCs immediately after isolation from different tissue sources adhere and grow differently after 14 days in culture (Magnification: 4x; scale bar: 100 µm). In particular, hMSCs from fetal annexes grow more distinctly in colonies than hMSCs from AT or BM. Chorion MSCs appear to form colonies resembling embryonic stem cells.

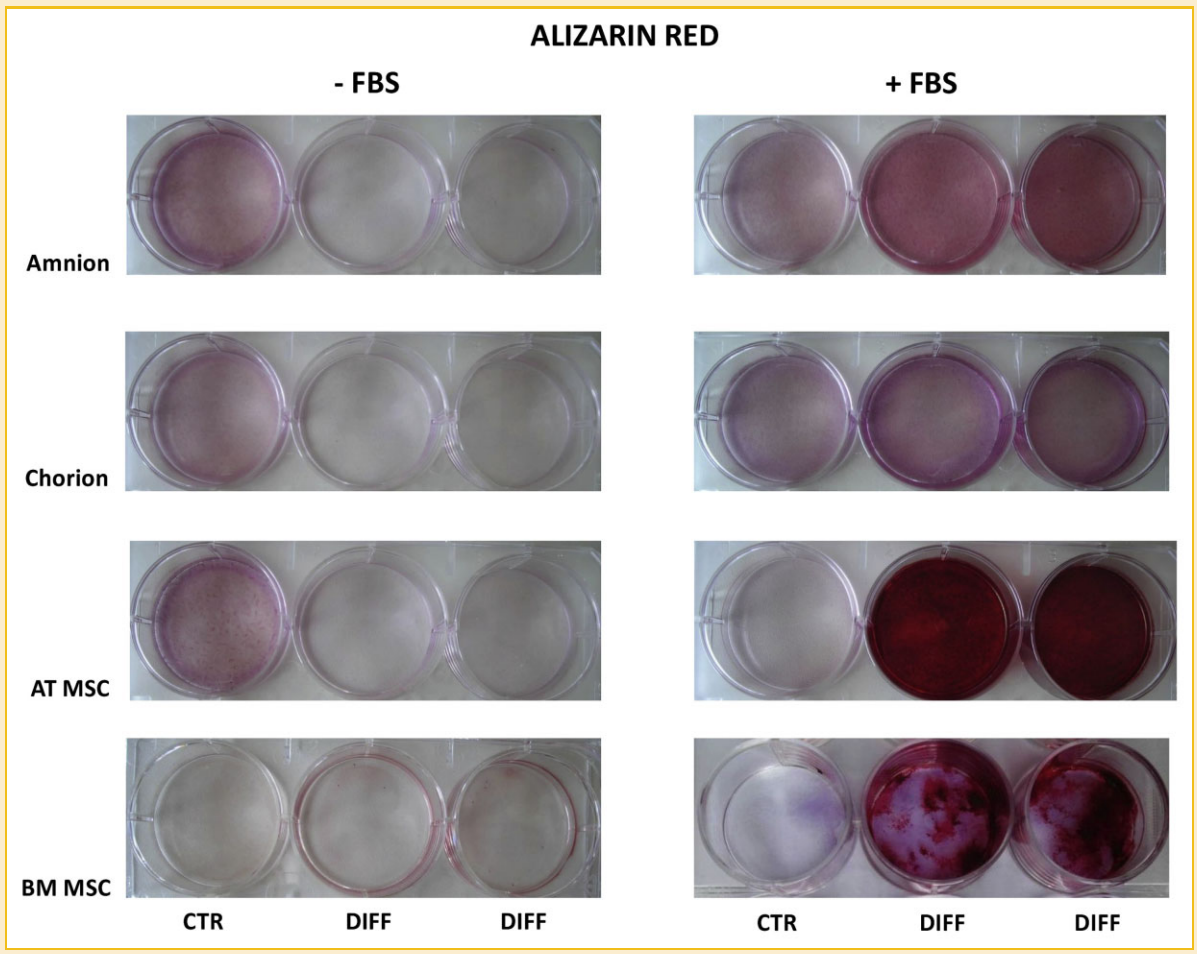

Fig. 5. hMSCs differentiation into the osteogenic lineage after 28 days observed by Alizarin red staining. FBS supply was crucial for osteogenic differentiation. Here, AT-MSCs and BM-MSCs show a remarkable enhanced mineralization capacity compared to other cell sources.

DISCUSSION

In this study we show for the first time that MSCs could be accessed from the iliac crest BM of cadaveric donors. These cells were harvested within 24 h after death. Although it is known that the number of precursor cells present in the BM decreases with time from death to harvest, it was difficult to obtain cells at earlier times. Legal autopsy, pathological examinations, and transportation logistic are insurmountable and necessary steps before obtaining a biopsy. Yet, further studies should include a thorough analysis on cell damage, senescence, and apoptosis tendencies before translating BM MSCs from cadaveric sources into clinical settings. These cells appeared and performed similarly to the more conventional BM MSCs isolated from the iliac crest of patients undergoing surgery, except that they expressed negative HLA-ABC and HLA-DR surface markers. A difference in the specific expression of CD166 could be also observed. It is possible, then, that cadaveric BM MSCs may display reduced immunogenicity, as this marker is associated with cell adhesion of activated leukocytes. We speculate that this might be attributed to the depletion of the immunological system in the donor BM after death, which can vary depending on parameters like time of disconnection from a respirator and storing time. This was also confirmed in the study by Ahrens et al. [2004] where BM MSCs from cadaveric vertebral BM were isolated and displayed typical MSCs surface markers, similar musculoskeletal differentiation potential, a higher frequency of colony forming units and lower immunogenicity compared to iliac crest BM MSCs of healthy donors and to peripheral blood progenitor cells. These findings highlight the potential use of these MSCs in allogeneic cell and matrix-assisted therapies for regenerative medicine applications with limited risks of rejection.

We also compared MSCs from different sources in terms of isolation yield, immunophenotypical profile, proliferation, and differentiation capacities in standardized culturing conditions. The selected MSCs were isolated from tissues that holds great potential for cell banking purposes as both AT, placenta, and UC tissue are all otherwise discarded and are easily accessible after surgeries or newborn deliveries. Although MSCs from different tissue sources have been isolated and characterized by several researchers, the number of studies comparing their yield, proliferation, and differentiation capacities have been somewhat restricted to few cell sources. A

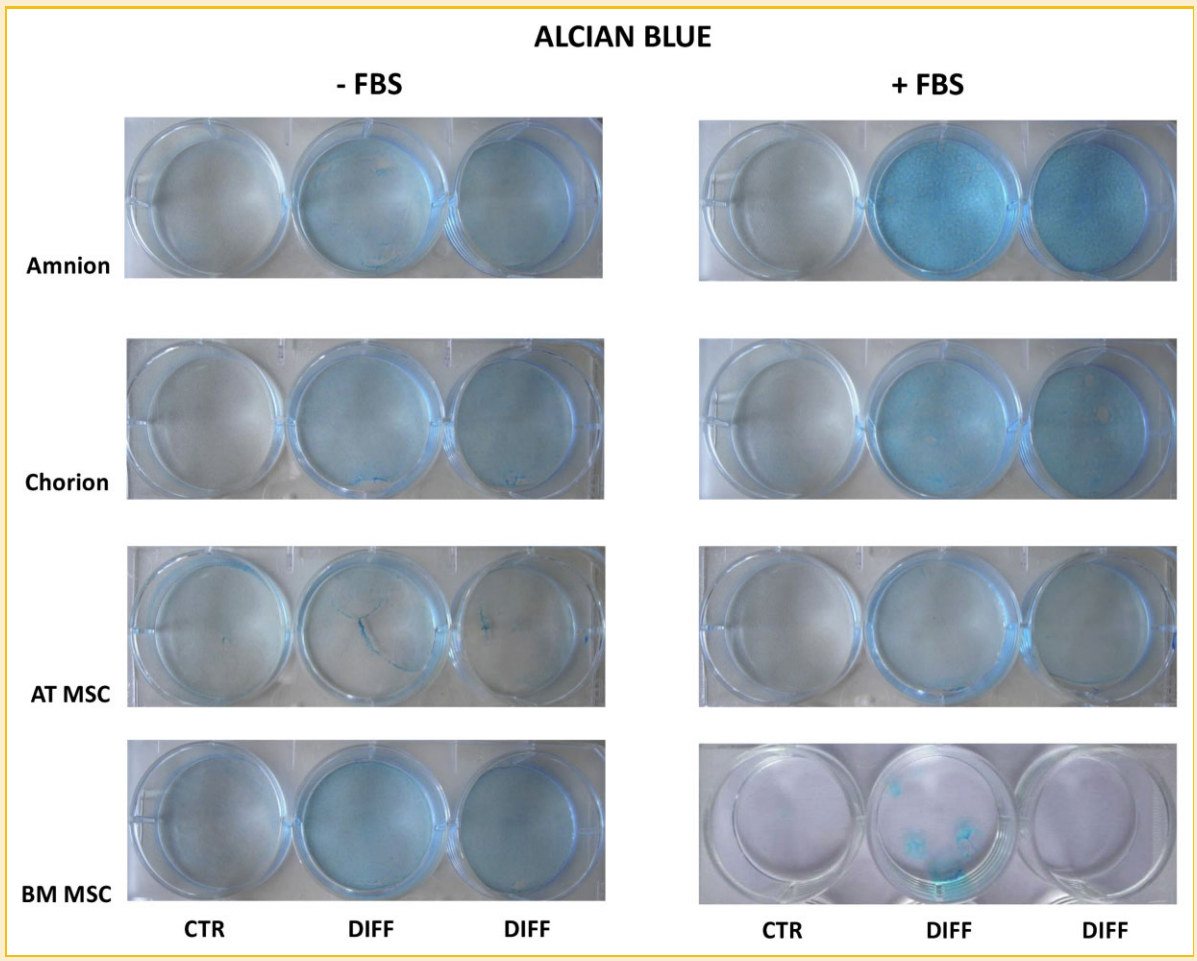

Fig. 6. hMSCs differentiation into the chondrogenic lineage after 28 days observed by Alcian blue staining. The presence of serum negatively influenced chondrogenic capacity which is comparable in all MSCs sources.

comparison of MSCs activity and differentiation potential in standardized and equal culturing conditions is of particular importance to understanding the potential of each of these cell sources for clinical translation and if existing standard operating protocols in cell and tissue banks should be changed. Im et al. [2005] compared BM and AT MSCs and demonstrated that the latter supported inferior osteogenic and chondrogenic differentiation potential. Yet, cells were not isolated from tissues of the same donors. Conversely, Kern et al. [2006] showed similar musculoskeletal differentiation capacities of BM, AT, and UC blood derived MSCs, with AT MSCs displaying the highest frequency of colony forming units. Similar contradictory studies have been performed for placenta derived MSCs in comparison with BM MSCs. Bernardo et al. [2007] compared the chondrogenic potential of fetal and adult BM MSCS with fetal lung and placenta derived MSCs and found that BM derived cells displayed better differentiation independent of their fetal or adult origin. Conversely, Miao et al. [2006] showed that human term placenta derived MSCs were similar to BM MSCs for surface stemness marker expression, proliferation, and differentiation capacities. Battula et al. [2007] extended the comparison a step further and observed that culture in medium optimized for embryonic stem cells increased the proliferation rate of both placenta and BM derived MSCs and expanded their differentiation potential to multiple lineages.

In this study, BM MSCs were isolated at a cell density 5 times higher than placenta and AT derived cells, and 2.5 times higher than Wharton's jelly. This indirectly implied a lower number of MSCs present in the marrow-isolated MNCs component isolated from the BM. The immunophenotypical profile of the isolated MNCs showed a highly heterogeneous population, while culture of the adhered cells resulted in positive expression of typical MSCs surface markers and negative expression of haematopoietic markers CD45 and CD117. Interestingly, these cells did not express HLA-DR already immediately after MNCs isolation nor at later passages, thus suggesting their potential for allogeneic therapies on HLA-screened donors [Lazarus et al., 2005]. This is particularly appealing as the number, frequency, and differentiation potential of BM MSCs from healthy patients is known to decrease with increasing age [Stenderup et al., 2003; Siddappa et al., 2007]. Surprisingly, chorionic, UC, and AT MSCs maintained a highly positive expression of HLA-ABC, while amnion derived MSCs minimally expressed the major histocompatibility complex of class I (<25%). Low risks of

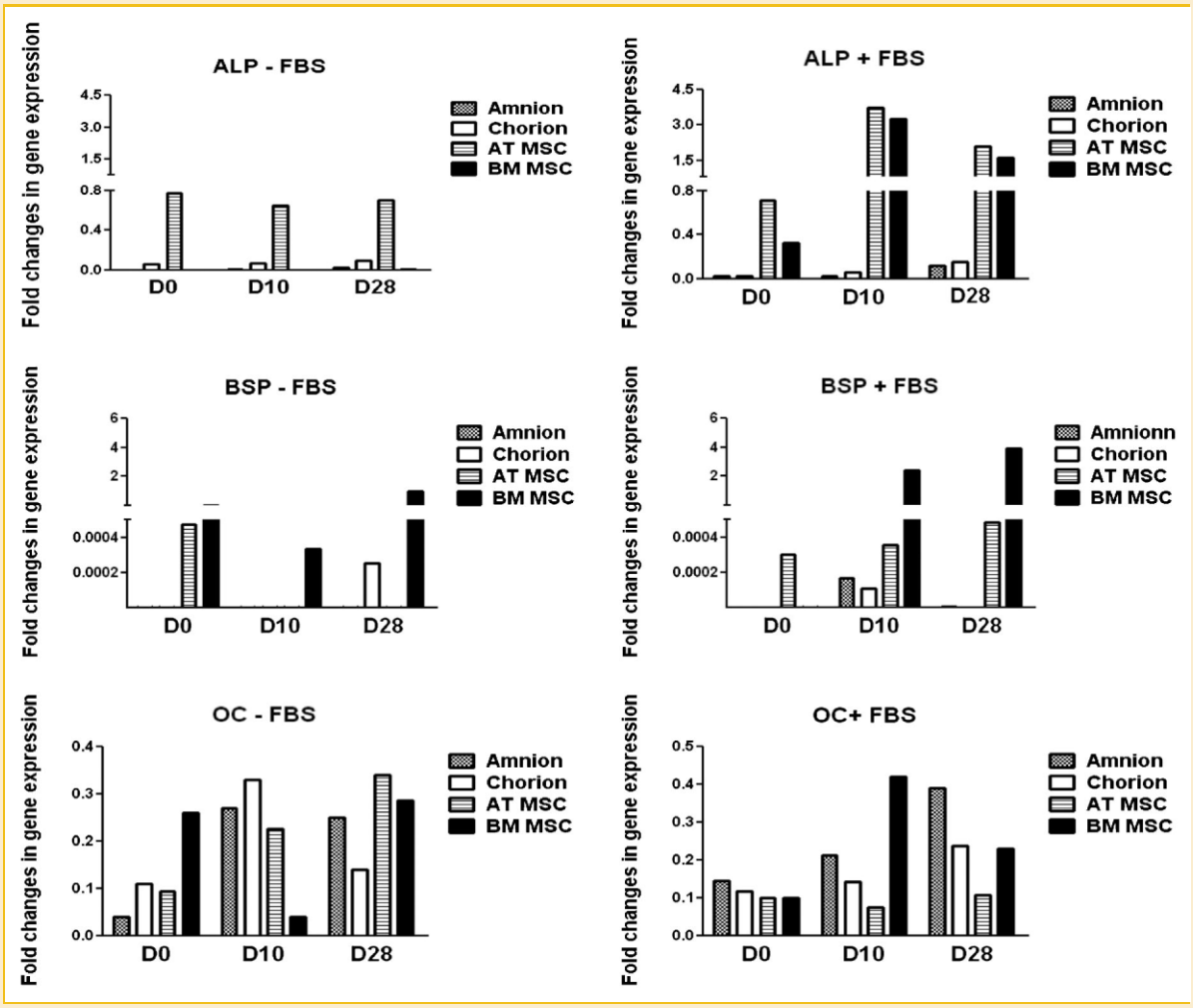

Fig. 7. ALP (A,B), BSP (C,D), and OC (E,F) mRNA expression profiles in Amnion, Chorion, AT MSCs, and BM MSCs grown in the absence or presence of serum. Samples were analyzed by Real-time PCR and mRNA levels were normalized using the reference gene GAPDH.

rejection were also demonstrated for fetal and placenta derived cells by Gotherstrom et al. [2004], which showed that these MSCs did not induce allogeneic lymphocytes proliferation and suppressed inflammatory response. This may again implicate the amniotic membrane together with cadaveric BM as preferred alternative MSCs sources for allogeneic use compared to the other analyzed tissues.

Although MSCs could be successfully isolated from Wharton's jelly following a similar protocol to Seshareddy et al. [2008] and maintained in culture for different passages, it was not possible to attain enough cells to perform the differentiation assays. Other groups have shown that MSCs derived from the UC stroma have a greater capacity for expansion than BM MSCs, thus suggesting a more primitive character [Troyer and Weiss, 2008; Moretti et al., 2009]. Despite that the UC MSCs in this study maintained a mesenchymal immune phenotype, the adhered cells were larger (Figure S1) and showed a much more spread morphology with highly elongated filopodia resembling neural-like cells compared to the other isolated MSCs. Karahuseyinoglu et al. [2007] studied in more details the biological characteristics of UC derived MSCs and found that they express neuronal surface markers and may have a preferential capacity to differentiate into the neuronal lineage compared to the musculoskeletal ones. Compared to Seshareddy, we cultured UC MSCs in a different proliferation medium in order to maintain the same cell culture conditions for all MSCs tested. Furthermore, we chose to expand MSCs at a relatively high cell seeding density. This might have induced premature senescence as reported by Sekiya et al. [2002]. Although the same authors also concluded that a high cell seeding density was beneficial to enhance hMSCs chondrogenic differentiation, we cannot exclude that these differences are the cause of the observed increase in UC cell morphology spreading and consequent limited expansion capacity. For all the other isolated hMSCs, we have shown that differentiation is still possible after 4 passages. Our main concern was safety: plating MSCs at a lower density might have prevented cells from entering potential early senescence, but would have resulted in higher population doublings in order to reach clinically relevant cell numbers.

All the analyzed MSCs were able to differentiate into the osteogenic and chondrogenic lineages. The presence of FBS in the

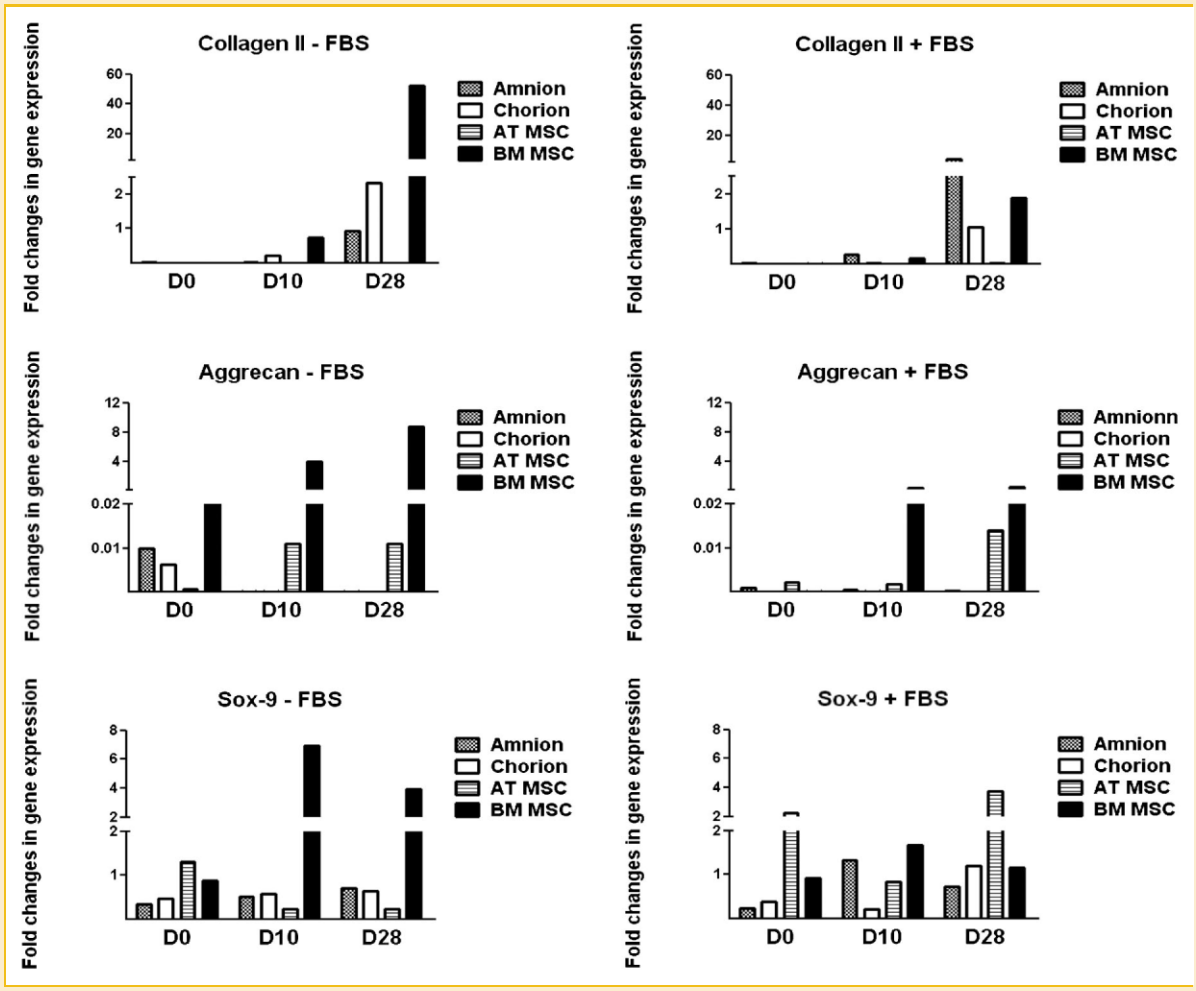

Fig. 8. Collagen type II (A,B), Aggrecan (C,D), and Sox-9 (E,F) mRNA expression in Amnion, Chorion, AT MSCs, and BM MSCs grown in the absence or presence of serum. Samples were analyzed by Real-time PCR and the mRNA levels were normalized using the reference gene GAPDH.

differentiation medium was critical for successful osteogenic differentiation while it negatively affected chondrogenic differentiation. This suggests that bone regenerating constructs may not be effectively fabricated in vitro without the addition of serum proteins, while cartilage regenerating constructs would be better applied without serum proteins. It is generally known in MSCs culture practices that the presence and composition of FBS have a significant influence on cell differentiation capacities. This typically results in specific FBS lot selection. Yet, a limited amount of studies addressed the effect of FBS presence in osteogenic or chondrogenic MSCs culture media. Malpeli et al.[2004 also observed that culturing MSCs in presence of FBS has a negative influence on chondrogenic differentiation. Further studies should be conducted to confirm these features and to highlight alternative autologous replacement of FBS for in vitro protocols [Mannello and Tonti, 2007]. Kocaoemer et al. [2007] cultured AT MSCs in media containing human serum or thrombin-activated platelet-rich plasma and observed an increase in cell proliferation and sustained osteogenic differentiation capacity compared to MSCs cultured with fetal calf serum. Similar to the study of Battula et al. [2007], Hudson et al. [2011] demonstrated that the use of a defined embryonic stem cell expansion media in the absence of FBS resulted in enhanced osteogenic and chondrogenic differentiation.

Despite these attempts to better define in vitro culture conditions, it is also possible that such cells may undergo osteogenic or chondrogenic differentiation in situ when applied to environments where ample serum proteins are present. Although MSCs from AT, placenta, and Wharton's jelly have been shown to differentiate into musculoskeletal lineages when cultured singularly in optimized conditions [Knippenberg et al., 2006; Parolini et al., 2007; Wang et al., 2009a,b], here we have observed that AT and BM MSCs displayed a remarkably stronger staining for mineralization and a comparable staining for alcian blue, thus suggesting their preferential use for bone tissue engineering applications. Yet, when PCR analysis was performed BM MSCs still maintained a significantly higher expression of both osteogenic and chondrogenic markers highlighting a stronger differentiation potential compared to alternative mesenchymal sources for these specific

lineages. Therefore, MSCs from other sources can be equivalently isolated and expanded for cell banking purposes, but further analysis into their musculoskeletal differentiation potential should be addressed in proper in vivo studies.

CONCLUSION

Multipotent MSCs could be isolated from the iliac crest BM of cadaveric donors. These cells displayed typical MSCs surface markers, similar or better musculoskeletal differentiation potential, but a lower frequency of colony forming units when compared to MSCs derived from adipose and term placenta tissues. MSCs from placenta annexes and ATs demonstrated a comparable immunophenotypical profile and proliferation capacity versus the more conventional BM derived MSCs when cultured in standardized medium conditions. All the cells did not express HLA-DR. Furthermore, amnion and BM MSCs minimally expressed also HLA-ABC. Although differences in their musculoskeletal differentiation potential were observed in vitro, these MSCs should be further analyzed in comparative pre-clinical studies, especially for personalized and ad hoc cell therapy applications.

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