IJBMS

Original article

Ex vivo Expansion and Differentiation of Mesenchymal Stem Cells from Goat Bone Marrow

*[1]Mohamadreza Baghaban Eslaminejad, [1]Hamid Nazarian, [1]Fahimeh Falahi, [1]Leila Taghiyar, [2]Mohamad Taghi Daneshzadeh

Abstract

Objective(s)
Mesenchymal stem cells (MSCs) from large animals as goat which is genetically more closely related to human have rarely been gained attentions. The present study tried to isolate and characterize MSCs from goat bone marrow.
Materials and Methods
Fibroblastic cells appeared in goat marrow cell culture were expanded through several subcultures. Passaged-3 cells were then differentiated among the osteogenic, adipogenic and chondrogenic cell lineages to determine their MSC nature. Differentiations were determined by RT-PCR analysis of related gene expression. To identify the best culture conditions for propagation, passage-3 cells were plated either at varying cell densities or different fetal bovine serum (FBS) concentrations for a week, at the end of which the cultures were statistically compared with respect to the cell proliferation. In this study, we also determined goat MSC population doubling time (PDT) as the index of their in vitro expansion rate.
Results
Passage-3 fibroblastic cells tended to differentiate into skeletal cell lineages. This was evident in both specific staining as well as the specific gene expression profile. Moreover, there appeared to be more expansion when the cultures were initiated at 100 cells/cm^2 in a medium supplemented with 15% FBS. A relatively short PDT (24.94±2.67 hr) was a reflection of the goat MSC rapid rate of expansion.
Conclusion
Taken together, fibroblastic cells developed at goat marrow cell culture are able to differentiate into skeletal cell lineages. They undergo extensive proliferation when being plated at low cell density in 15% FBS concentration.

Keywords: Adipogenesis, Bovine serum, Cell seeding density, Chondrogenesis, Goat mesenchymal stem cells, Osteogenesis

1- Stem Cell Department, Cell Science Research Centre, Royan Institute, ACECR, Tehran, Iran
*Corresponding Author: Tell: +98-21-22413790; Fax: +98-21-22409314; email: eslami@royaninstitute.org
2-Royan Animal Facility, Karaj, Iran

Introduction

Mesenchymal stem cells (MSCs) possess two fundamental characteristics: the ability of extensive replication and the capacity of multilineage differentiation among bone, cartilage and adipose cell lineages. These cells have been reported to be present in bone marrow and several other tissues in adult (1). MSCs were first described by Friedenstein and co-workers who have reported the isolation of the cells from rat bone marrow tissue, using the cell plastic-adherent property (2). Since then, this property has been used to isolate and characterize MSCs from a number of species including dog, rabbit, pig, baboon and human (3-7). Having the ability of self-renewal for long-term along with multilineage differentiation potential have rendered MSCs as significant cellular materials for tissue regeneration applications (8).

Two important challenges associated with MSCs would be the topics of their safety and efficacy in use as therapeutic tool for tissue regeneration. These subjects need sufficient investigation in animal models, which in turn requires MSCs isolation and characterization, as the first step, from the interest animal. In this regards, most related investigation has so far been carried out in small animal as rodents (9). MSCs from large animals as goat which is more similar to human than rodents has rarely gained attention. MSCs biology in this animal has indeed remained to be fully investigated. In those research works associated with goat MSCs, the cells isolated from the animal marrow have mostly been used to establish 3D cultures in scaffolds without taking into account the cell *in vitro* characteristics as well as culture requirements. In this regards, Nair *et al* (2008) have manufactured a triphasic scaffolds consisting of hydroxyapatite, tricalcium phosphate and calcium silicate and evaluated the scaffold biocompatibility with goat MSCs 3D culture (10). In a similar investigation, Li and colleagues have used goat MSCs for biocompatibility test of newly manufactured hydrogel scaffolds (11). Williams *et al* have investigated the chondrogenic potential of goat MSCs within hydrogel *in vitro* (9). Dai *et al* have used goats as animal model to evaluate the regenerative capacity of goat MSCs-loaded scaffolds in bone large defects (12). Neither of these experiments has examined the multipotent differentiation capacity of the isolated cells which MSC committee of international society of cell therapy has announced it as defining criterion for MSCs (13). In the present study, this subject was examined.

Carrying out the differentiation analysis with RT-PCR, we noticed that a number of specific genes for goat bone, cartilage and adipose cell were not registered in gene database of NCBI. As a solution, we designed and used the primers for equine corresponding genes (which were available in gene database) to amplify the goat genes; and then the amplification product was extracted, sequenced and investigated whether or not they were similar to corresponding genes in close species to goat. All sequences were then registered in NCBI gene database. Accession numbers were assigned to the sequences which are currently available. Moreover, in this study, culture conditions for the maximum proliferation of goat MSCs were optimized.

Materials and Methods

Bone marrow cell culture

Four male adult goats of Najdi strain were used in this study. Before experiments, approval of the animal use was obtained from the Ethic Committee of Royan Institute. The animals were kept in the facility of Royan institute (Karaj, Iran) till become acclimatized to housing and diet. Under general anesthesia by Ketamine (22 mg/kg) and xylazine (0.2 mg/kg) IM injection, bone marrow aspirate (about 10 ml) were drawn from the tibia, collected into 50 ml tube containing 7500 unit heparin and shipped on the ice to cell culture facility of Royan Institute. Bone marrow aspirates were added to 5 ml DMEM medium (Dulbeccoo's modified eagle medium, Sigma, USA) supplemented with 100 IU/ml penicillin (Sigma, USA), 100 IU/ml streptomycin

(Sigma, USA), 10% FBS (Fetal Bovine Serum) and centrifuged at 300 g for 5 min. Supernatant was discarded, cell pellet resuspended in 1 ml DMEM medium and plated at 5×10^6 cells/cm^2 in 75-cm^2 culture flasks in an atmosphere of 5% CO$_2$ and 37 °C. About 3-4 days after culture initiation, culture medium was replaced with fresh DMEM and the flasks were incubated with a medium replacement of 2 times a week until the cultures became confluent. At this stage, cells were trypsinized and subcultured at 5×10^5 cells/cm^2. Cell passages were repeated up to the subculture 3 when sufficient cells were available to continue the experiments described below.

Differentiation potential

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

To induce osteogenic differentiation, confluenced passaged-3 cells were cultured in the 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 3 weeks (14-15). 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 min and then subjected to alizarine red solution for 2 min.

For adipogenesis, DMEM medium containing 100 nM dexamethasone (Sigma, USA) and 50 mg/ml indomethacin (Sigma, USA) was used to induce the differentiation in the confluent culture of passaged-3 cells (14, 15). Three weeks after culture initiation, the cells were fixed with 4% formalin at room temperature, washed by 70% ethanol and stained, using oil red solution in 99% isopropanol for 15 min.

To induce the cartilage differentiation, micro mass culture system was used. For this purpose, 2.5×10^5 passaged-3 cells were pelleted under 300 g for 5 min and cultured in a DMEM medium supplemented 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 (15,16). Three weeks after initiation of the culture, the pellets were removed and subjected to the following: fixing in 10% formalin; dehydrating in ascending concentrations of ethanol; clearing in xylene; embedding in paraffin wax and sectioning at 5 μm by microtome. The sections were then stained, using toluidine blue for 30 sec at room temperature.

RT-PCR analysis
Primers

Because of the lack of a number of specific gene sequences for goat osteocyte, chondrocyte and adipocyte (except for GAPDH and Osteocalcin), we designed the primers for equine bone, cartilage and adipose-specific genes which were available in NCBI gene Database and used them in order to amplify the genes in goat differentiated cultures. The bands of amplified fragments were recovered from the gel by QIA quick$^®$ Gel Extraction kit and then sequenced by Chromas Software version 2.31. BLAST results of these sequences, showed that there are many similarities between them and sequences related to the genes of other species close to goat. Therefore, it confirmed that the equine specific primers were able to amplify our interest fragments in goat genome. Sequenced gene fragments were registered in NCBI gene database and they were assigned the accession numbers. The similarity percentage of these sequences to the sequences of other species and also primer sequences used to amplify the interest genes are indicated in Table 1 and 2, respectively. The sequences that have been registered at NCBI gene database for goat are shown in Table 3.

Table 1. The similarity percentage of amplified sequences from ovine differentiated cultures, using equine primers compared with those of other species.

Gene name	Other species	Similarity percentage	Other species	Similarity percentage
Aggrecan	Bos taurus	97	Equus caballus	91
Collagen II	Bos taurus	98	Equus caballus	100
Peroxisome proliferator-activated receptor alpha (PPARA)	Bos taurus	93	Equus caballus	90
Peroxisome proliferator-activated receptor gamma (PPARG)	Bos taurus	99	Equus caballus	93
Lipoprotein lipase	Bos taurus	99	Equus caballus	90
Collagen 1	Bos taurus	97	Equus caballus	97

Table 2. Primers used in RT-PCR analysis, * indicates the genes that were not found in NCBI gene database. These genes were sequenced in this study and registered at NCBI gene database. Their accession numbers indicated in the left column are now available in the database.

	Accession number	Gene name	Primer sequence	A.T
1	FJ178373	Aggrecan*	F: 5' ttg gac ttt ggc aga ata cc 3' R: 5' ctt cca cca atg tcg tat cc 3'	59
2	FJ178374	Collagen II*	F: 5' gcg gag act act gga ttg R: 5' ttt ctt gtc ctt gct ctt gc 3'	58
3	FJ200435	peroxisome proliferator-activated receptor alpha (PPAR a)*	F: 5' agaacaaggaagcggaagtc 3' R: 5' atcccgtctttgttcatcac 3'	60
4	FJ178372	peroxisome proliferator-activated receptor gamma(PPAR g)	F: 5' aagagcagagcaaagaggtg 3' R: 5' gggcttcacattcaacaaac 3'	60
5	FJ200436	Collagen1*	F: 5' ccc aga aca tca cct acc ac 3' R: 5' gga ggg agt tta cag gaa gc 3'	58
6	FJ200437	LPL*	F: 5' tct ctt ggg ata cag cct tg 3' R: 5' atg ccc tac tgg ttt ctg 3'	59
7	XM001504101.1	Collagen type X	F: 5' act gag cga tac caa aca cc 3' R: 5' ggt cca ttt agt cct ctc tcc 3'	62
8	DQ152956.1	GAPDH	F: 5' gga gaa acc tgc caa gta tg 3' R: 5' tga gtg tcg ctg ttg aa gtc 3'	60
9	NM_001040009.1	Osteocalcin	F: 5' agc gag gtg gtg aag aga c 3' R: 5' gct cat cac agt cag ggt tg 3'	61
10	XM_001917620.1	Collagen type III	F: 5' ttg atg gtg cta ctt tga a 3' R: 5' aac atc ctc ctt caa cag c 3'	54

RT-PCR Procedure

Total RNA was isolated, using the RNX[TM] (-Plus) (RN7713C; CinnaGen Inc., Tehran, Iran). Before RT, a sample of the isolated RNA was treated with 1U/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; Fermentasm, Germany) and 1×reaction buffer with MgCl$_2$ for 30 min at 37 $^\circ$C. To inactivate the DNaseI, 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 Random hexamer as a primer designed for equine genes and a designed for equine genes 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 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 2 ml cDNA, 1×PCR buffer (AMS[TM]; CinnaGen Co., Tehran, Iran), 200 mM dNTPs, 0.5 mM of each antisense and sense primer, and 1U Taq DNA polymerase. PCR was performed, using Mastercycler Gradient 5331, Ependorf, Hamburg, Germany.

Table 3. The sequences of goat bone, cartilage and adipose genes that have been registered in NCBI gene database by the authors. The Accession numbers that have been devoted to these sequences are indicated in Table 2.

Aggrecan	ttggactttg gcagaatacc ttctgtaact ttaggaagtg ggcaagctcc agaagcaagt ggtcttcctt ctggatttag tggtgagtat tctggggtgg accttggaag tggcccatcc tctggccttc ctgatctaag tggacttcca tctgggttcc caactgtctc tctggtggat acgacattgg tggaaga
Collagen II	cggagactac tggatcgacc ccaaccaggg ctgcaccctg gatgccatga aggttttctg caacatggag actggcgaga cctgcgtcta ccccaacccg gccagcgtcc ccaagaagaa ctggtggagc agcaagagca aggacaagaaa
Collagen I	ggtgggaggt gacaggaagt cccagaacat cacctaccac tgcaagaaca gcgtggccta catggaccag cagaccggca acctcaagaa ggccctgctc ctccagggct ccaacgagat cgagattcgg gccgagggca acagccgctt cacctacagc gtcacctacg atggctgcac gagtcacacc ggagcctggg gcaagacagt gatcgaatac aaaaccacca agacctcccg cttgcccatc atcgatgtgg ctcccttgga cgttggcgcc ccagaccagg aattcggctt cgacatcggc tctgtctgct tcctgtaaac tccctcca
LPL	ttctcttggg atacagcctt ggggcccatg ctgctggtat tgcaggaagt ctgaccaata agaaggtcaa caggattacc ggcctagatc cggctggacc taacttcgag tatgcagaag ctccaagtcg cctttctccc gatgacgcgg attttgtaga tgttttacac acgttcacca gggggtcacc tggccgaagt atcggaatcc agaaaccagt agggcatt
PPARgamma	aagagcagag caaagaggtg gccatccgca tcttccaggg gtgtcagttt cgctccgtgg aagccgtgca ggagatcaca gagtacgcca agaatatccc cggctttgtg aaccttgact tgaacgacca agtaactctc ctaaaatacg gcgtgcacga gatcatttac acgatgctgg cctccttgat gaacaaggat ggggtcctca tatccgaggg ccaaggattc atgacaaggg agtttctaaa gagcctgcga aagccctttg gtgacttat ggagcccaag ttcgagtttg ctgtgaagtt caacgcactg gaattagatg acagcgactt agcaatattt atagctgtca ttattctcag tggagaccgc ccaggttttgt tgaatgtgaa gccc
PPAR alpha	Agaacaagga agcggaagtc cgcatcttcc actgctgcca gtgcacgtct gtggagaccg tcaccgagct cacggagttc gccaagtcca tcccagggtt tgcaagcttg gacttgaacg accaggtcac cctgctgaag tatggcgttt acgaagccat atttgcgatg ctgtcttctg tgatgaacaa agacgggata

Optimizing the culture condition for maximum proliferation

In this study, initiating cell seeding density and FBS concentration in medium were optimized for extensive proliferation of the cells. For this purpose, the isolated cells (passage-3) were plated in a 100-mm dish at varying densities of 10, 50, 100, 200 and 500 cell/cm^2 in DMEM, supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin and 10% fetal calf serum for a week. At the end of this period, the cells were lifted, counted and the fold increase number was calculated and compared statistically (One-way ANOVA). Likewise, the cultures of passage-3 cells were established with 100 cell/cm^2 in DMEM, supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin and such varying concentrations of FCS as 5%, 10%, 15% and 20% for one week. Subsequently, the fold increase number of the cells was calculated in each group and compared by means of One-way ANOVA.

CFU- F (colony forming unit- fibroblast) assay

Hundred cells of the passage-3 culture were plated in a 25-cm^2 plastic flask in DMEM, supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin and 10% fetal calf serum before they were incubated at 37 °C and 5% CO_2 for 6 days. At the end of the cultivation period the number of the clones was counted under an inverted microscope.

Population doubling time (PDT)

After the optimal cell seeding density and FBS concentrations were determined, the cells cultivated under these conditions and their PDT was calculated. For this purpose, passaged-3 cells were plated at 100 cells/cm^2 in a DMEM supplemented with 15% FBS and incubated for a period of 7 days, when the cultures terminated, the cells lifted and counted with hemocytometer. Using the following equation, PDT was determined. In this equation N is the initiating cell number, N_0 the harvesting cell number and CT stands for culture time.

$$\textbf{(PDT)} = \frac{CT}{Log\dfrac{N}{N_0} \times 3.31}$$

Results

Cell culture

The primary cultures of the goat bone marrow cells contained mainly fibroblastic cells as well as a few small round cells. The number of latter was reduced by performing subcultures, during which the fibroblastic MSCs were purified and became dominant in the culture (Figure 1).
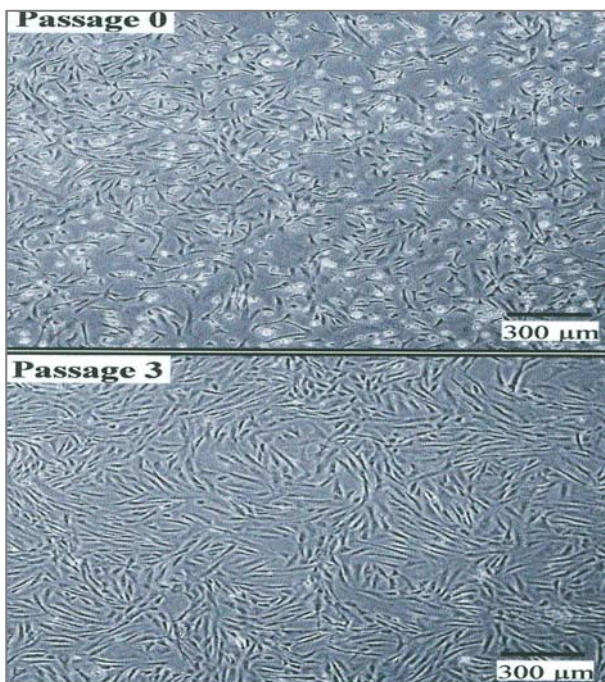
Figure 1. Goat marrow cell culture. At primaty culture, fibroblastic as well as small clear cells can be observed. The number of clear cells was decreased during the passages and fibroblastic cells became dominant in culture.

Multi-lineage differentiation

In osteoinductive cultures, in some areas, nodule-like structures were observed. Following alizarin red staining, red mineralizing areas of cultures appeared (Figure 2A). Differentiation was further demonstrated by RT-PCR analysis of the osteocytic markers. After a 3-week induction period, osteocalcin and collagen I and III mRNA levels were largely produced (Figure 2B).

Nodular structures were not observed at adipogenic culture. Small lipid droplets appeared within the cytoplasm of the cells first on days 3 to 4; they gradually occupied the whole cells by day 21. The lipid droplets turned into red when stained by the oil red staining method (Figure 2C). RT-PCR analysis was also indicative of the expression of PPAR alpha (Peroxisome proliferator's activated-receptor alpha), PPAR gamma (Peroxisome proliferator's activated-receptor gamma), LPL and LPL (lipoprotein lipase) genes (Figure 2D).

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 2E), and its marker molecules including aggrecan, Collagen II and Collagen X were examined by RT-PCR analysis (Figure 2F).
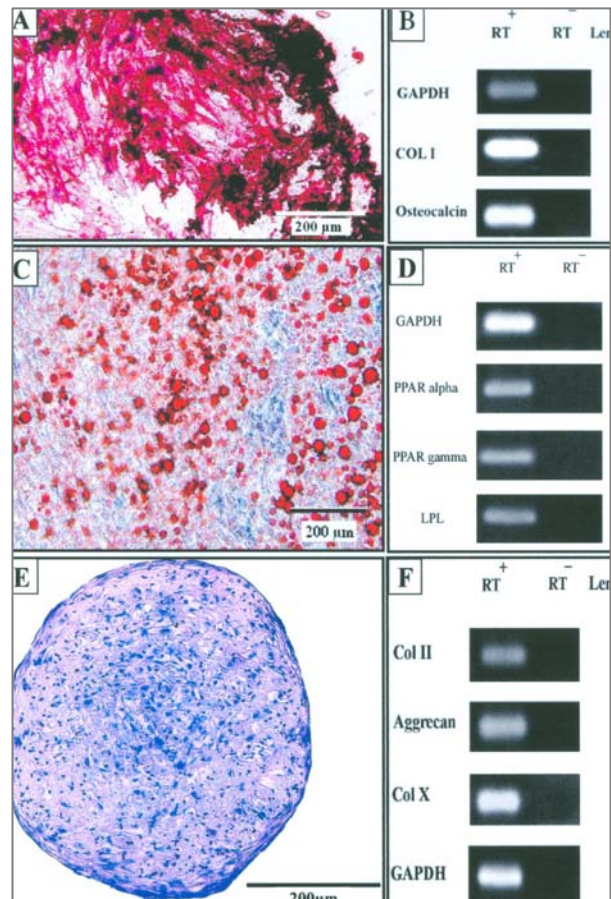

Figure 2. Multilineage differentiation potential of the isolated cells. Passage-3 cultures were easily differentiated among bone (A-B), cartilage (C-D) and adipose (E-F) cells. Differentiation into the skeletal cell lineage is considered to be as defining criterion for the cells.

Culture optimization
Seeding density

The maximum fold increase in cell number was achieved when the cells were plated at 100 cell/cm^2 (Figure 3, upper graph). At this point, their difference in comparison to the groups of 10, 50, 200 and 500 cell/cm^2 was statistically significant ($P<0.01$).
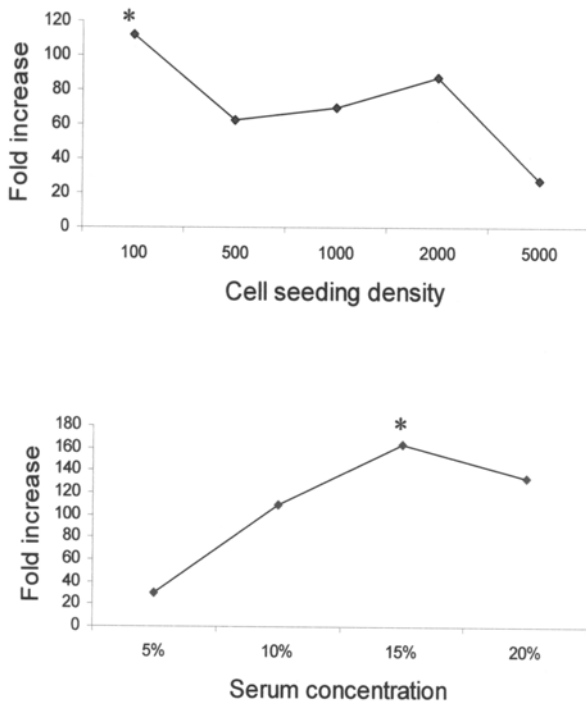
Figure 3. Optimization of the culture conditions for extensive proliferation of the cells. Passage-3 goat MSCs possessed maximum proliferation when they were plated at 100 cells/ cm^2 (upper graph) in a medium supplemented with 15% FBS (lower graph). * indicates a statistically higher value (*P*<0.05).

Serum concentration
The cells having been plated with 15% FBS had a significantly larger fold increase in cell number as compared to those having been plated with 5%, 10% and 20% FBS (Figure 3, lower graph).

CFU-F Assay Results
Almost 58.8 clones, each consisting of 20-70 cells, formed by per 100 cells plated in 25-cm^2 flasks for 6 days

Population doubling time (PDT)
Population of passaged-3 goat MSCs appeared to have a rather high expansion rate, so that their number was doubled in average of 24.94±2.67 hr when plated at 100 cells/cm^2 in a medium supplemented with 15% FBS.

Discussion
In this study, we culture-expanded the cells from goat marrow and evaluated them whether or not they were able to produce specialized lineages as bone, cartilage and adipose cells.

The studied cells appeared to be mesenchymal stem cells in nature because they successfully differentiated into the skeletal cell lineages. It should be mentioned that since investigators have reported studies of MSC, using different methods of isolation and expansion, and different approaches to characterizing the cells, thus it is increasingly difficult to compare and contrast study outcomes which hinders progress in the field. To address this issue mesenchymal and tissue stem cell committee of international society for cellular therapy has proposed the ability of multilineage differentiation as defining criterion for MSCs (13). In a few related investigations that have previously been conducted, these important defining characteristics have been ignored; hence, it was not clear that the cells having been used by those researchers were the MSC in nature. In the present study multilineage differentiation capacity of marrow-derived goat MSCs were examined and confirmed.

In addition to its significance as a defining criterion for MSCs, *in vitro* differentiation of MSCs would be of great clinical value. In cell therapy strategies; it is believed that transplanted cells should be of terminally differentiated lineages otherwise they would produce non specific cells in defect site (17-18). Therefore, *in vitro* differentiations of the cells into interest cells could be a necessary step prior to their transplantation. In this study the potential of goat MSC *in vitro* differentiation which has not gained attention at former investigations were explored.

The other point of the present study was the issue of the lack of a number of goat bones, cartilage and adipose cell-specific gene sequences in NCBI gene database. To solve this problem, we used the primers designed for equine corresponding genes and amplified the genes in differentiated cultures of goat MSC and then extracted the products and sequenced them. Our further examination by BLAST software indicated the similarity of the sequenced products with known sequence of the genes in several species. Therefore, we concluded that they would be the sequence that would be conserved in the species. The sequences were then registered at NCBI gene

database and were gained an accession number indicated in Table 1.

MSCs are being considered as valuable tool for regenerative medicine. The preclinical development of therapeutic procedures, using MSCs requires sufficient animal studies both for safety and for the investigation of efficacy of the cells. In this regard, the investigation of MSC in large animals would be of great importance since in contrast to rodents they are genetically more closely related to humans. Results from large animal models have been much more predictive with regard to human clinical trials than have results from *in vitro* assays or rodent models. Furthermore, one limitation associated with mice as a model is their limited life expectancies that preclude long-term follow-up (19, 20). Given the significance of large animal model in stem cell investigation, in this study MSCs from goat bone marrow were isolated and characterized. This has not been performed at related previous investigations.

MSCs occur at low frequencies in bone marrow samples (21). On the other hand they are needed in large numbers in most experimental set up. As a solution, MSCs should be expanded in culture before they are being used at any application. MSC *ex vivo* propagation is largely dependent on the presence of FBS in culture medium. According to former researches, MSCs that are developed in mediums containing FBS would be immunogenic and may transfer bovine pathogens upon transplantation (19). In spite of extensive attempt that has been made to substitute the bovine serum, less success has been achieved (21, 22). In this study, therefore, goat MSCs were cultivated in different concentrations of FBS to determine the least concentration with the highest mitogenic effect, which this was achieved by 15% FBS in the medium.

In the present study, the best cell density at culture initiation which resulted in extensive proliferation of the cells was determined. This is of utmost importance especially at cell therapy strategies where the rapid expansion of cells would be desired. According to our data, goat MSCs possessed the highest fold increase

when being cultivated at 100 cells/ cm^2. In a study by Bartmann *et al* it has been reported that there would be an inverse correlation between human MSC seeding density and their proliferation (23). Our results seem to be differed from those of Bartmann *et al* in that, although, the culture with the least cell concentration showed the highest proliferations, but in culture with 2000 cells/ cm^2 there appeared to be more proliferation than in culture with 500 and 1000 cells/cm^2. This discrepancy would be the results of different species from which the cells obtained (human versus goat). Such results, however, can be used to optimize the goat culture condition for maximum proliferation of the cells.

In spite of the considerable attempts that have been made to define the antigenic profile of human MSCs, no definitive single marker has so 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 (24-26). However, the identification of MSCs, especially those from animal source would be a difficult task owing to the lack of distinct specific marker. To overcome this problem, MSCs committee of the international society for cell therapy has proposed that MSCs from animal source should possess two characteristic criteria: a) they must be adherent on plastic surfaces of culture dish. b) They must be able to produce bone, cartilage and adipose cell lineages (13). Since the isolated cells of the present study were easily differentiated into bone, cartilage and adipose cells and also they were plastic adherent cells, we convinced that they were the MSCs described elsewhere.

Conclusion

Taken together, fibroblastic cells developed at marrow cell culture of goat are able to differentiate into skeletal linage as bone cartilage and adipose cells, hence, they are MSCs described elsewhere. According to our findings, the best culture condition for maximum proliferation of goat MSCs would

be achieved when the cells were plated at 100 cells/cm^2 in a medium containing 15% FBS. Furthermore, in this study, we identified the sequences of a number of goat genes which were not present in NCBI gene database. The genes were registered and gained an accession number.

Acknowledgment

References
1. Prochop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissue. Science 1997; 276:71-74.
2. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966;16:381-390.
3. Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 1999; 107:275–281.
4. Javazon EH, Colter DC, Schwarz EJ, Prockop DJ. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. Stem Cells 2001; 19:219–225.
5. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 1970; 3:393–403.
6. Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I. Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Biol 1994; 161:218–228.
7. Awad HA, Butler DL, Boivin GP, Smith FN, Malaviya P, Huibregtse B, *et al*. Autologous mesenchymal stem cell-mediated repair of tendon. Tissue Eng 1999; 5:267-277.
8. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells:characterization, differentiation and application in cell therapy. Mol Med 2004; 8:301-136.
9. Williams CG, Kim TK, Taboas A, Malik A, Manson P, Elisseeff J. *In vitro* chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. Tissue Eng 2003; 9:679-688.
10. Nair MB, Suresh Babu S, Varma HK, John A. A triphasic ceramic-coated porous hydroxyapatite for tissue engineering application. Acta Biomater 2008; 4:173-181.
11. Li Q, Wang J, Shahani S, Sun DD, Sharma B, Elisseeff JH, *et al*. Biodegradable and photocrosslinkable polyphosphoester hydrogel. Biomaterials 2006; 27:1027-10134.
12. Dai KR, Xu XL, Tang TT, Zhu ZA, Yu CF, Lou JR, *et al*. Repairing of goat tibial bone defects with BMP-2 gene-modified tissue-engineered bone. Calcif Tissue Int 2005; 77:55-61.
13. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The international societry for cellular therapy position statement. Cytotherapy 2006; 4:315-317.
14. Eslaminejad MB, Nikmahzar A, Thagiyar L, Nadri S, Massumi M. Murine mesenchymal stem cells isolated by low density primary culture system. Dev Growth Differ 2006; 48: 361-370.
15. Eslaminejed MB, Mirzadeh H, Mohamadi Y, Nickmahzar A. Bone differentiation of the marrow-derived mesenchymal stem cells using tricalcium phosphate/alginate/gelatin scaffolds. J Tissue Eng Regen Med 2007; 6:417-424.
16. Eslaminejad MB, Nadri S, Hosseini RH. Expression of Thy 1.2 surface antigen increases significantly during the murine MSCs cultivation period. Dev Growth Differ 2007; 49:351-364.
17. Pioletti D, Montjovent MC, Zambelli PY, Applegate L. Bone tissue engineering using fetal cell therapy. Swiss Med Wkly 2007; 136:557-560.
18. Weissman IL. Translating stem and progenitor cell biology to the clinic: barrier and opportunities. Science 2000; 287:1442-1446.
19. Donahue RE, Dunbar CE. An update on the use of non-human primate models for preclinical testing of gene therapy approaches targeting hemotopoiesis cells. Hum Gene Ther 2001; 12:607-617.
20. Horn PA, Thomasson BM, Wood BL, Andrews RG, Morris JC, Kiem HP. Distinct hematopoietic stem/progenitor cell populations are responsible for repopulating NOD/SCID mice compared with nonhuman primates. Blood 2003; 102:4329-4335.
21. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al*. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284:143-147.
22. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, *et al*. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 2004; 95:747-756.

23. Bartmann C, Rohde E, Schallmoser K, Pürstner P, Lanzer G, Linkesch W, *et al*. Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation. Proc Natl Acad Sci USA 1998; 95:10614-10619.

24. Kuçi S, Wessels JT, Bühring HJ, Schilbach K, Schumm M, Seitz G, *et al*. Identification of a novel class of human adherent CD34- stem cells that give rise to SCID-repopulating cells. Blood 2003; 3:869-876.

25. Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 2002; 7:783-791.

26. Gronthose S, Simmons PJ. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-derived condisions *in vitro*. Blood 1995; 85:929-940.