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Mesenchymal stromal cells multipotency and plasticity: induction toward the hepatic lineage

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Abstract. – *Background:* **Human mesenchymal stromal cells (MSCs) can be isolated from a variety of adult and perinatal tissues and exert multipotency and self renewal properties which make them suitable for cell-based therapy. Their potential plasticity extended to non-mesodermal-derived tissues has been indicated, although it is still a debated issue. In this study we have isolated MSCs from both adult and fetal tissues. Their growth, immunophenotype and multi-lineage differentiation potentials have been analyzed, focusing, in particular, on the hepatic differentiation.**

Methods: **Cells were isolated from bone marrow (BMSC), adipose tissue (ATSC) and second trimester amniotic fluid (AFSC), upon a written informed consent obtained from donor patients. Cells were expanded and growth kinetics was assessed by means of proliferation assay. Their immunophenotype was analyzed using cytometry and multi-lineage differentiation potential was evaluated by means of in vitro differentiation assays. Finally, the expression of tissue-specific markers was also assessed by mean of semi-quantitative PCR.**

Results: **Bipolar spindle-shaped cells were successfully isolated from all these tissues. Interestingly, ATSCs and AFSCs showed a higher proliferation potential than BMSCs. Mesodermal differentiation capacity was verified in all MSC populations, even if AFSCs were not able to undergo adipogenesis in our culture conditions. Furthermore, we showed that MSC cultured in appropriate conditions were able to induce hepatic-associated genes, such as ALB and TDO2.**

Conclusion: **Taken together the data here reported suggest that MSCs from both adult and fetal tissues are capable of tissue-specific commitment along mesodermal and non-mesodermal lineages. In particular we have demonstrated that a specific hepatogenic commitment can be efficiently induced, proposing these cells as suitable tool for cell-based applications aimed at liver regeneration.**

Key Words:

Mesenchymal stromal cell, Bone marrow, Adipose tissue, Amniotic fluid; Mesodermal lineage; Autologous MSC-based therapy.

Introduction

Mesenchymal stromal cells (MSCs) were first isolated by Friedenstein et al[1] more than 40 years ago, as a fibroblastoid cell population in the bone marrow stroma (BM). Numerous works have further demonstrated that these cells were able to differentiate into all the elements of the mesodermal lineage, including osteocytes, chondrocytes, adipocytes and stromal cells[2,3]. Furthermore, in the past few years, several studies have reported that in addition to their mesenchymal-related multipotency, MSCs were also able to give rise to ectodermal neural cells[4] and endodermal hepatic elements[5]. Owing to their cellular plasticity, along with their extensive self-renewal property, MSCs represent a promising candidate for cell-based therapy and tissue engineering. BM-derived MSCs have been introduced in the clinical setting, showing promising results in the orthopaedic field[6] and for the treatment of hematological malignancies[7]. However, BM harvesting procedure may be distressful for the patient, and the number of MSCs decrease drastically in older patients. These limits have led to the

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investigation of alternative sources of MSCs. To date, similar cell populations have been identified in numerous adult tissues, including muscle[8], adipose tissue[9], trabecular bone[10], synovial fluid[11] and perinatal tissues such as umbilical cord blood[12], amniotic fluid[13], placenta[14]. In particular, adipose tissue and amniotic fluid have been proposed as new attractive tissue sources for MSC isolation, considering their growth and plasticity properties.

Adipose tissue (AT), as well as BM, derives from the embryonic mesenchyme and possess a stromal cell population able to differentiate along the mesodermal lineage. These cells have been termed alternatively processed lipoaspirates[9], adipose tissue-derived stromal cells[15], human multipotent adipose-derived stem cells[16]. Due to its abundance and easy retrieval from a simple lipoaspirate, AT presents unquestionable advantages over BM and other adult tissues. In addition, adipose-derived stromal cells possess a longest culture period and a higher proliferation capacity than bone marrow derived stromal cells[17]. Thus, AT may represent an opportune source to obtain a large number of stem cells for autologous stem cell-based therapy.

Growing evidences in the recent years have been supporting the idea of higher growing and plasticity properties exerted by developmental younger tissues, such as placenta, umbilical cord and second trimester amniotic fluid (AF)[18]. AF, in particular, contains a mixture of cell types from various fetal tissues and have proved to be a successful source of MSCs, exerting an ontologically immature phenotype (postembryonic stem cells) which suggests a broader degree of multilineage plasticity[19,20]. Moreover, the concept of *in utero* stem cell transplantation has provided hope as a new therapeutic fetal strategy to treat a genetic disease prenatally before the onset of irreversible organ damage.

The aim of this study was to isolate, expand and comparatively analyze the immunophenotype along with the growth and plasticity properties of MSCs isolated from human bone marrow, adipose tissue, and amniotic fluid. In particular, we focused on their differentiation potential along the hepatic lineage.

Material and Methods

Cell Isolation and Culture

Bone marrow aspirates were obtained from healthy donors. Subcutaneous adipose tissue specimens were acquired from exceeding tissues removed during partial abdominoplasty. Amniotic fluid samples were obtained by amniocentesis performed between 16 and 20 weeks of gestation for fetal karyotyping. All the procedures employed in this study have been approved by the Ethical Committee of the Catholic University of Rome, and each patient signed a written informed consent. Cell culture media, serum and supplements were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise specified.

BMSC

BM mononuclear cells were separated by centrifugation in a Ficoll-Hypaque gradient (density = 1.077 g/cm^3) and resuspended in Mesenchymal Stem Cell Medium (MSCGM) (Lonza, Basel, Switzerland). Cells were seeded in T25 tissue culture flask at a seeding density of 1x10^5 cells/cm^2 and incubated overnight in a humidified incubator at 37°C and 5% CO_2. The following day, medium was replaced to remove non-adherent cells. When reaching 80% confluency, cells were detached with trypsin/EDTA and expanded in culture flasks.

ATSC

AT was extensively washed with PBS, mechanically fragmented, and digested with 0.1% collagenase for 30 minutes at 37°C under gentle agitation. Enzymatic digestion was blocked by adding 10% FBS and the solution was filtered through a 100-μm mesh filter to remove all residual tissue. Cell suspension was then centrifuged at 200G for 5 minutes and the stromal vascular fraction was resuspended in MSCGM before plating into a T75 tissue culture flask. The following day, non adherent cells were removed and medium refreshed. ATSCs were expanded as previously described for BMSCs.

AFSC

AFSCs were isolated from diagnostic specimens using a two-step culture protocol, saving the amniocytes needed for chromosome analysis[21]. Briefly, non-adherent cells were collected by centrifugation from the culture medium saved from amniocytes after 7 days of primary culture. Cells were then plated and cultured in MSCGM as previously described.

Growth Kinetics

For the assessment of growth characteristics of MSCs derived from BM, AT, and AF, 3x10^4 cells were seeded in 6 well plates. The number of cells was counted in triplicate twice a week over 3 weeks, using trypan blue staining.

Flow Cytometry Analysis

The specific surface antigens of MSCs derived from BM, AT and AF (passage 4-5) were investigated by flow cytometry analysis. The cells were trypsinised and stained with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies: CD29-PE, CD44-PE, CD105-PE, CD73-PE, CD45-FITC, CD117-PE, CD90-FITC and CD133-PE. Thereafter, cells were acquired and analyzed using a FACS Coulter-EPICS XL-100 equipped with a 488nm nm Argon Laser lamp. Mouse isotype antibodies were added to calculate the fluorescence background.

Differentiation Potential of MSCs

Osteogenic Differentiation

Confluent cells were cultured in osteogenic medium consisting of LG-DMDM supplemented with 10% FBS, 10 mM α-glycerophosphate, 0.1 μM dexamethasone, and 50 μM ascorbate, for 3 weeks. Osteogenic differentiation was assessed by alizarin red staining.

Adipogenic Differentiation

Confluent cells were cultured in adipogenic medium prepared as follow: LG-DMEM with 10%FBS, and adipogenic supplements: 1 μM dexamethasone, 0.5 μM 3-isobuthyl-1-methylxanthine, 60 μM indomethacin, and 1 μg/ml insulin, for 3 weeks. The presence of intracytoplasmic lipid droplets was assessed by staining cells with oil red O.

Hepatogenic Differentiation

MSC cultures (passage 4) were used for differentiation assays. Cells were plated on fibronectin-coated plates (2.5 $\mu g/cm^2$) and grown until confluence. Then, cells were serum-deprived for 24 hours to stop proliferation before initiating a two-step differentiation protocol, including the sequential addition of growth factors. Step-1 induction medium consisting of LG-DMEM supplemented with 2% FBS, nicotinamide (4.9 mM), hepatocyte growth factor (HGF) (20 ng/ml) and fibroblast growth factor 4 (FGF4) (12.5 ng/ml) for 2 weeks. Step-2 maturation medium consisting of LG-DMEM supplemented with 2% FBS, nicotinamide (4.9 mM) and oncostatin M (OSM) (20 ng/ml). Medium was changed twice weekly and hepatic differentiation was assessed at the end of the treatment by RT-PCR for liver-associated gene.

RNA Isolation and Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

RNA was extracted from BMSC, ATSC and AFSC cultures and following 4 weeks of *in vitro* differentiation. Total RNA was isolated from each sample using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's procedures. An additional on-column DNase incubation step was performed, allowing the selective removal of genomic DNA during the isolation process. RNA was quantified using a UV spectrophotometer and RNA quality and integrity was assessed by electrophoresis on agarose gel. One microgram of RNA was reverse transcribed into cDNA using the Superscript III (Invitrogen, Carlsbad, CA, USA). PCR was performed by using the Taq polymerase (Roche) for a total of 35 amplification cycles. Primers used are listed in Table I.

Table I. Primer list. (F: Forward sequence; R: Reverse sequence).

Genes	Symbols		5'-3'sequences
Tryptophan 2,3 dioxygenase	TDO2	F	GGGAACTACCTGCATTTGGA
		R	GTGCATCCGAGAAACAACCT
Cytochrome P450, family 3	CYP3A4	F	GAAACACAGATCCCCCTGAA
		R	CTGGTGTTCTCAGGCACAGA
Albumin	ALB	F	CTTCCTGGGCATGTTTTTGT
		R	TGGCATAGCATTCATGAGGA
Cytokeratin 18	CK18	F	CACAGTCTGCTGAGGTTGGA
		R	GAGCTGCTCCATCTGTAGGG
Glyceraldehyde -3 phosphate dehydrogenase	gapdh	F	ATGTTCGTCATGGGTGTGAA
		R	GTCTTCTGGGTGGCAGTGAT

Results

Isolation and Characterization of MSCs

Bipolar, spindle-shaped cells were efficiently isolated from the three tissues. Adherent cells were visible 24 hours after sample plating for AT, whereas 3 to 5 days were necessary to detect fibroblastoid cells within BM and AF cultures (Figure 1). In all culture conditions, the primary cellular population was heterogeneous with elongated thin cells and flat rounded cells, while upon the first passage of culture, both AT and BM cultures displayed homogeneous adherent fibroblast-like cells that could be grown until the 18th and 15th passage respectively in our culture conditions (1B, 1D). The expansion of AFSCs was quite more laborious, as we successfully expanded spindle-shaped MSCs from 3 of 10 samples until the 15th passage (1C). In the other cases, cells became large and flat and ceased proliferating at the 3rd passage (1D).

Characteristics of MSCs

Flow cytometry was performed to determine the surface protein expression of MSCs derived from the 3 tissues (Figure 2). The analysis showed similar antigenic characteristics for ATSCs and BMSCs. Cells were strongly positive for CD29, CD44, CD105, CD73, CD90, while negative for CD45, c-KIT, and CD133. The immunophenotype of AFSCs was quite different as these cells are CD105 and CD90 low positive and we identified a subpopulation expressing CD133.

The proliferation potential of the 3 cellular populations was evaluated *in vitro*, by calculating the number of viable cells twice a week over 21 days (Figure 3). Growth kinetics of ATSCs and AFSCs were quite similar, whereas the proliferation potential of BMSCs was significantly lower.

The differentiation potential towards the osteogenic and adipogenic lineages of the 3 cell populations (ATSC, BMSC, AFSC) was then assessed *in vitro* (Figure 4). Cells were cultured with specific induction factors over 3 weeks. To confirm if MSCs underwent osteogenesis, calcification of the extracellular matrix was evaluated using alizarin red staining. All cultures exhibited a strong red-coloured mineralized matrix after differentiation, whereas no calcification was observed in undifferentiated cells. The adipogenesis was determined by staining treated cells with oil red O. A significant number of treated ATSCs and BMSCs contained multiple lipid-filled droplets that accumulated oil red O, whereas no lipid droplets were observed in treated AFSCs.

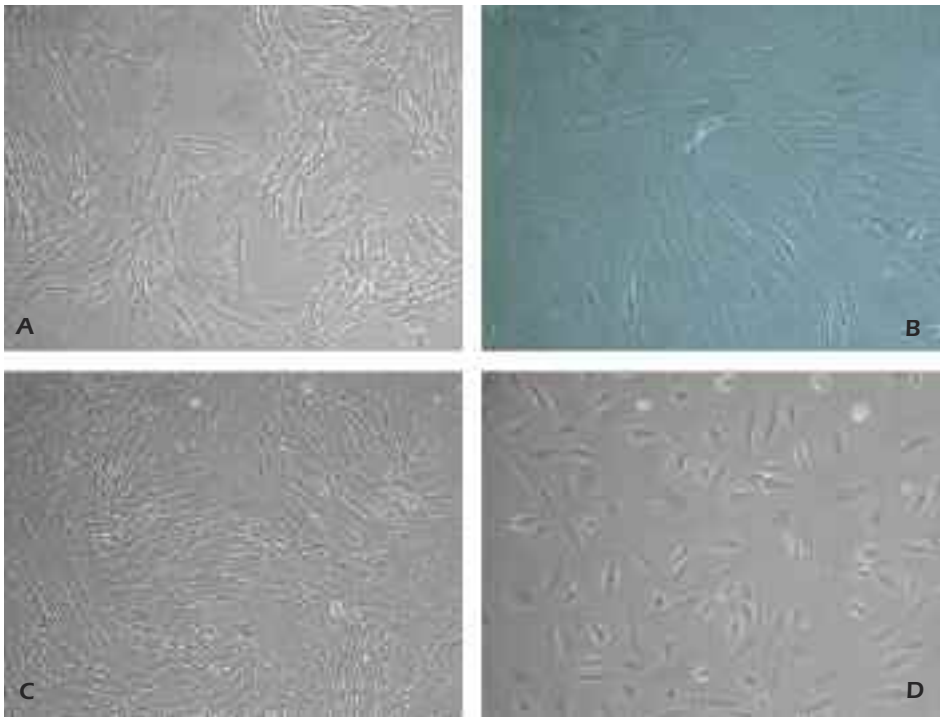

Figure 1. ***A,*** Morphological characteristics of BMSCs. ATSCs. ***B,*** AFSC. ***C,*** In the 4th passage growth culture. ***D,*** Appearance of the non growing amniotic fluid-derived cells that were discarded.

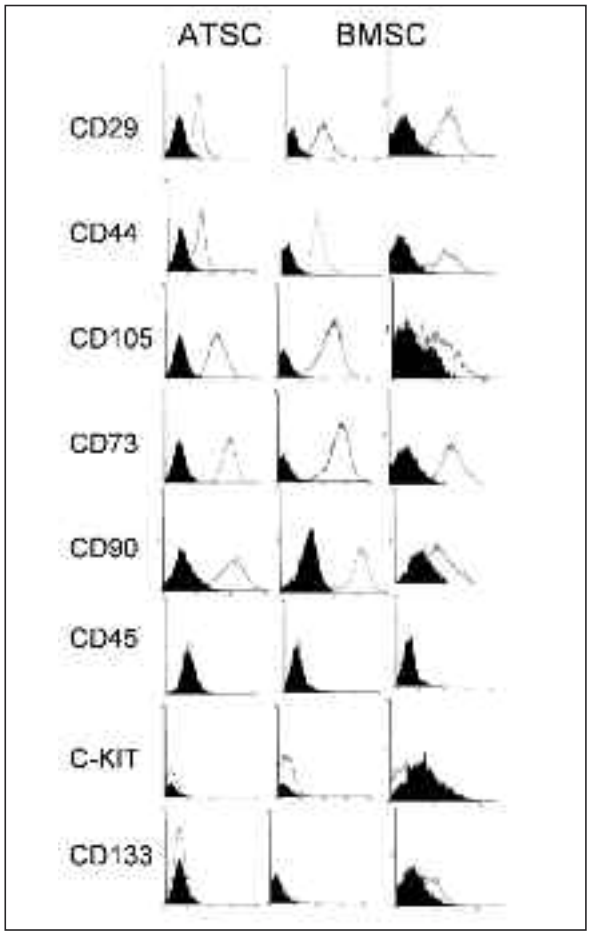

Figure 2. Cytometry analysis of surface protein markers of ATSC, BMSC, and AFSC. Data are representative of 3 independent experiments for each tissue. Black histogram represent the control isotype; White histogram , the specific antibodies.

In Vitro Hepatic Differentiation

We examined the morphological changes of the cells during the different steps of the hepatogenic induction. Following serum depletion, cells ceased growing and lost progressively their fibroblast-like morphology to exhibit round epithelial cell-like shape at the end of the protocol (Figure 5). Differentiated cells showed a clear nucleus; a few number were also binucleated. The expression of hepatic markers was then assessed by RT-PCR (Figure 6). We detected a constitutive expression of CK18 within ATSCs, while this gene was induced after *in vitro* treatment in BMSCs and AFSCs. Results showed that the transcript coding for the mature hepatic marker TDO2 was strongly induced following 1 month of culture with the hepatogenic medium. Furthermore, we detected also a slight expression of ALB and CYP3A4 in differentiated cells.

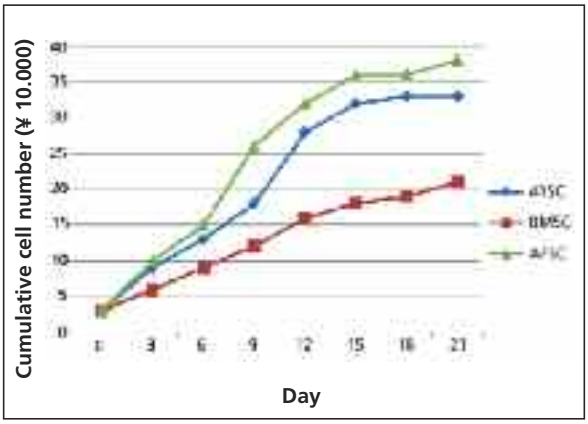

Figure 3. Growth kinetics of BMSC, ATSC, and AFSC. Cells were counted every 3 days, using the trypan blue exclusion assay. An average population doubling time was estimated to be around 36h for ATSC, 32h for AFSC, and 72h for BMSC.

Discussion

Stem cell transplantation can be considered among the most promising approaches in regenerative medicine, gene therapy trials and all clinical situations requiring damaged tissue/organ replacement. To date, MSCs isolated from bone marrow have been widely used in the clinical settings, due to their multipotency and their immunomodulatory properties[22]. In particular, being isolated from an adult tissue, their use in autologous cell transplantation could be considered among the most interesting features. Nonetheless, their limited frequency within bone marrow and the risk and pain associated to BM harvesting, make them less attractive for therapeutic transplantation. In this study, we have investigated other sources of MSCs, reporting the presence of promising stromal cell populations within adipose tissue and amniotic fluid. MSCs derived from these three tissues exhibited a similar expression pattern of surface antigens, even if some discrepancy has been found for AFSCs. In particular, these cells seem to be more heterogeneous, as we identified a significant subpopulation of CD133+ AFSCs. CD133 is a common marker of various stem cell populations, including the hematopoietic stem cells[23] and neuronal stem cells[24]. Prusa & al reported the presence of a CD133+ fraction in amniotic fluid, harboring the potential to differentiate into neurogenic cells[25]. Thus, our results are in line with recent reports, showing the existence of a wide variety of fetal-derived cells in the human amniotic fluid that retain an ontogenetically immature phenotype of post-embyonic stem cells[18].

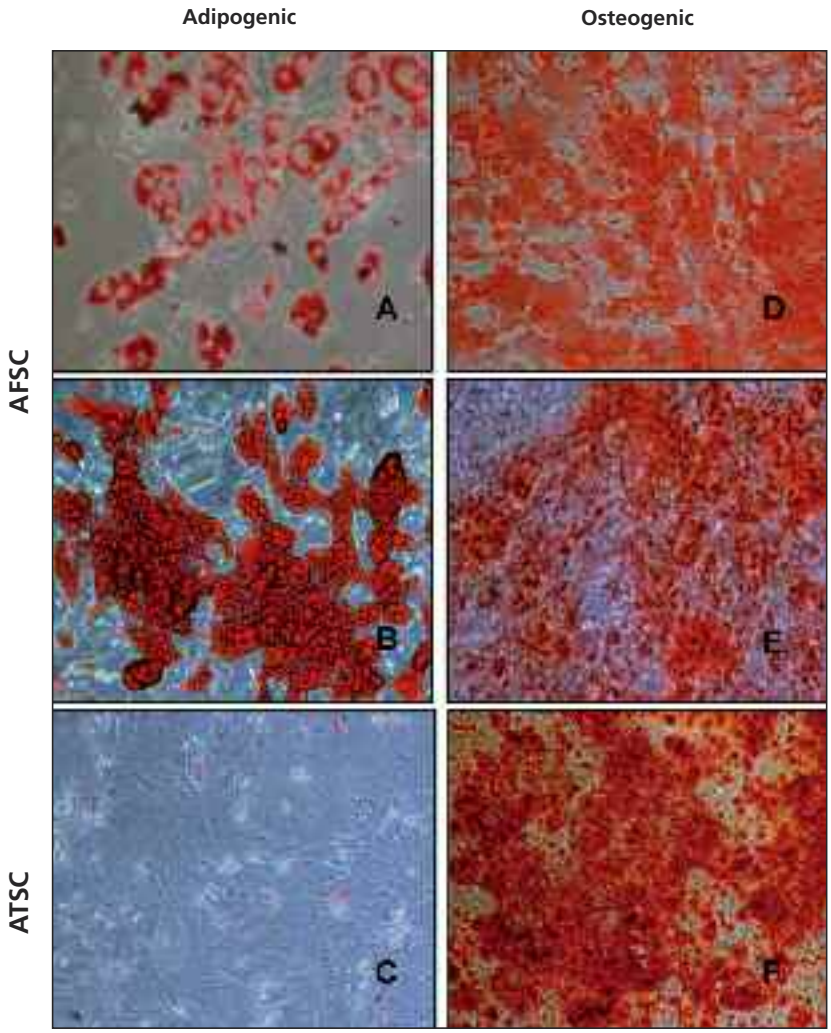

Figure 4. Differentiation potential of BMSC, ATSC, and ATSC towards the adipogenic and osteogenic lineages. Lipid droplets formation was evaluated by oil red O staining in adipogenic-induced BMSCs ***(A)*** and ATSCs ***(B)***, while no staining was visible in treated AFSCs (C). Osteogenesis was assessed by intense positive staining with alizarin red, showing red-coloured calcified matrix in all the three treated stromal populations ***(D-F)***. Pictures were taken at 400X magnification.

The multipotency of adult stromal cells have been widely demonstrated, proposing MSCs as suitable tool for cell-based therapy as well as optimal vehicle for gene delivery[26]. In this study the differentiation potential of the MSC populations towards the adipogenic and osteogenic lineages was assessed *in vitro*. Similar results were obtained for ATSCs and BMSCs, showing morphological evidences of differentiation after 3 weeks of culture in appropriate supplemented media. Surprisingly, in contrast with previous published results[27], AFSCs failed to differentiate along the adipogenic lineage.

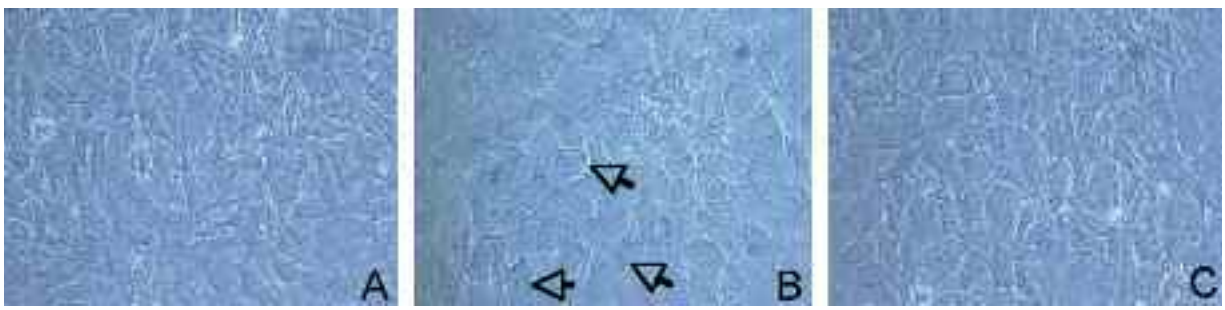

Figure 5. Morphological change following hepatogenic differentiation. ATSC, BMSC, and AFSC were induced to differentiate by using a two-step protocol with sequential addition of growth factors. At the end of the protocol, cells developed a round to polygonal hepatocyte-like shape. Arrows indicate the binucleated cells. (Magnification 100X for all pictures).

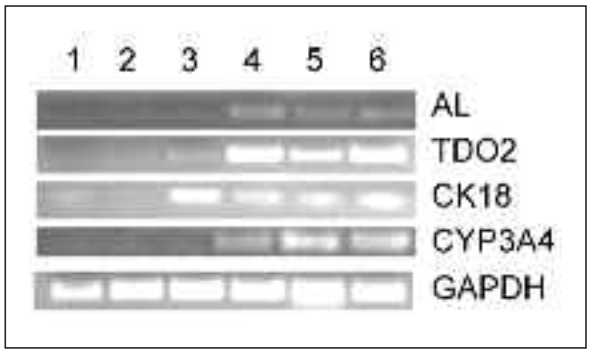

Figure 6. RT-PCR of hepatic-associated genes. Total RNA was extracted from ATSC, BMSC, AFSC before differentiation and after 4 weeks within hepatogenic medium. Differentiated cells express the mature hepatocyte markers ALB, TDO2 and CYP3A4. ATSC express constitutively CK18. Abbreviations of the genes are listed in Table 1. (1: AFSC; 2: BMSC; 3: ATSC; 4: differentiated AFSC; 5: differentiated BMSC; 6: differentiated ATSC).

This inconsistency with the literature suggest that, despite the overlapping pattern of marker expressions between AF cells described in this study and those previously reported, different populations with various degree of plasticity can be isolated, using slightly different protocols[19]. This data suggests that the isolation procedure of AFSCs has to be improved (microbeads selection, FACS sorting) using specific surface antigens.

A broader degree of plasticity has been also pointed out for MSCs during the last decades, as in addition to their mesodermal commitment, MSCs can differentiate towards elements of other embryonic germ layer. The results obtained in this study, confirm this evidence, indicating that MSCs cultured under appropriate conditions are able to express markers of the hepatic lineage. A two-stage protocol, including the sequential addition of selected growth factors, was employed for the hepatic induction, based on protocols provided by data in the literature, showing successful hepatogenic differentiation of BMSCs and ATSCs[28]. Our results proved that also AFSCs are able to express hepatic-related genes following this culture protocol.

The undifferentiated fetal condition of AFSCs in particular, make them suitable for *in utero* cell-based therapy of congenital disorders. Moreover, in this report AFSCs were isolated from a single AF specimen without interfering with the process of fetal karyotyping, keeping down the risk associated with AF sampling[19]. Intrauterine transplantation for congenital disorders (including Rh alloimmunization, Chediak-Higashi disease, leukodystrophy, bare lymphocyte syndrome, among others) has been attempted using allogeneic hemopoietic stem cells. Nonetheless successful results have been achieved only for immunodeficiency syndromes, while failure of engraftment occurred in most cases[29]. Thus, the use of an autologous source of stem cells for *in utero* therapy should largely overcome the immunological obstacle. Moreover, the immunomodulatory properties characteristically described for AFSCs, along with other tissue-derived MSCs, supports their use for intrauterine co-transplantation in conjunction with allogeneic haematopoietic stem cells aimed at decreasing the risk for graft rejection[13].

Taken together, these results suggest that MSCs are capable of tissue-specific commitment along mesodermal and non-mesodermal lineages. In particular we have demonstrated that a specific hepatogenic commitment can be efficiently induced in MSCs from both adult (bone marrow, adipose tissue) and fetal (second trimester amniotic fluid) tissues. Autologous MSC-based therapy could be suitable in the case of several acquired and congenital diseases requiring partial or total hepatic tissue replacement, including metabolic disorders, trauma and non infectious liver failure, in both adult and prenatal settings.

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