

Original Article

Mesenchymal Stem Cells from Murine Amniotic Fluid as A Model for Preclinical Investigation

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Abstract

Background: Despite the suitability of a mouse model for preclinical investigations; little is known regarding mesenchymal stem cells derived from murine amniotic fluid. This is the subject of the present study.

Methods: Amniotic fluid was collected from NMRI mice during the second weeks of pregnancy and plated. The cells that adhered to the culture surfaces were propagated with three successive subcultures and then characterized. To determine the differentiation potential, the cells were cultivated under osteogenic, adipogenic, and chondrogenic conditions, and followed by specific staining and RT-PCR analysis for differentiation. The proliferative potential of the cells were measured with clonogenic assays, population doubling time and number and by growth curve plotting. Cellular aging was investigated with the senescence-associated β -galactosidase staining method.

Results: The amniotic fluid primary cell culture was composed of round flattened and fibroblastic cells. The latter dominated the culture after several passages. Successful tripotent differentiation of the isolated cells into bone, cartilage and adipose cells were indicative of their mesenchymal stem cells nature. The isolated cells appeared to be relatively proliferative cells as confirmed by the population doubling time value which was equal to about 69 hours. Furthermore, the cells were relatively clonogenic and they tended to initiate proliferation immediately after plating (there was no lag phase in their growth curve). β -galactosidase positive cells were first observed at passage 3 and increased in number with subsequent passages.

Conclusions: Collectively it was concluded that murine amniotic fluid contained mesenchymal stem cells with relatively high proliferation property and typical tripotent differentiation potential.

Keywords: amniotic fluid, bone, cartilage and adipose differentiation, mesenchymal stem cells, proliferation

Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic cells that have originally been reported as bone precursor cells, which reside in bone marrow tissue.¹ A detailed description of the cells has been provided by Friedenstein et al. (1970) who reported the cells to be a small fraction of marrow adherent cells with spindle-shaped morphology capable of producing aggregations of 2 – 4 cells.² According to observations by Friedenstein et al., the colonies remain inactive for two to four days after which they undergo rapid propagation, resulting in a homogenous cell population. The most important characteristics of MSCs are their ability to produce bone and cartilage-like tissue in culture. This capacity has been reported to be maintained even after a 20 – 30 population doubling.² These observations

have later been confirmed and further developed by Piersma et al. and Owen et al. during the 1980s.^{3,4}

According to previous research, MSCs are capable of giving rise to a variety of cell types in addition to their well-recognized skeletal cell lineages. It has thus far been reported that MSCs can generate neurons, keratinocytes, lung epithelial cells, liver cells, intestinal epithelial cells as well as kidney and spleen cells.^{5,6} This property of MSCs, e.g., production of cells other than mesenchymal cell lineages, is referred to as MSCs transdifferentiation.

MSCs have been considered as an appropriate source for cell and gene therapy due to their extensive self-renewal property as well as multilineage differentiation potential.⁷ The efficacy of the cells in treating some tissue defects has, by date, been well established. Namely, MSC efficacy has been established in curing osteogenesis imperfecta, regenerating bone and cardiac muscle, and resurfacing articular cartilage as well as restoring hematopoiesis in patients who have received chemotherapy.^{8–13}

The first step in the preparation of MSCs for any application is to obtain marrow aspirates. Marrow aspiration is an invasive procedure that involves inserting a needle into the patient's iliac crest, hence causing pain and morbidity. For this reason, the attention of investigators has been directed

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to an easily-obtainable MSC sources including peripheral and umbilical cord blood.¹⁴⁻¹⁶ Another potential source for MSCs would be amniotic fluid (AF), which is routinely discarded during child birth.

AF which fills the amniotic sac and surrounds the developing embryo has been reported to include a heterogeneous population of cells. These cells are routinely used to diagnose embryonic chromosomal, biochemical, structural, and genetic anomalies. In spite of great advances that have been made in use of AF cells as diagnostic tools, the cellular origin has remained to be clarified. It is believed that AF cells may originate from skin, urogenital, respiratory, digestive tract and membranes of the developing embryo.^{17,18}

Some research has suggested that there could be stem-like cells in AF. In this regard, the presence of small, nucleated hematopoietic precursors most likely originating from the yolk sac has been confirmed.¹⁹ Furthermore, the probability of the presence of non-hematopoietic cells in the AF has previously been suggested.²⁰ In general there are a number of investigations that have isolated and characterized human AF-derived MSCs,²¹⁻²⁴ however, little is known regarding murine AF-derived MSCs, which would be a good model for preclinical investigations. The present study deals with the isolation, proliferation, differentiation, and aging of MSCs from murine AF.

Materials and Methods

Cell culture

In this study, 10 NMRI mice, ages 3 – 5 weeks old were used in strict adherence to protocols approved by the Animal Care and Use Committee of Royan Institute (Tehran, Iran). The mice were killed by cervical dislocations at 14 – 16 days of pregnancy. The abdomen was opened and the entire uterus dissected and placed in petri dishes. An average of 500 – 1000 μ L of AF was aspirated from each mouse with 22 gauge needles and collected in 15 mL tubes. AF from each mice was added to 2 mL of DMEM medium (Dulbecco's modified eagle medium, Gibco, Germany) supplemented with 10% FBS (fetal bovine serum, Gibco, Germany), 10 IU/mL penicillin/streptomycin (Gibco, Germany) and cultivated in 25 cm² culture flasks. The cultures were then incubated in an atmosphere of 5% CO₂ at a temperature of 37°C. Three to four days after culture initiation, the medium was discarded; the cultures were washed with phosphate buffer saline (PBS) and fresh medium was added. Medium changes were performed every 2 – 3 days until the culture reached 70 – 80% confluence. At this time, cells were trypsinized and lifted. About 2 \times 10⁵ cells were counted and re-plated in 25 cm² flasks (passage 1). Two additional successive subcultures were performed and the resulted cells (passage 3) were used to conduct the following experiments.

Flow cytometry

Flow cytometric analysis was used to characterize the isolated cells with respect to their surface antigen profile. For this purpose, about 1.5 \times 10⁵ passage-3 cells were suspended in 100 μ L of PBS and placed in 5 mL tubes. Then, 5 μ L of FITC-conjugated antibodies including CD34, MHC II, CD11b, CD45, CD31, CD44, and CD90 (all purchased from Becton Dickinson, USA) were added to the cell suspension, followed by incubation at 4°C for 30 min in the dark. The solutions were centrifuged at 1200 rpm for 4 min, cells were dispersed in 300-500 μ L washing buffer and analyzed by flow cytometry (FACScalibur cytometer equipped with 488 nm argon lasers). In this study, IGG2 and IGG1 were taken as isotope controls. WinMDI software was used to analyze the flow cytometric results.

Differentiation potential

To evaluate the MSC nature, isolated cells were differentiated into osteogenic, chondrogenic, and adipogenic cell lineages.

To induce osteogenic differentiation, confluent passage-3 cells were cultured in DMEM medium supplemented with 50 mg/mL ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethasone (Sigma, USA) and 10 mM β glycerol phosphate (Sigma, USA) for three weeks. At the end of this period, alizarin red staining was used to observe matrix mineralization. For staining, the cultures were first fixed by methanol for 10 min and then subjected to alizarin red solution for 2 min.

For adipogenesis, DMEM medium containing 100 nM dexamethasone (Sigma, USA) and 50 mg/mL indomethacin (Sigma, USA) was added to the confluent culture of passage-3 cells. Three weeks after culture initiation, cells were fixed with 4% formalin at room temperature, washed by 70% ethanol and stained by oil red solution in 99% isopropanol for 15 min.

To induce cartilage differentiation, a micro mass culture system was used. For this purpose, 2.5 \times 10⁵ passage-3 cells were pelleted at 300 g for 5 min and cultured in a DMEM medium supplemented by 10 ng/mL TGF- β 3 (transforming growth factor- β 3, Sigma, Germany), 10 ng/mL BMP6 (bone morphogenetic protein-6, Sigma, Germany), 50mg/mL insulin-transferring- selenium+premix (Sigma, Germany), 1.25 mg bovine serum albumin (Sigma, Germany) and 1% FBS. Three weeks after culture initiation, pellets were removed and subjected to the following: fixation in 10% formalin; dehydration in ascending concentrations of ethanol; clearing in xylene; embedding in paraffin wax and sectioning in 5 μ m sections by microtome. The sections were then stained by toluidine blue for 30 s at room temperature.

RT-PCR analysis

For RT-PCR, total RNA was isolated from differentiated cells with the RNXTM(-Plus) (RN7713C; CinnaGen Inc., Tehran, Iran). Before RT, a sample of the isolated RNA

Table 1. Primers used in RT-PCR.

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

was treated with 1 U/mL of RNase-free DNaseI (EN0521; Fermentas, Opelstrasse 9, Germany) per 1 mg of RNA in order to eliminate residual DNA in the presence of 40 U/mL of ribonuclease inhibitor (E00311; Fermentas, Germany) and 1× reaction buffer with MgCl₂ for 30 min at 37°C. To inactivate DNaseI, 1 mL of 25 mM EDTA was added and incubated at 65°C for 10 min. Standard RT reactions were performed with 2 µg total RNA using oligo(dt) as a primer and a RevertAid™ 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™M-MuLV Reverse Transcriptase (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. Reaction mixtures for PCR included 2 mL cDNA, 1× PCR buffer (AMST™; Cinnagen Co., Tehran, Iran), 200 mM dNTPs, 0.5 mM of each antisense and sense primers (Table 1), and 1U Taq DNA polymerase.

Clonogenic assay

About 10⁴ passage-3 cells were plated in 100 mm² dishes for a period of 14 days at the end of which the cells were washed twice with PBS and stained with crystal violet for 5 min. The colonies produced by AF cells were then observed with an inverted light microscope and counted. This procedure was separately performed for each of the ten mice and the mean colony number was reported. The diameters of the colonies were also measured and recorded.

Population doubling numbers (PDN) and time (PDT)

To examine cell growth rate, the number of population

doubling (PDN) as well as the time required by cells for each population doubling (PDT) were determined during three successive subcultures. These were calculated from hemocytometer counts for each passage according to the following formula:

$PDN = \log(N1/N0) \times 3.31$ and $PDT = CT/PDN$ (25). Where N1 is the cell number at the end of the cultivation period, N0 the cell number at culture initiation and CT, the cell culture time.

Growth curve

To plot the growth curve, 10⁴ passage-3 cells were plated in 24 well culture plates for 7 days during, which some wells were trypsinized daily, the cells were lifted and counted. The growth curve was then plotted using the cell counting data.

Senescence-associated β-galactosidase (SA-β-gal) staining

The senescence status of the isolated cells was verified by *in situ* staining for SA-β-galactosidase.²⁶ Briefly, the cells from passages 1, 2, 3, 5 and 7 grown on 4-well culture plates were washed twice with PBS and fixed with 3% formaldehyde for 4 min. Cells were washed again and incubated with β-galactosidase substrate staining solution (150 mM NaCl, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid and 40mM sodium phosphate at pH 6 containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-D-β-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 were counted in 10 random fields to determine the percentage of SA-β-galactosidase-positive cells.

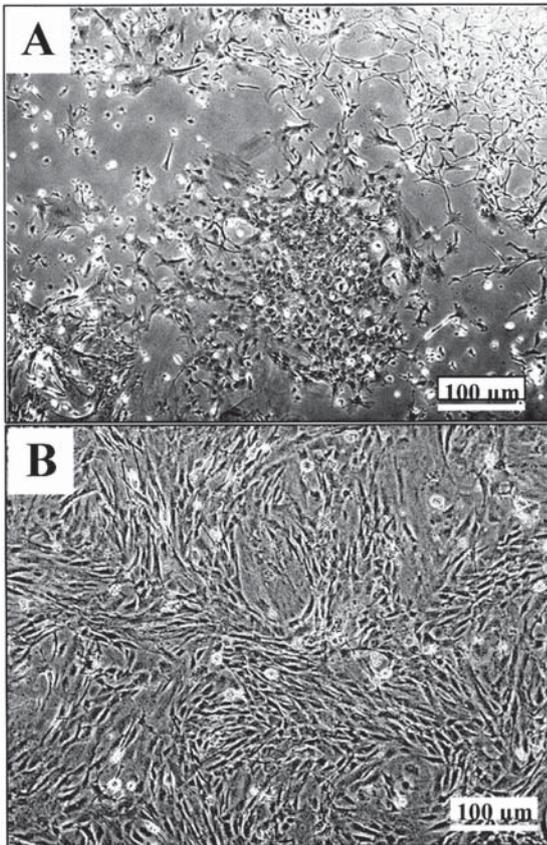


Figure 1. Culture of murine amniotic fluid cells. **A)** Primary culture. Various morphologies can be seen in the culture. **B)** A population of cells with almost fibroblastic morphology dominates the culture after three successive subcultures, bar=100µm.

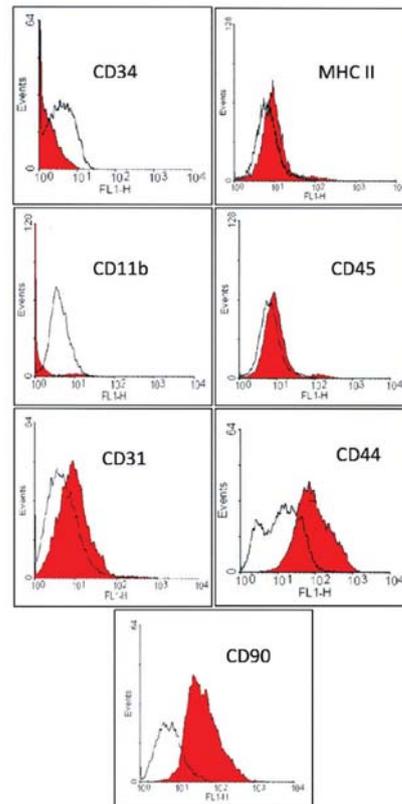


Figure 2. Flow cytometric analysis of some surface antigens in murine amniotic fluid-derived MSCs in passage 3 cultures. While the majority of the cells expressed CD 44 and CD 90, other antigens were expressed in the minority of the cells.

Results

Cell culture

The primary culture tended to reach confluence 7 – 10 days after culture initiation. At this culture stage, a variety of cell morphology, which included elongated, round flattened and triangular cells were observed (Figure 1A). The population of spindle-shaped cells became dominant by subculture three (Figure 1B).

Flow cytometry

According to the flow cytometry results, the majority of cells isolated from the murine AF tended to express CD90 and CD44 surface antigens. The other studied antigens which included CD34, MHC II, CD 11b, CD 45, and CD 31 were expressed in very low percentages of the studied cells (Figure 2).

Multi-lineage differentiation

In some areas of the osteoinductive cultures, nodule-like structures were observed. Following alizarin red staining,

red mineralizing areas within the cultures appeared (Figure 3A). Differentiation was further demonstrated by RT-PCR analysis of the osteocyte markers. After a three week induction period, osteocalcin and PTHR mRNA were largely produced (Figure 3B).

Nodular structures were not observed in the adipogenic culture. Small lipid droplets appeared within the cytoplasm a few days after culture initiation and gradually occupied the entire cells by day 21. The lipid droplets turned red when stained by the oil red O staining method (Figure 3C). RT-PCR analysis was also indicative of expressions of LPL, Adipsin and PPAR gamma in the adipogenic culture (Figure 3D).

In the micro mass culture system for chondrocyte differentiation, the size of the pellet seemed to have increased during the culture period, probably as a result of matrix production and secretion. Metachromatic nature of the matrix was demonstrated by the toluidine blue staining method (Figure 3E), and the expression of its marker molecules including aggrecan, collagen II and collagen X were examined and confirmed by RT-PCR analysis (Figure 3F).

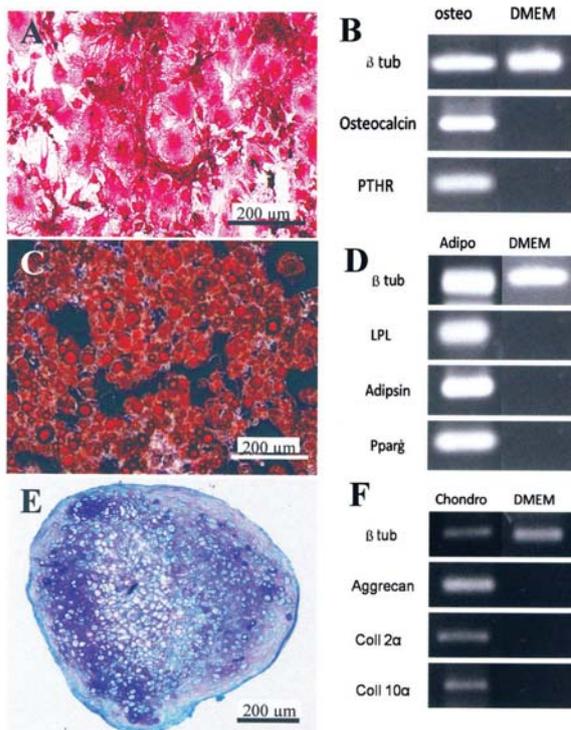


Figure 3. Differentiation potential of the isolated cells from murine amniotic fluid. **A)** Osteogenic culture stained red following alizarin red staining. **B)** In this culture, bone related genes including osteocalcin and PTRH were expressed. **C)** Adipogenic culture stained red following oil red staining. **D)** Adipogenic genes were expressed in adipogenic cultures. **E)** Sections prepared from micromass culture for chondrogenesis stained purple following toluidin blue staining. **F)** Cartilage specific genes were expressed at the end of chondrogenic cultures.

Colony numbers

The colonies produced by AF-derived MSCs stained purple upon crystal violet staining (Figure 4A). A mean of 236 ± 22.8 colonies were observed at AF MSCs culture per 10^4 initiating cells. The mean diameter of each colony was 2.61 ± 0.96 mm.

PDN and PDT

According to our results, the cells possessed a total PDN value of 4.4 ± 1.1 . Population doubling time (PDT) for our isolated cells, e.g., the time during which the cell population doubled appeared to be about 69.695 ± 14.5 hr.

Growth curve

According to the growth curve plotted for the isolated cells, there was no lag stage (Figure 4B), indicating that the cells were able to begin proliferation without the need for an adaptation period. This implies that isolated MSCs from AF were resistant cells that had a rapid recovery ability from the stressful situation of the culture conditions.

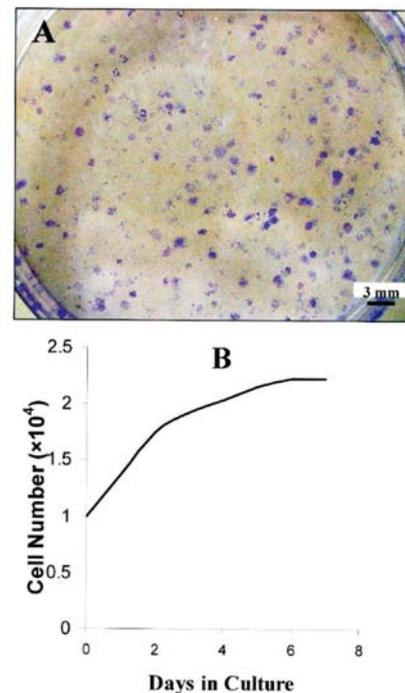


Figure 4. **A)** Crystal violet staining of murine amniotic fluid-derived MSCs colonies. **B)** Growth curve that was plotted for the cells. The cells possessed no lag (adaptation) phase meaning that they began proliferation immediately after being plated.

Senescence

According to the SA- β -galactosidase staining results, no positively-stained cells were observed at passages-1 and -2 cultures. β -galactosidase positive cells were first observed at passage 3, which composed about $21.8\% \pm 6.43$ of the cells. The value tended to increase to $29.4\% \pm 3.6$ at passage 5 and $38.8\% \pm 4.14$ at passage 7 (Figure 5).

Discussion

In the present study, a fibroblastic population of cells from murine AF was isolated and culture expanded through several successive passages. In the next step, we characterized the cells in terms of differentiation potential, *in vitro* proliferation rate as well as the onset of replicative senescence in culture. Since the cells succeeded to differentiate into bone, cartilage and adipose cell lineages, we concluded that they were the MSCs isolated and described elsewhere.²⁷⁻²⁹ A study such as this would be of significance since it describes the isolation of tripotent MSCs from murine AF, which would be a good model for preclinical investigations. In spite of MSCs' potential importance in future cell and gene therapy, their safety for transplantation purposes still remains to be clarified. More research must therefore be carried out, particularly in animal models such as mice, before MSCs can be used routinely for therapeutic purposes.

In a study conducted by Nadri et al., murine AF-derived

MSCs were isolated and compared with marrow-derived MSCs. According to their results, AF-derived murine MSCs were not able to generate adipogenic cell lineages.³⁰ However, 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 most in accordance with the definition of MSCs as described elsewhere.³¹ According to previous research, marrow contains MSCs with varying differentiation potentials, including cells with unipotent, bipotent, and tripotent differentiation capacities.⁷ One possible explanation for the bipotent nature of the cells isolated by Nadri et al. is that AF like marrow would be contained MSCs with varying differentiation potentials and the isolated cells by Nadri et al. would be those with bipotent differentiation ability.

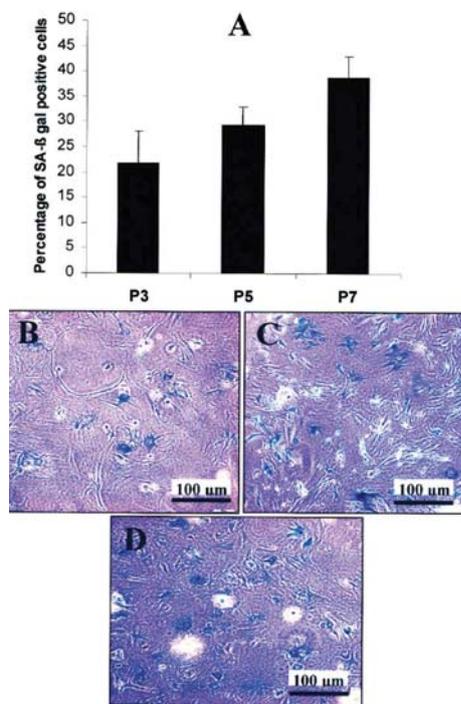


Figure 5. Senescence-associated β -galactosidase (SA- β -gal) staining. **A)** Graph indicating the percentages of senescence cells in different passages. **B–D)** representative images of blue-stained senescence cells in passages 3, 5, and 7, respectively.

In spite of the considerable attempts that have been made to define the antigenic profile of human MSCs, no definitive single marker has thus far been introduced. In this regard, several markers including CD133, LNGFR (low affinity nerve growth factor receptor) and STRO-1 have been proposed as markers of human MSCs.^{32–34} In a guideline published in *Cytotherapy* it has been proposed that for human MSCs the expression of some surface antigens, including: CD105, CD73, CD90, CD45, CD34, CD14, CD19, and HLA-DR should be checked. According to that guideline, in the case of MSCs from non-human systems, surface an-

tigen expression is not universally well characterized and the antigens recommended for human cells may not apply. To overcome this problem, they have proposed two characteristic criteria for recognizing MSCs from non-human tissues: MSCs from animal systems must be adherent on plastic surfaces of culture dishes and they must be able to produce bone, cartilage and adipose cell lineages.³¹ Since the isolated cells of the present study were easily differentiated into bone, cartilage, and adipose cells and were also plastic adherent cells, we concluded that they were the MSCs described elsewhere. Furthermore, in the present study, to determine the surface antigenic profile of AF-derived cells, we examined the expression of some surface antigens and found that the majority of the cells tended to express CD44 and CD90. Antigens including CD34, MHC II, CD11b, CD45, and CD31 were observed to be expressed in very low numbers of the studied cells. Peister et al. have examined the expression of some surface markers on marrow-derived MSCs from different strains of inbred mice including B1/6, FVB/N, BALB/c, and DBA1.²⁸ These investigators have concluded that there was a variation in surface marker expressions among the studied mice. The result of the present study, considering the AF-derived MSCs from NMRI mice, is in agreement with the findings by Peister et al. in that the expression of some surface antigens on MSCs of NMRI mice appeared to be different from those on strains studied by Peister et al. For instance, CD90 surface antigen, which is expressed on the majority of MSCs from NMRI mice did not express on MSCs derived from B1/6, FVB/N, BALB/c, and DBA1 mice. Furthermore, CD34 being expressed on the minority of NMRI cells had been found to be expressed on more than 75% of B1/6 cells.

According to the previous investigations, MSCs occur at low frequencies in source tissues.³⁵ Therefore, any experimental work with MSCs requires *in vitro* expansion of the cells. In the present study, the isolated cells were characterized in terms of their *in vitro* proliferation. Clonogenic assays are among those assays in frequent use to determine cell proliferation potential,^{27–29,36,37} but this assay measures only the colony number, but not the colony size. For this reason, in addition to the clonogenic assay that was performed in this study, the colony size was also measured.

The other indices indicating the cell rate of proliferation are population doubling number (PDN) and population doubling time (PDT). In this study, PDN and PDT were calculated for three successive subcultures and the primary culture was excluded from the calculation, because only a small portion of the cells used at culture initiation became the origin of culture since the majority of those cells were discarded during medium replacement. The other indices that indicated the high proliferative property of the isolated cells were their growth curve characteristics. According to this curve, there was no lag phase, which implied that the cells rapidly recovered from the damage that occurred dur-

ing trypsinization.

Acid β -D-galactosidase is a hydrolase located in eukaryotic cell lysosomes. The activity of this enzyme can be detected *in situ* in most mammalian cells by means of a cytochemical assay normally carried out at pH 4. The other version of this enzyme that is active at pH 6 has been reported to be found in senescent fibroblastic cultures.²⁶ Since then, the SA- β -galactosidase assay has been used to examine the senescence of a variety of cells in culture.³⁸⁻⁴⁰ In the present study, this method was used to determine the onset of replicative senescence of AF-derived MSCs. According to our findings, β -galactosidase positive cells were first observed at passage three and their number increased along with an increase in passage number. The senescence of MSCs have already been described by Bonab et al. on human marrow-derived MSC cultures⁴¹ but this is the first report on aging of murine AF-derived MSCs in culture.

Taken together, mice AF contains MSCs with relatively high proliferation property and typical tripotent differentiation potential. These cells can be used as model cells to conduct investigations aimed to clarify MSCs safety and efficiency for clinical use.

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Sepidan, around 100 Km from Shiraz, winter 2011. (Photo by M.H. Azizi MD)