

Mesenchymal Stem Cells Derived from Rat Epicardial Versus Epididymal Adipose Tissue

*¹Mohamadreza Baghaban Eslaminejad, ²Soura Mardpour, ¹Marzieh Ebrahimi

Abstract

Objective(s)

Some investigation has indicated that adipose-derived stem cells possess different surface epitopes and differentiation potential according to the localization of fat pad from which the cells were derived. In the present study proliferation capacity and aging of such cells were explored.

Materials and Methods

Adherent cells were isolated from the collagenase digests of adipose tissues excised from rat epicardial and epididymal regions and propagated with several subcultures. The cells were then investigated whether or not they were able to differentiate into bone, cartilage and adipose cell lineages. Studied cells from two adipose tissues were also compared with respect to their *in vitro* proliferation capacity. The presence of senescent cells in the culture was determined and compared using senescence-associated (SA) β -galactosidase staining method.

Results

Successful differentiations of the cells were indicative of their mesenchymal stem cells (MSCs) identity. Epicardial adipose-derived cells tended to have a short population doubling time (45 ± 9.6 hr) than the epididymal adipose-derived stem cells (69 ± 16 hr, $P < 0.05$). Colonogenic activity and the growth curve characteristics were all better in the culture of stem cells derived from epicardial compared to epididymal adipose tissue. Comparatively more percentage of senescent cells was present at the cultures derived from epididymal adipose tissue ($P < 0.05$).

Conclusion

Our data emphasize on the differences existed between the stem cells derived from adipose depots of different anatomical sites in terms of their proliferative capacity and *in vitro* aging. Such data can help understand varying results reported by different laboratories involved in adipose stem cell investigations.

Keywords: Adipose tissue, Cell aging, Cell differentiation, Cell proliferation, Mesenchymal stem cell

1- Department of Stem Cell and Developmental Biology, Royan Institute for Stem Cell Biology and Technology, Iranian Academic Center for Education, Culture and Research (ACECR), Tehran, Iran

* Corresponding author: Tel: +98-21-23562000; Fax: +98-21-22310406; email: eslami@royaninstitute.org

2- Department of Developmental Biology, University of Science and Culture, Tehran, Iran

Introduction

Mesenchymal stem cells (MSCs) are defined as multipotent cells possessing a differentiation potential primarily among adipocytic, chondrocytic and osteocytic cell lineages and having the capacity of self-renewal replication for long-term. MSCs have first been recognized by Petrakova and Friedenstein who have isolated and described bone precursor cells from rat bone marrow tissue (1). Definitive evidences on the presence of MSCs in bone marrow have been provided by Friedenstein *et al* (1970) who have found a fibroblastic colonogenic population of bone marrow cells capable of producing bone and cartilage deposits in culture (2).

Having the potential of multipotent differentiation and capacity of self-renewal replication, MSCs are considered as promising candidates for cell-replacement treatment of specifically skeletal tissue defects (3). Up to now multiple tissues have been found to contain MSCs in addition to the well-known bone marrow tissue (4-9). Adipose tissue is among those sources that have gained specific attentions probably because it is abundant, relatively accessible with minimal invasion and replenishable. Stem cells from adipose tissue have first been isolated and described by Radbell who have collected fragments of rodent adipose tissue through an open surgery (10). Later, several groups have reported the isolation of stem cells from human adipose tissue taking advantage of liposuction aspirates as a tissue source (11, 12).

The procedure used to isolate stem cells from adipose tissue includes collagenase digestion of the tissue which produces a digest with two distinct fractions: a floating mature adipocytes fraction and a pletted stromal vascular fraction (SVF). Former investigations have indicated that SVF is consisted of a heterogeneous cell population, including blood cells, fibroblast, pericytes and endothelial cells as well as preadipocyte progenitor/stem cells (10-12). Using SVF culture, investigators have isolated a population of stem cells with differentiation potential into bone and cartilage cell lineages (13, 14). Furthermore it has been

indicated that SVF contains stem cells capable of giving rise to endothelial cells *in vivo* and *in vitro* (15). Moreover, spontaneous cardiomyocyte differentiation has been described in SVF cultures (16).

Some limited data indicated that stem cells from adipose tissue of different anatomic sites present different properties. In an interesting study conducted by Prunet-Marcassus *et al* (2006), human adipose tissues were collected from inguinal, epididymal and interscapular regions and it was indicated that surface epitopes and differentiation potentials were varying among the stem cells of those fat depots (17). The other important aspect that is worth to be investigated is to compare *in vitro* proliferation capacity and senescence of stem cells derived from adipose tissue of different localizations. Addressing this issue, in the present study we collected tissues from both subcutaneous (epididymal) and internal (epicardial) adipose depots of Wistar rats and compared their stem cells with respect to their *in vitro* proliferation and aging characteristics of the cells. Study like this which addresses probable differences among stem cells derived from tissues distributed at various anatomical sites would help to understand the varying results reported by different laboratories involved in adipose stem cell investigating.

Materials and Methods

Cell culture

The use of 10 male Wistar rats, age 2-4 weeks, was approved by the ethic committee of Royan Institute (number: 87/251 date: 29 Nov 2008). The cell isolation was done according to the previously-published procedure with some modifications (17). In brief, the animals were sacrificed by cervical dislocation, the adipose tissue from scrotal and epicardial regions were dissected off and collected in 15-ml sterile tubes containing phosphate buffer solution (PBS). Under laminair hood, the specimen was minced into small pieces and then subjected to enzymatic digestion using 0.075% collagenase type I at 37 °C for 2 hr. At the end of this time, the floating cells were separated from the vascular stromal fraction by centrifugation (1200 rpm) for 5 min. The pellet (stromal

vascular fraction) was then filtered through a 200 μm nylon mesh to remove undigested tissue. The digest was suspended in 1 ml proliferation medium including DMEM (Dulbecco Modified Eagle Medium, Sigma, Germany) containing 10% FBS (fetal bovine serum, Gibco, Germany) and 10 IU/ml penicillin/streptomycin (Gibco, Germany) and plated at 10^6 cells/ml in 25 cm^2 -culture flasks. The cultures were incubated in an atmosphere of 5% CO_2 and 37 °C. Three days after culture initiation, the medium was discarded and the cells were washed with PBS and fed with fresh medium. Medium changes were performed each 3 days until the culture became confluent. At this time, the cultures were trypsinized using 0.05% trypsin/1 mM EDTA and passaged at 1:2 ratios into fresh 25- cm^2 culture flasks. Subculture was repeated till passage 3 when sufficient cells were provided for the next stage of experiment.

Differentiation potential

Osteogenic culture

The cells from passage 3 were counted and plated at 2×10^5 cells in 25- cm^2 culture flask in proliferation medium. When the culture was at approximately 80% confluency, the proliferation medium was replaced by differentiating 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). The cultures were incubated for 21 days with medium changes of twice weekly. At the end of this period, osteogenesis was evaluated with RT-PCR analysis for bone related gene expression as well as alizarin red (Sigma, USA) staining for mineralized matrix deposition.

Adipogenic culture

Passaged-3 cells were plated at 2×10^5 cells in 25- cm^2 culture flasks with proliferation medium and allowed to become confluent. The medium was then removed and the cultures were provided with adipogenic differentiating medium composing of DMEM supplemented with 50 $\mu\text{g}/\text{ml}$ ascorbic acid 3-phosphate, 100 nM dexamethasone and 50 $\mu\text{g}/\text{ml}$

indomethacin. The cultures were incubated for 21 days during which the medium was changed twice weekly. At the end of this period the cultures were examined for adipogenic differentiation using oil red staining for lipid droplets as well as RT-PCR analysis for detection of adipocyte specific gene expression.

Chondrogenesis

Micro mass culture system was used to investigate the chondrogenic potential of the isolated cells. For this purpose, 2.5×10^5 passaged-3 cells were pelleted under 300 g for 5 minutes and provided with 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), 50 mg/ml insulin transferin selenium+ premix (Sigma, Germany), 1.25 mg bovine serum albumin (Sigma, Germany) and 1% fetal bovine serum. The cultures were incubated at 37 °C and 5% CO_2 for three weeks with medium changes of twice weekly. To examine chondrogenesis, the pellets were fixed with 10% formalin, dehydrated in ascending concentrations of ethanol, cleared in xylene, embedded in paraffin and cut into 5 μm -thick sections which were stained by alcian blue (Sigma, USA) for cartilage specific matrix detection. Furthermore, RT-PCR analysis was used to examine the production of cartilage specific mRNA in the differentiating cells.

RT-PCR analysis

RT-PCR procedure

Total RNA was isolated from the differentiated cells using the RNXTM (-Plus) (RN7713C; CinnaGen Inc., Tehran, Iran). Before RT, a sample of the isolated RNA was treated with 1U/ μl of RNase-free DNaseI (EN0521; Fermentas, Opelstrasse 9, Germany) per 1 μg of RNA in order to eliminate residual DNA in the presence of 40 U/ μl of ribonuclease inhibitor (E00311; Fermentas, Germany) and 1 \times reaction buffer with MgCl_2 for 30 min at 37 °C. To inactivate the DNaseI, 3 μl 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 set up were reverse transcribed simultaneously. Reaction mixtures for PCR included 20 ng cDNA, 10×PCR buffer (AMS™; CinnaGen Co., Tehran, Iran), 200 mM dNTPs, 0.5 mM of each antisense and sense primer (Table 1), and 1U Taq DNA polymerase.

Colonogenic assays

The passaged-3 cells obtained from both epididymal and epicardial adipose tissues were counted using hemacytometer, plated at 10⁴ cells in 10-cm petri dish and allowed to grow for 7 days. At the end of this time, the cultures were terminated and stained with crystal violet for visualizing colonies produced at cultures. The petri dishes were then observed with a light microscope to determine the number of produced colonies by each studied cells. We also measured the size of colonies in both groups.

Calculation of population doubling time (PDT)

PDT is defined as the time by which cell

population doubles in number. Determination of PDT would be an important index indicating the cell capacity to undergo proliferation at given culture conditions. Population doubling number (PDN), in this study was calculated according to the equation PDT= culture time (CT)/PDN. To determine PDN, the formulae PDN= log N/N₀×3.31 was used (18). In this equation N stands for the cell number at culture end and N₀ the number of the cells at culture initiation. To determine the culture time and N and N₀, passaged-3 cells were counted and plated at 10⁴ cells/cm² in 25-cm² culture flasks for a period when one of the cultures reaches confluent (which in this study it was the epicardial cells that achieved confluency in about 6 days). At this time the cells were trypsinized and counted. Using the data, PDT was calculated for either studied cell culture.

Growth curve

The cells in culture usually grew with specific pattern having three characteristics phases including lag, log and plateau phase.

To determine this pattern for stem cells isolated either from epididymal or epicardial adipose tissue, growth curves were plotted. For this purpose, 5×10⁴ cells/well were plated in 12-well plate and allowed to become confluent. On a regular daily basis, some of the wells were trypsinized and cells were counted. Using the data, growth curve was plotted.

Table 1. Primers used in RT-PCR analysis.

Genes	Primer Sequence (5'-3')	Annealing Temperature (°C)
ALP	ACA CGG ACA AGA AGC CCT	60
	GTG AAG CAG GTG AGC CAT AG	
Osteocalcin	GTC CCA CAC AGC AAC TGC	60
	CCA AAG GCT GAA GCT GCC G	
C/EBP-alpha	GCA GAA GGT GTT GGA GTT GA	61.5
	AGC GAC CCT AAA CCA TCC TC	
PPAR-alpha	CCC TGC CTT CCC TGT GAA CTG AC	63
	GGG ACT CAT CTG TAC TGG TGG GGA C	
PPAR-gamma 2	GGT GAA ACT CTG GGA GAT CC	62
	TGA GGG AGT TTG AAG ACT CTT C	
Agc	ACTTTGACCGAGCCCAGCGG	61.5
	CTGAAGAGATGGAGGGTGAG	
COLII	GCCGAGGGCAACAGCAGGTT	61.5
	CCTTCCCATTGTTGACATTG	
Beta-tubulin	CTCACTGTGCCTGAACTC	60
	GGAACATTGCCGTAACACTGC	

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

To determine the percentages of senescent cells in the cultures of both epididymal and epicardial adipose-derived stem cells, SA-β-galactosidase staining was used. The staining was performed according to the previously-described procedure (19). Briefly, the cells from passage 1, 2, 3, 5 and 7 growing on 4-wells culture plates were washed with PBS for 2 times, and fixed with 3% formaldehyde for 4 min. The cells were then incubated with beta-galactosidase substrate staining solution including 150 mM NaCl, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid, and 40 mM sodium phosphate, pH 6, 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-β-galactosidase at 37 °C for 14 hr. A minimum of 100 cells was counted in 10 random fields to determine the percentage of SA-β-gal-positive cells which were appeared as blue-stained cells.

Statistical analysis

Each experiment described above was replicated for 10 rats. All values were expressed as means ± standard deviations. The results were analyzed by student t-test. A *P*-value of <0.05 was considered to be statistically significant.

Results

Cell culture

At primary cultures, the cells from both adipose tissues appeared as small spindle-shaped cells. A few small round cells were also observed at both cultures (Figure 1A-B). Primary cultures reached 70-80% confluence in approximately 8-10 days. The cell growth tended to accelerate during the passages, so that epicardial adipose-derived stem cell subcultures reached confluence in 4-5 days while those of epididymal adipose-derived stem cells attained confluency in 6-7 days. After several passages both cultures were composed of a homogeneously fibroblastic cell monolayer (Figure 1C-D).

Differentiation

The stem cells derived from SVF of both epicardial and epididymal adipose tissue tended to differentiate into bone, cartilage and adipose

cell lineages. The differentiations were evident in specific staining and RT-PCR analysis. At osteogenic cultures, mineralized matrix was stained red following alizarin red staining (Figure 2A-B) and the expression of alkaline phosphatase and osteocalcin genes were detected by RT-PCR analysis (Figure 3).

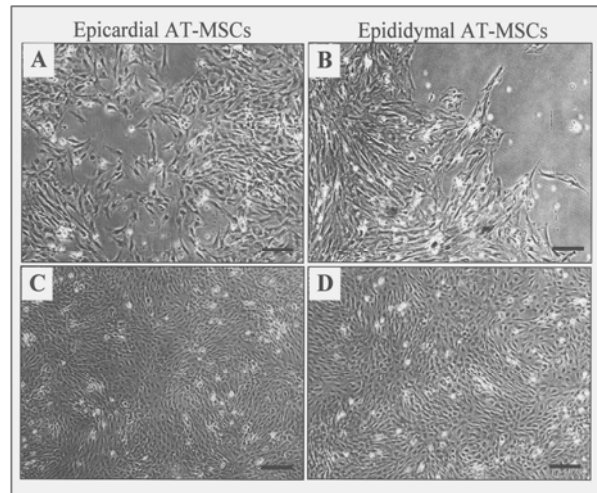


Figure 1. The cell culture prepared from stromal vascular fraction (SVF) of rat adipose tissue. At primary cultures, the majority of cells, either from epicardial (A, bar= 100 μm) or epididymal (B, bar= 100 μm) adipose tissues (AT), appeared to be small spindle-shaped cells. A few small round cells were also observed at both cultures. After a three round of passages, epicardial (C, bar= 200 μm) and epididymal (D, bar = 200 μm) derived cell culture were both composed of homogenous population of fibroblastic cells.

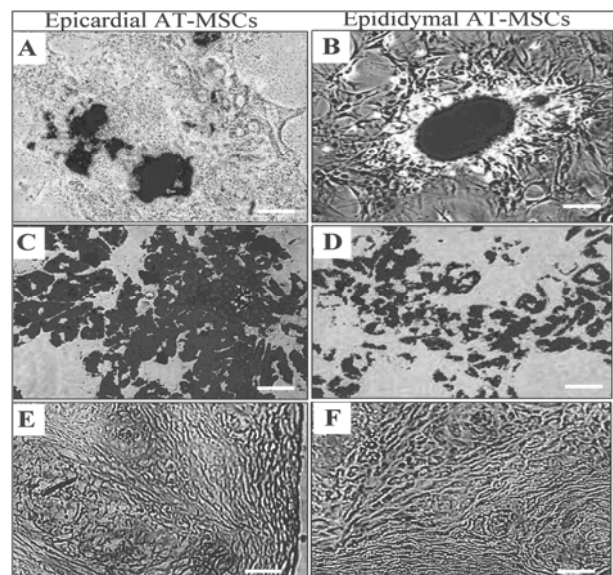


Figure 2. Differentiation potential of the rat stem cells isolated from epicardial and epididymal adipose tissues (AT). Osteogenic cultures stained with alizarin red. C-D) Adipogenic cultures stained with oil red. Chondrogenic cultures stained with alcian blue. The bar in all parts is equal to 100 μm.

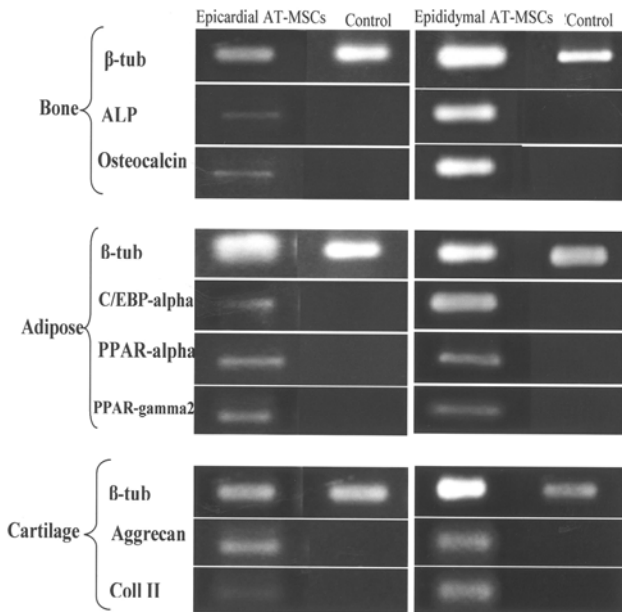


Figure 3. RT-PCR analysis for specific gene expression at osteogenic, adipogenic and chondrogenic differentiation cultures of the mesenchymal stem cells (MSCs) derived from epicardial and epididymal adipose tissue (AT).

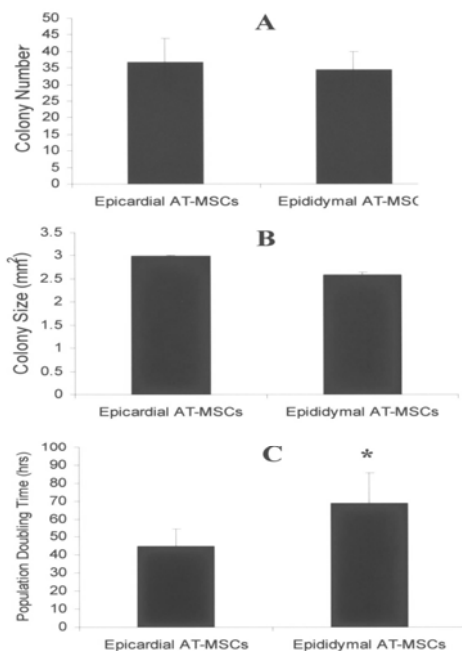


Figure 4. Proliferation capacity of adipose derived MSCs in culture. A) Epicardial AT-MSCs (Adipose tissue-mesenchymal stem cells) tended to produce more colonies than epididymal AT-MSCs did. B) The size of colonies generated at epicardial AT-MSCs culture were larger than those at epididymal AT-MSCs. C) In accordance with colonogenic results, the PDT was significantly shorter in epicardial AT-MSCs culture than that of epididymal AT-MSCs culture.* indicates a statistically significant difference ($P < 0.05$).

At adipogenic cultures, lipid droplet produced in cell cytoplasm was stained red upon oil red staining (Figure 2C-D) and the expression of C/EBP (CCAAT/enhancer binding protein)-alpha, PPAR (peroxisome proliferators activated receptor)-alpha and PPAR-gamma2 genes were confirmed by RT-PCR detection (Figure 3). At chondrogenic cultures, cartilage matrix was successfully stained with alcian blue (Figure 2E-F) and the expression of cartilage specific genes including aggrecan and collagen II was observed by RT-PCR data (Figure 3). This tripotent differentiation capacity indicated that both stem cells were originated from MSC population.

Colonogenic assays

According to our results epicardial adipose-derived stem cells produced 36.9 ± 7 colonies (Figure 4A) with average size of 2.98 ± 0.7 mm² (Figure 4B), while epididymal adipose-derived stem cells generated 34.62 ± 5.2 colonies (Figure 4A) with average size of 2.58 ± 0.21 mm² (Figure 4B). The differences were not statistically significant.

Population doubling time (PDT)

PDT value was 69 ± 16 hr for epididymal adipose-derived stem cells while this value was recorded as 45 ± 9.6 hr for epicardial adipose-derived stem cells. The difference was significant ($P < 0.05$).

Growth curve

According to the plotted curve, the cells in both culture started proliferating immediately after being plated. The culture reached plateau in approximately 5 days after initiation (Figure 5A).

SA- β -gal staining

According to the SA- β -gal staining results, the passage 1 and 2 culture of both groups exhibited no β -galactosidase positive cells. These cells were first observed at passage 3 accounting for about $22.4\% \pm 2.98$ of the cells in epicardial cultures and $35.7\% \pm 2.43$ of the cells in epididymal cultures. The values were increased proportional to the passage number reaching into $27\% \pm 0.97$ of the cells in epicardial cultures and $45.2\% \pm 1.75$ of the cells in epididymal cultures at

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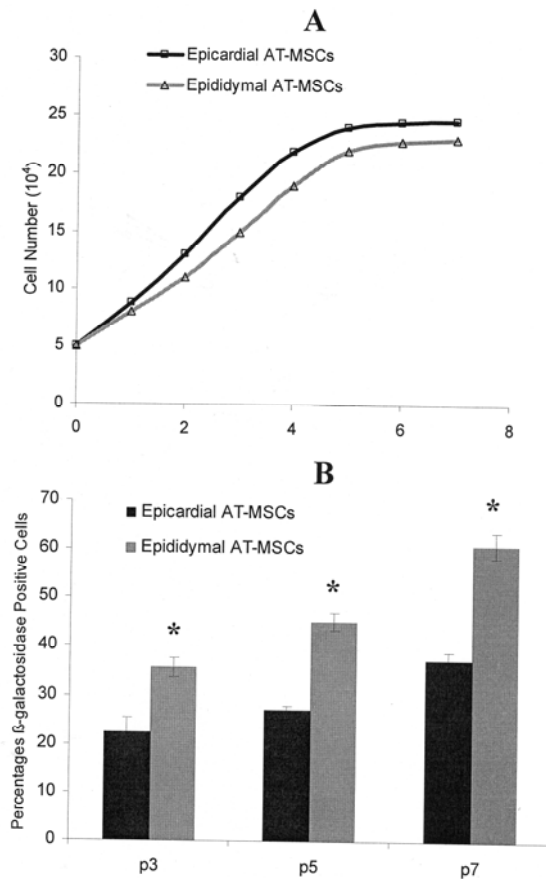


Figure 5. A) growth curve plotted for adipose MSCs. The cells, either epicardial or epididymal-MSCs, started proliferation immediately after being plated. B) The presence of senescent cells at culture. Senescent cells were first observed at passage 3 and their number was increased as the passage number advanced. The percentages of β-galactosidase positive cells were significantly lower in epicardial AT-MSCs culture as compared to that of epididymal AT-MSCs cultures. *indicates a statistically significant difference ($P < 0.05$).

passage 5; and 37.45%±1.5 of the cells in epicardial cultures and 61±2.71 of the cells in epididymal cultures at passage 7 (Figure 4 B). The differences were statistically significant ($P < 0.05$).

Discussion

In this study, SVF from epididymal and epicardial adipose depots were plated and their adherent cell population was culture-expanded through several successive passages. According to our findings, MSCs from different adipose depots were varying in terms of their expansion rate and the senescence in culture. In this regards Prunet-Marcassus *et al* (2006) have previously reported some

heterogeneity of the cells with respect to their surface markers and differentiation capabilities as well (17). Studies like this would help scientist understand and interpret the varying data reported by different labs studying the biological characteristics as well as regenerative capacity of the cells.

A similar investigation has previously been conducted by Schipper *et al* (2008) on comparing the adipose stem cells from 5 human different subcutaneous regions. They have concluded that differences in cell proliferations among the stem cells from different sites were related to the age of individual from which the stem cells were derived. In other words they did not find any difference of proliferative potential among the cells derived from individuals of similar age (21) while we found such differences in rats of similar age. This discrepancy in data would be explained in terms of different type of adipose tissue used in the two studies. In the present study we used adipose tissue from subcutaneous and internal regions whereas in above-mentioned study subcutaneous adipose tissues of five different regions have been used. This data emphasize on differences existed among the stem cells of subcutaneous fat compared to those from internally-located adipose tissue.

According to the previous researches SVF from collagenase digest of adipose tissue is heterogeneous with respect to its cellular composition having a variety of stem cells including MSCs, hematopoietic stem cells and non-stem cells such as endothelial cells, fibroblast, blood cells, preadipocyte and pericytes. To isolate and purify adipose stem cells from other cell types, one method would be using the extended plastic culture which has been used in majority of the related studies (10-12). Plating on plastic surface, the cells with anchorage dependent properties can survive and stay in culture while those growing in suspension (hematopoietic cells) cannot survive and would be eventually eliminated. As the culture period is extended by performing several round of passages, the cells with limited proliferation and life span are gradually lost (i.e. endothelial cells) while

those with an extensive self-renewal capacity would dominate the culture by propagation. In this study such strategy was used to purify MSCs cells from other cell types present in SVF.

In stem cell experiments the other challenge is the confirmation of stem cells identity of the isolated cells. In this case, the best method would be to evaluate the antigenic profile of isolated cells if there was an established specific profile for the given cells. In this regard, studies have indicated no specific surface marker for adipose-derived mesenchymal stem cells although a number of non specific epitopes have been reported (21-27). To overcome this problem MSCs committee of international society for cell therapy has proposed several criteria by which MSCs can be recognized. These are as follows: the cells must be plastic adherent and they must be capable of differentiating along three skeletal lineages of bone, cartilage and adipose cells (28). In this study, we used these criteria to recognize adipose derived stem cells as MSC population.

In the present study, the isolated cells were characterized in terms of their *in vitro* proliferation. Colonogenic assays are among those methods that frequently being employed to determine the cell proliferation potential (29), but this assay measures only the colony number while the colony size is ignored. For this reason the present investigation was taken into consideration the colony's size as well. The other indices indicating the cell proliferation rate is PDT (population doubling time) which is defined as the time by which the given cell population is doubled in number by undergoing proliferation. Growth curve being plotted for adipose-derived cells can offer valuable data concerning the growing cells *in vitro*. According to our data, epicardial adipose derived stem cells appeared to be growing faster than epididymal adipose tissue-derived stem cells. The origin of all of these differences is unknown for us. In this regard the type of adipose tissue would have some

impact. Epicardial adipose tissue is indeed categorized as brown adipose type, while the fat layer around epididymal tissue is of white adipose tissue type (30). This issue however needs further investigation.

Acid β -D-galactosidase as a hydrolase is 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. Recently, a pH 6 β -galactosidase activity has been reported, which was found specifically in senescent fibroblastic cultures (31) and referred to as senescence associated (SA)- β -galactosidase. Since then, SA- β -galactosidase assay was used to examine the senescence of variety of cells in culture (32, 33). In the present study, this method was used to determine the onset of replicative senescence of adipose tissue-derived MSCs. According to our findings, comparatively more percentage of senescent cells was present at the cultures derived from epididymal adipose tissue.

Conclusion

Taken together, this study indicates that some differences existed between stem cells harvested from adipose tissue of different anatomical sites. According to our findings, mesenchymal stem cells derived from rat epicardial adipose tissue displayed more *in vitro* proliferation rate than their counterparts in epididymal adipose tissue. Furthermore, it was indicated that less percentage of senescent cells occur at epicardial derived cell cultures compared to that of epididymal cell culture. Such data helps understand the varying results of those experiments dealing with adipose derived stem cells.

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