**ORIGINAL ARTICLE**

# Comparison of Proliferation, Senescence and Differentiation into Skeletal Cell Lineages of Murine Bone Marrow-Derived and Amniotic Fluid Mesenchymal Stem Cells

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## Abstract

**Background**: While mesenchymal stem cells (MSCs) have been isolated from multiple tissue sources, the differences existed between the cells from different tissues have still remained to be clarified. This study compares MSCs from murine amniotic fluid (AF) with those from bone marrow (BM) tissues.

**Methods:** AF and BM cells were collected from 10 NMRI mice at second weeks of their pregnancy and the culture was expanded. The isolated MSCs were then compared in terms of in vitro differentiation capacity, expansion rate and the percentages of senescence-associated ß-galactosidase (SA- ß-gal) positive-cells in their cultures.

**Results:** Either cell appeared to be able to differentiate into bone, cartilage and adipose cell lineage. AF-cells were observed to be more proliferative than BM-cells. The population doubling time (PDT) of AF-cells was 92.6±13.9 hours compared to 168± 40 hours that was recorded for BM-cells. The percentage of SA- ß-gal positive-cells in AF-cell culture appeared to be significantly lower than that in BM-cell culture.

**Conclusions:** Collectively, it seems that murine AF housed MSCs with a relatively higher proliferation property than BM-derived MSCs and a typical tripotent differentiation potential comparable with marrow MSCs, hence it would be as an appropriate source of MSCs for use in regenerative medicine related studies.

**Keywords:** Amniotic fluid; Bone marrow; Mesenchymal stem cells; Proliferation; Differentiation

## Introduction

Mesenchymal stem cells are known to possess two crucial properties of the capacity of self renewal for a relatively long period and the potential of differentiation into skeletal cell lineages.[1] Having these two characteristics, MSCs are considered as appropriate candidates to be used in regenerative medicine. By date, the efficiency of the cells has been confirmed in curing some skeletal tissue defects and diseases.[2-7] Furthermore, it has been indicated that MSCs are able to generate the non-skeletal cell lineages including intestine and kidney epithelial cells, keratinocytes, hepatocytes, neurons and liver cells.[8,9] This property is referred to as MSCs plasticity.

In spite of the great promise that MSCs have created in cell-based treatment of tissues, their exact identity, developmental origin and in vivo function yet need to be investigated.[10] Furthermore, MSCs safety for transplantation purposes still remains to be clarified. Many more researches must, therefore, be carried out, particularly in animal models, before

MSCs can be used routinely for therapeutic purposes.

MSCs have first been isolated and described from bone marrow tissue.[11,12] The following studies have then indicated that multiple tissues would contain MSCs. These are reported to include peripheral blood, umbilical cord blood, bone, cartilage, adipose and much other tissues.[13-19] Amniotic fluid is among those sources that have recently been known to contain MSCs. This fluid which fills the amniotic sac and surround developing embryo contains a heterogeneous population of cells which are routinely being used to diagnose the embryonic chromosomal, biochemical, structural and genetical anomalies. In general there are a number of investigations isolating and characterizing human AF-derived MSCs.[20-23]

With isolation and presentation of MSCs from different sources, investigators have attempted to compare MSCs from different sources in order to clarify the differences existed between the cells occurred in different tissues.[24-26] The present study was designed to fulfill this purpose with trying to compare MSCs from amniotic fluid with those from bone marrow tissue. In this study mouse was used as an animal model which is a good model for preclinical investigations. While MSCs from murine bone marrow are relatively well-studied cells,[25-28] those from their amniotic fluid have rarely been a subject of investigations. In the present study, MSCs obtained from amniotic fluid and bone marrow tissue of NMRI mice were compared in terms of in vitro differentiation potential into skeletal cell lineages, proliferation rate as well as their senescence in culture conditions to determine the best tissue source of MSCs for clinical application.

## Materials and Methods

The use of 10 NMRI mice with 6-8 weeks age was approved by Royan Institute Ethics Committee. Female mice were sacrificed by cervical dislocation in the second weeks of pregnancy. The abdomen was opened, the uterus was dissected off and placed in a sterilized culture dish under a laminar hood. About 0.5-1 ml amniotic fluid was then aspirated from each mouse using a syringe with a 23 gauge needle. The amniotic fluid from each mouse was separately mixed with 3 ml of DMEM (Dolbeccos' Modified Eagle Medium, Gibco, Germany) supplemented with 10% FBS (Fetal Bovine Serum, Gibco, Germay), 10 IU/ml penicillin/streptomycin (Gibco, Gemany) and plated in 25 cm$^2$ culture flask that was incubated in an at-mosphere of 5% CO2, and 37 °C temperature. Three days after culture initiation, culture medium was removed; the cells were washed with PBS (Phosphate buffer solution, Gibco, Germany) and added with fresh medium. Medium changes were performed twice a week till all available surfaces of the culture dish was covered by the cells (about day 10). At this time, the cells were lifted by adding 0.5 ml of 1 mM EDTA/0.25 trypsin and passaged at 1:2 ratios. The cultures were then propagated with two additional successive subcultures.

Bone marrow collection was started after amniotic fluid collection was finished. For this purpose, tibia and femurs of the mice were dissected off and the surrounded soft tissue was precisely cleaned. The long bones were then placed in DMEM medium supplemented with 10% FBS and antibiotics. Under the sterilized conditions, the ends of bones were cut, a 27-gauge needle was placed into the bone medullary canal from one end and bone marrow was flushed out into a 15-ml tube containing DMEM supplemented with 10% FBS and antibiotics. The samples were washed with two successive round of centrifugation at 1200 RPM for 5 min and the resultant pellet was suspended in fresh medium. The cells were then counted and plated at $10^6$ cell/cm$^2$ in 25-cm$^2$ culture dishes which were incubated in an atmosphere of 5% CO$_2$ and 37 °C temperature. Three days after culture initiation, the medium was discarded and the adherent cells were washed with PBS and fed with fresh medium. Culture medium changes was done twice weekly till confluency (about day 15). At this time, the culture was tripsinzed and the cells were subcultured in 1:2 ratio. Two additional subcultures were done before proceeding to the next stage of the study.

To evaluate the differentiation potential, the isolated cells were cultivated at differentiating conditions into osteogenic, chondrogenic and adipogenic cell lineages.

For osteogenic differentiation, confluent passaged-3 cells from either amniotic fluid or bone marrow tissue were added with DMEM medium supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethazone (Sigma, USA) and 10 mM β glycerol phosphate ( Sigma, USA) for 3 weeks. At the end of this period, alizarin red staining was used to observe the matrix mineralization. For staining, the cultures were first fixed by methanol for 10 minutes and then subjected to alizarin red solution for 2 minutes.

To examine adipogenic differentiation of the isolated cells, DMEM medium containing 100 nM dex-

amethazone (Sigma, USA) and 50 mg/ml indometha-cin (Sigma, USA) was added to the confluent cultures of both amniotic fluid - and bone marrow-derived cells. Three weeks after culture initiation, the cells were fixed with 4% formalin at room temperature, washed by 70% ethanol and stained by oil red solu-tion in 99% isopropanol for 15 minute.

Micro mass culture system was used to induce the cartilage differentiation of both amniotic fluid - and bone marrow-derived cells. For micro mass culture, $2\times10^5$ passaged-3 cells were pelleted under 300 g for 5 minutes and added with DMEM medium supple-mented by 10 ng/ml TGF- ß3 (transforming growth factor-ß3) (Sigma, Germany), 10 ng/ml BMP6 (bone morphogenetic protein-6)(Sigma, Germany), 50 mg/ml insulin transferin selenium+premix (Sigma, Germany) and 1.25 mg bovine serum albumin (Sigma, Germany) and 1% fetal bovine serum. Three weeks after initiation of the culture, the pellets were removed, fixed in 10% formalin, dehydrated in as-cending concentrations of ethanol, cleared in xylene, embedded in paraffin wax and finally sectioned at 5 μm by microtome. The sections were then stained by toluidine blue for 30 seconds at room temperature.

Total RNA was isolated from the differentiated cells using the RNX$^{TM}$ (-Plus) (RN7713C; CinnaGen Inc., Tehran, Iran). Before RT, a sample of the iso-lated RNA was treated with 1 U/ml of RNase-free DNase I (EN0521; Fermentas, Opelstrasse 9, Ger-many) per 1 mg of RNA in order to eliminate residual DNA in the presence of 40 U/ml of ribonuclease in-hibitor (E00311; Fermentasm, Germany) and

1×reaction buffer with MgCl2 for 30 min at 37$^{\circ}$C. To inactivate the DNase I, 1 ml of 25 mM EDTA was added and incubated at 65$^{\circ}$C for 10 min. Standard RT reactions were performed with 2 μg total RNA using oligo (dt) as a primer and a RevertAid$^{TM}$ First Strand cDNA Synthesis Kit (K1622; Fermentas, Germany) according to the manufacturer's instructions. For every reaction set, one RNA sample was prepared without RevertAid$^{TM}$ M-MuLV Reverse Transcrip-tase (RT reaction) to provide a negative control in the subsequent PCR. To minimize variation in the RT reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneously. Reac-tion mixtures for PCR included 2 ml cDNA, 1×PCR buffer (AMS$^{TM}$; CinnaGen Co., Tehran, Iran), 200 mM dNTPs, 0.5 mM of each antisense and sense primer (Table 1), and 1U Taq DNA polymerase.

To compare the colonogenic activity of the amni-otic fluid- and bone marrow-derived cells, colono-genic assays was performed. For this purpose, about $10^4$ passaged-3 cells from either source were plated in 100 mm$^2$ culture dishes for a period of 10 days at the end of which the cells were washed twice with PBS and stained with crystal violet for 5 min. The dishes were then observed under invert light microscope and the number of colonies was counted. The diameter of colonies were also measured and recorded.

To compare the expansion rate of the MSCs ob-tained from amniotic fluid and bone marrow tissue, (PDN) population doubling time (the time required by the cells in order to double their population) was deter-mined. This was calculated according to the following

**Table 1:** Primers used in RT-PCR

| | | |
|---|---|---|
| B- tubulin | F: 5' TCA CTG TGC CTG AAC TTA CC 3'<br>R: 5'GGA ACA TAG CCG TAA ACT GC 3' | 320 bp |
| Osteocalcin (OC) | F: 5' GGC AAT AAG GTA GTG AAC AG 3'<br>R: 5' GGT CCT AAA TAG TGA TAC CGT 3' | 381 bp |
| PTHR | F: 3' GAC AAG CTG CTC AAG GAA GTT CTG 3'<br>R: 5' GGA ATA TCC CAC GGT GTA GAT CAT G 3' | 444 bp |
| Collagen type2 | F: 5' GGC TTA GGG CAG AGA GAG AAG G 3'<br>R: 5' TGG ACA GTA GAC GGA GGA AAG TC 3' | 475 bp |
| Collagen type10 | F: 5' CAG CAG CAT TAC GAC CCA AG 3'<br>R: 5' CCT GAG AAG GAC GAC GAG TGG AC 3' | 287 bp |
| Aggrecan | F: 5' CCA AGT TCC AGG GTC ACT GTT AC 3'<br>R: 5' TCC TCT CCG GTG GCA AAG AAG 3' | 264 bp |
| Pparg | F: 5' GAG CAC TTC ACA AGA AAT TAC C 3'<br>R: 5' AAT GCT GGA GAA ATC AAC TG 3' | 151 bp |
| Lipoprotein lipase (LPL) | F: 5' AAT TGT CCC ATG CTG TAA CC 3'<br>R: 5' CAG GAC ACA GGA AGC TAA GG 3' | 100 bp |
| Adipsin | F: 5' ATG GTA TGA TGT GCA GAG TGT AG 3'<br>R: 5' CAC ACA TCA TGT TAA TGG TGA C 3' | 307 bp |

formulae: PDT=CT/PDN,[26, 29] where CT is the culture time and PDN the population doubling number. To determine the PDN, following formula was used: PDN= log (N1/N0) ×3.31 [25, 28]. In this equation N1 is the cell number at the end of cultivation period, N0 the cell number at culture initiation. CT (culture time) was the passage 1-3.

To plot growth curve, $10^4$ passaged-3 cells from either groups were plated in 22-well culture plate and allowed to achieve confluence. During the culture time, cell number was daily determined with hemocytometer and growth curve was plotted for each culture.

The senescence status of the isolated cells was verified by in situ staining for senescence-associated ß-galactosidase (SA- ß-gal).[30] Briefly, the cells from passage 1, 2, 3, 5 and 7 growing on 4-wells culture plates were fixed with 3% formaldehyde for 4 min, washed with PBS and incubated with beta galactosidase substrate staining solution (150 mM NaCL, 2 mM MgCL2, 5mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid, and 40mM sodium phosphate, pH 6, containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-ß-galactosidase) for 14 h at 37 °C. Senescence cells were identified as blue-stained cells by standard light microscopy, and a minimum of 100 cells was counted in 10 random fields to determine the percentage of SA-ß-gal-positive cells.

Each experiment described in here was replicated for 10 mice. All values stated as means±standard deviations. The results were analyzed by student t-test using SPSS software (version 15, Chicago, IL, USA). A *p* value of <0.05 was considered to be statistically significant.

## Results

Amniotic fluid primary culture was reached confluence in about 10 days after culture initiation, while those from bone marrow achieved to this stage in a 15 days period after culture initiation. The cellular composition of both cultures observed to be heterogeneous at primary culture (Figures 1A and B). In both cases, fibroblastic cells were gradually dominated the culture as the subcultures were performed (Figures 1C and D).

The cells from both cultures were easily be able to differentiate along bone, cartilage and adipose cell lineages. This was confirmed by both specific stainings (Figures 2A-F) and RT-PCR analysis (Figures 3A-F).
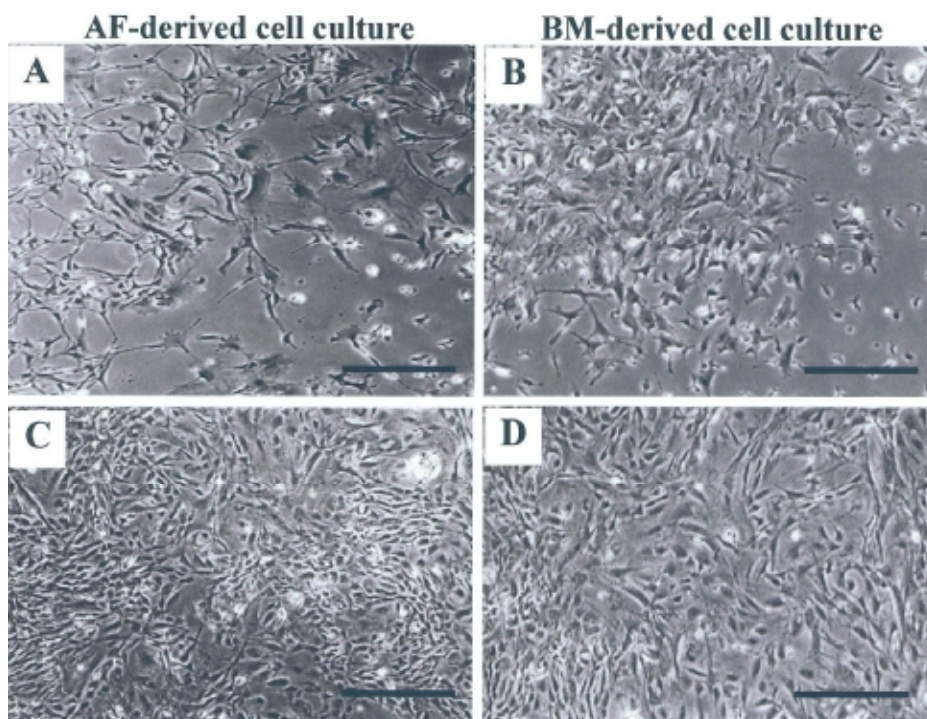


**Fig. 1:** Photomicrograph of the amniotic fluid – and marrow-derived cell cultures. A-B) the cells at primary cultures C-D) the cells at passage 1 culture. Bar= 100 µm
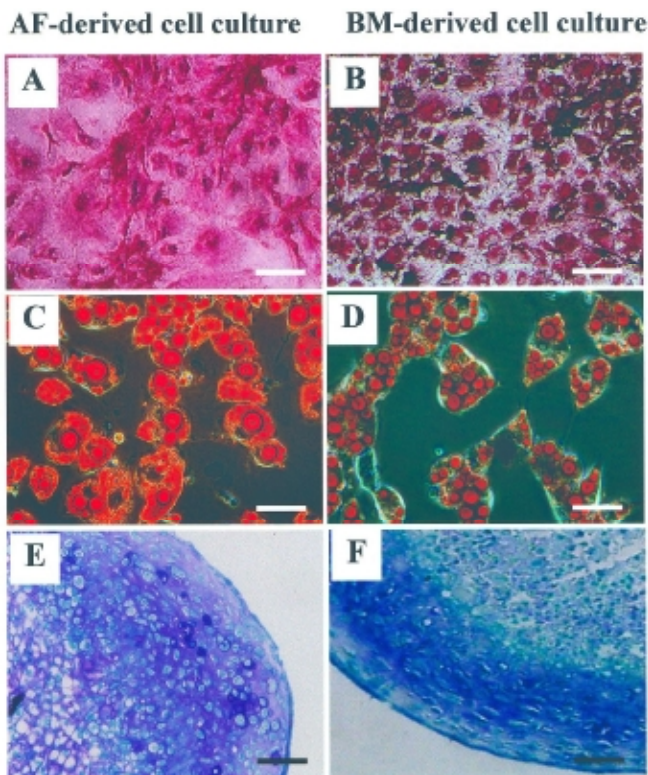
**Fig. 2:** Differentiation potential of AF- and BM-derived cells. A-B) Osteogenic differentiation culture that is stained by alizarin red. C-D) adipogenic cultures that is stained by Oil Red and E-F) sections from chondrogenic micromass culture which is stained by toluidine blue. Bar=50μm



**Fig. 3:** RT-PCR analysis of differentiation in AF-and bone marrow-derived MSCs. A-B) Bone specific genes C-D) Adipose-related genes and E-F) chondrocyte specific gene.

The mean colony number for amniotic fluid and bone marrow-derived cells was appeared to be different. While amniotic fluid-derived cells tended to produce 236±22.8 colonies, those of bone marrow generated 485±58.2 colonies (Figure 4A). The differences were statistically significant ($p$=0.002). In terms of colony size, those from amniotic fluid were appeared to be significantly larger than bone marrow-derived cells (Figure 4B). The mean size of colonies in amniotic fluid-derived cell culture was 2.6±0.96 versus 1.03±0.14 mm of colonies in BM-derived cultures ($p$=0.002).

According to our results, amniotic fluid-derived cell population tended to double in 69.695±14.5 hour, while those from marrow appeared to be doubled in 105.672±17.728 hours (Figure 5A). The difference was statistically significant ($p$=0.004).

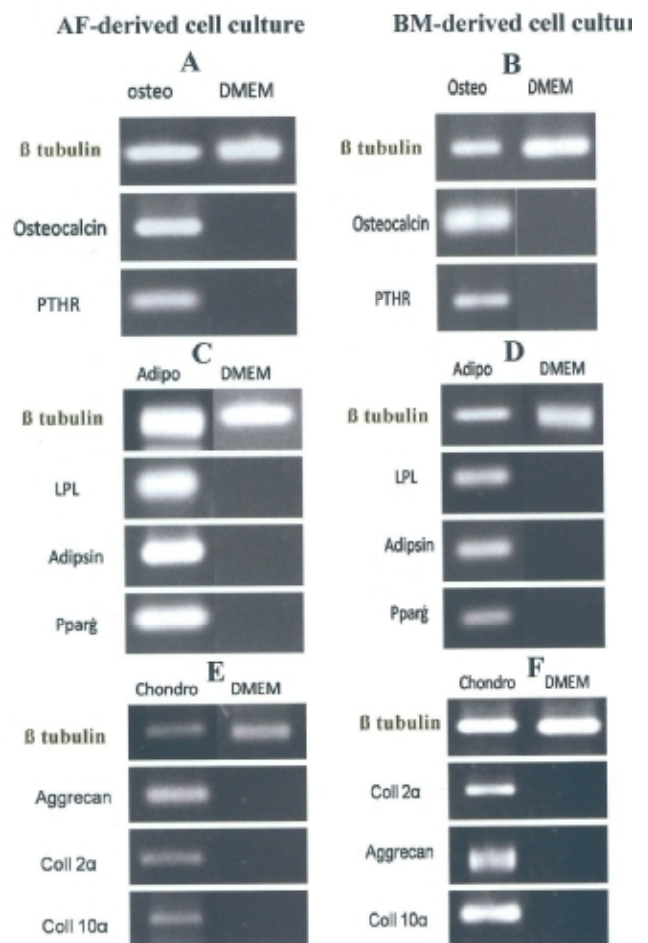Examination of the daily growth of the cells from two different sources was indicated that there was some difference specifically in terms of lag phase of growth curve. While amniotic fluid-derived cells started to proliferate immediately after plating, those of marrow had a short lag time (Figure 5B).

According to this result, no ß galactosidase positive cells were observed during passage 1 and 2 of either culture. Stained cells were first appeared in passage 3 and their number increased as the passage number went high. In general, amniotic fluid-derived cell culture contained less ß galactosidase positive cells than their marrow counterparts (Figure 6). The difference was statistically significant ($p$=0.008).

## Discussion

In the present study, it was indicated that MSCs from murine amniotic fluid possessed tripotent differentiation
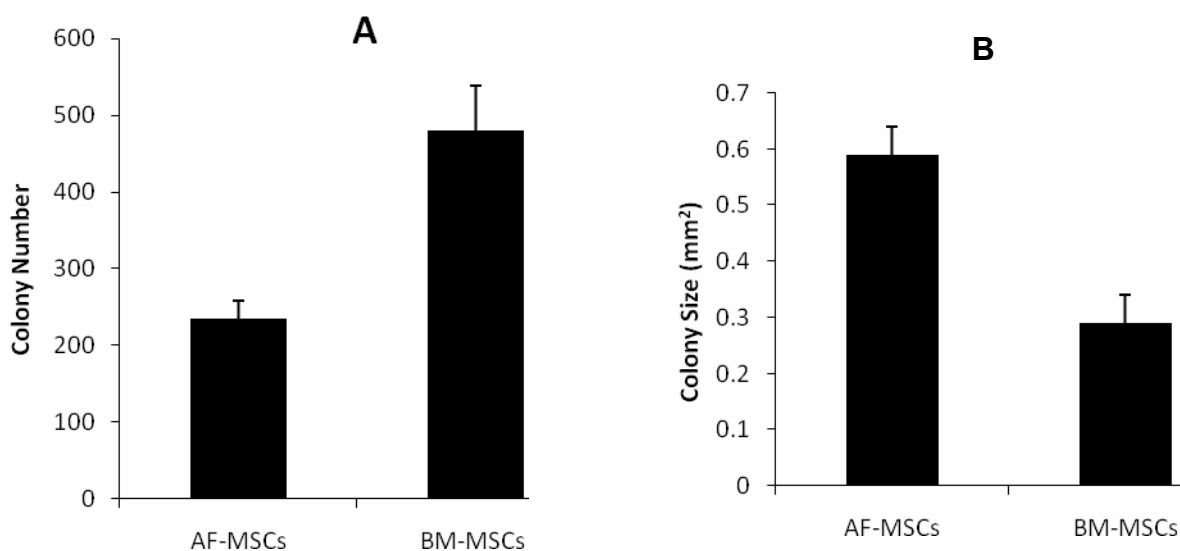
**Fig. 4:** The mean colony number and their average dimension in amniotic versus bone marrow-derived MSCs culture. A) The number of colony produced at BM-MSCs culture was significantly higher than that in AF-MSCs culture. *indicates a significant difference with *p*=0.002 B) the colonies produced at AF-derived cell culture were bigger than those at BM-derived cell cultures. * indicates a significant difference with *p*=0.002.
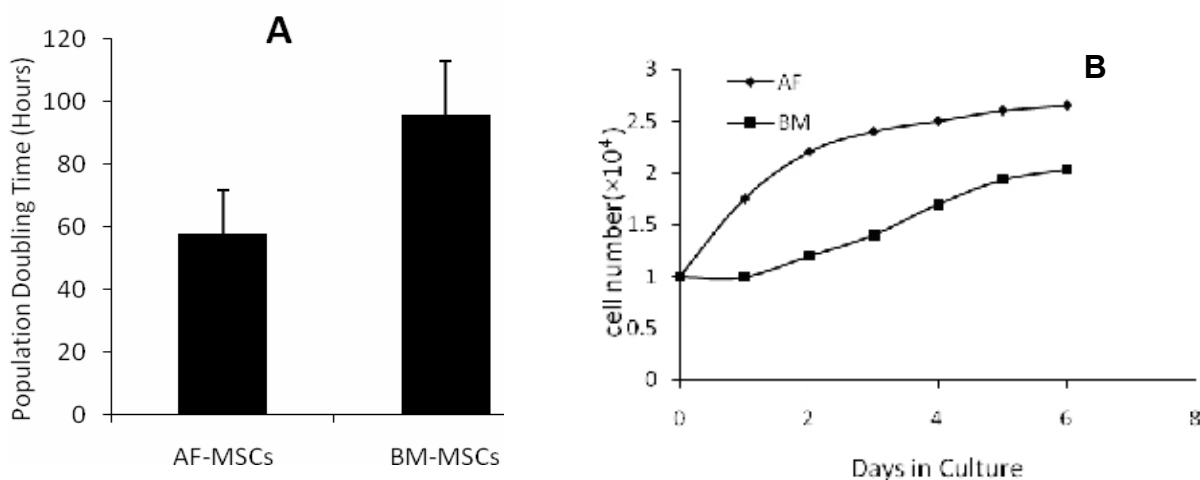


**Fig. 5:** Analysis of the cell proliferation in culture. A) Population doubling time (PDT): AF-derived MSCs appeared to double in rapid rate than their BM-derived counterparts. * indicates a significant difference with *p*=0.004. B) Growth curves plotted for the isolated cells from both AF and BM tissues. AF-cells tended to proliferate immediately after plating, while those from bone marrow possessed a 2 days lag time in culture.

potential into bone, cartilage and adipose cells like their bone marrow counterparts. The important point was that amniotic fluid-derived MSCs appeared to have more rapid proliferation rate than marrow-derived MSCs. The MSCs in vitro proliferation would be of great significance because any application of MSCs in experiments or clinic requires their in vitro expansion. According to previous research works, MSCs occur in very scarce number in tissue sources.[31] These data were obtained in murine system which is considered as an appropriate animal model for preclinical investigations.

Our study was not the only study in this context. In a study conducted by Nadri *et al.* amniotic fluid-
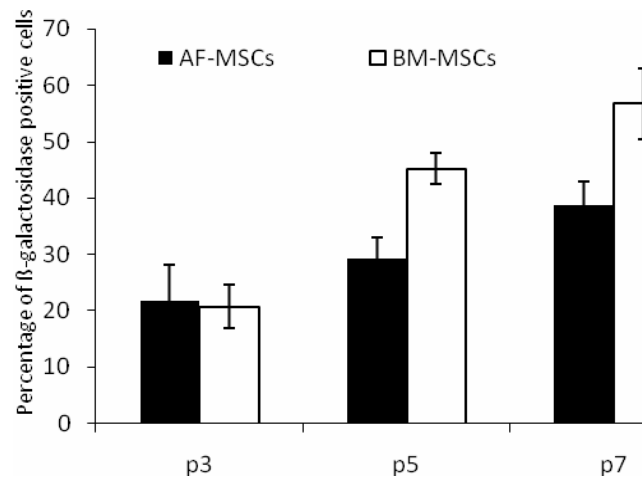
**Fig. 6:** Analysis of ß galactosidase positive cell percentage at the isolated cell culture. These cells were first observed at passage three and their number increased with passages. Comparatively, the percentages of ß galactosidase positive cell at AF-cells were lower than those in BM-MSCs at passage 5 (p5) and 7 (p7). * indicates a significant difference with $p$=0.008.

derived murine MSCs were isolated and compared with marrow-derived MSCs. According to their results, amniotic fluid-derived murine MSCs were not able to generate adipogenic cell lineages.[32] But our investigation indicated that these cells like their marrow counterparts were able to differentiate into adipose cells in addition to bone and cartilage cell lineages. Tripotent differentiation potential of our cells is the most accordance with MSCs definition described elsewhere.[33] According to previous research works, marrow contains mesenchymal stem cells with varying differentiation potential including the cells with unipotent, bipotent and tripotent differentiation capacities.[1] One possible explanation for bipotent nature of the cells isolated by *Nadri et al.*, could be to imagine that amniotic fluid like marrow contains MSCs with varying differentiation potential and the isolated cells by *Nadri et al.*, were those with bipotent differentiation ability. Furthermore *Nadri et al.*, compared growth ability of the MSCs from two sources using only their colonogenic ability which would not be a reliable method for determining MSCs growth potential because in this assay, the number of the cells are taken in consideration while their dimension are ignored. To thoroughly investigate the matter of amniotic fluid- versus marrow-derived MSCs proliferation capacity in culture, we used several precise assays including colonogenic sassy (with taking into account of the colony size), the cell population doubling time (PDT) and plotting a growth curve. The growth curve

of amniotic fluid-derived cells had an interesting difference with that of marrow-derived cells: there was no lag phase in amniotic fluid- derived MSCs culture. This implies that the amniotic fluid- derived MSCs were rapidly recovered from the damages occurred during tripsinization and that these cells were very resistant to stressful conditions of the culture.

PDT is an important index that frequently is being used to characterize cultures. In the present study, PDT was calculated during the subcultures and the primary culture was excluded because for calculation, there is a need for determining the number of cells at culture initiation, while this value was not calculable for primary culture. Bone marrow and amniotic fluid tissue are both heterogeneous in cellularity which a small fraction of their cells were able to adhere on culture surfaces and the majority of the cells were discarded upon first culture medium removal.

By date, several markers including CD133, LNGFR (low affinity nerve growth factor receptor) and STRO-1 have been proposed as the markers of human MSCs.[34-36] In this regard, the specific marker for animal MSCs has yet remained to be clarified making isolation and identification of them as a difficult task. To address this issue, mesenchymal and tissue stem cell committee of international society for cellular therapy has proposed the ability of multilineage differentiation and adherent property of the cells as defining criteria for MSCs identification.[35] Since the isolated cells of the present study were easily dif-

ferentiated into bone, cartilage and adipose cells and also they were plastic adherent cell, we convinced that they were the MSCs described elsewhere.

In this study we used beta galactosidase staining method to examine the percentages of the senescent cells in our studied cell culture. Acid ß-D-galactosidase which is located in eukaryotic cell lysosomes can be detected in situ by means of a cytochemical assay normally carried out at pH 4. The other version of this enzyme that has recently been described in senescent fibroblastic cultures found to be active at pH 6.[32] Since then, SA- ß-galactosidase assay was used to examine the senescence of variety of cells in culture.[39-41] According to our findings, ß galactosidase positive cells were first observed at passage three and their number increased as the passage number went high. This is the first report on murine amniotic fluid- derived MSCs aging which was not described by the former similar investigation.

Taken together, it seems that murine amniotic fluid housed MSCs with relatively high proliferation property than marrow-derived MSCs and a typical tripotent differentiation potential comparable with marrow MSCs. High proliferation capacity of amniotic fluid MSCs as well as their lower percentages of senescent cells during the subcultures compared to their marrow-derived counterparts render them as an appropriate candidate for use in studies related to regenerative medicine of specially skeletal defects where there are a tremendous need for highly viable proliferating cells.

## Acknowledgments

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