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The Proliferation and Differentiation Capacity of Bone Marrow Derived- Human Mesenchymal Stem Cells in Early and Late Doubling

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ABSTRACT

Bone marrow derived-Human Mesenchymal stem cells (hMSCs) are non-hematopoetic, stromal cells that demonstrate multilineage differentiation capacity and being capable to give rise to diverse tissues, including bone and cartilage. Due to this capability, hMSCs are currently evaluated for regenerative medicine, repopulating injured tissues and clinically ablated diseased tissues with healthy, terminally differentiated cells. Thus, for therapeutic applications, enough numbers of homogenous MSCs are required. In this study, the population doubling of bone marrow derived hMSCs was assessed in early and late passages. It was noted that in healthy cells, generally, the population doubling increases over time due to the slower rate of cell growth. Subsequently, the mesengenic multipotency of BM-MSCs for chondrogenesis, osteogenesis and adipogenesis was investigated in early and late doublings. According to our findings, the early passage hMSCs treated with the differentiation agents exhibited approximately 100, 48±10.33 and 28.6±6.62% osteocytes, chondrocytes and adipocytes, respectively. Whereas, the late passage hMSCs subjected to the differentiation agents only demonstrated the high degree of osteogenicity but they revealed neither chondrogenicity nor adipogenicity. Furthermore, the Expression of Oct4, Sox2 and Nanog genes in undifferentiated human BM-derived MSCs was studied. The result revealed the Oct4 is expressed at very low levels in early passage MSCs and disappeared at late passage however Nanog and Sox2 were almost undetected in MSCs. In conclusion, the proliferation rates and other properties of the cells gradually change during expansion and therefore, it is recommended to not expand hMSCs beyond four or five passages.

Key words: Human mesechymal stem cells, bone marrow, chondrogenesis, osteogenesis, adipogenesis, Oct4, Sox2, Nanog

INTRODUCTION

Stem cells are regarded as undifferentiated cells that can undergo both proliferation and differentiation (Fuchs and Segre, 2000; Mirzapour *et al*., 2011). Mesenchymal Stem Cells (MSCs) are non-hematopoetic, stromal cells that demonstrate the differentiation capacity to create various tissues including bone, cartilage and adipose tissue (Pountos and Giannoudis, 2005). MSCs can be found in bone marrow (Bianco *et al*., 2001), adipose tissue (Zuk *et al*., 2001), cord blood, amniotic fluid (In't Anker *et al*., 2003) and placental tissue.

MSCs have been detected as plastic adherent multipotent cells and referred by diverse terms such as colony-forming fibroblastic cells (Kuznetsov *et al*., 1997), Bone Marrow Stromal Sells (BMSC, Baddoo *et al*., 2003; Short *et al*., 2001; Sun *et al*., 2003; Peister *et al*., 2004), Multipotent Adult Progenitor Cells (MAPC, Reyes *et al*., 2001; Jiang *et al*., 2002) and Marrow Isolated Adult Multilineage Inducible Cells (MIAMI, D'Ippolito *et al*., 2004).

The employment of MSCs in the tissue regeneration has been gaining great interest as therapeutic agents especially in bone graft systems (Joseph *et al*., 2011). Moreover, these cells are capable to treat a wide range of diseases, including spinal cord injury (Hofstetter *et al*., 2002) and myocardial infarction (Shake *et al*., 2002; Toma *et al*., 2002). There are various methods reported in order to optimize the expansion rate. It is of paramount importance to recognize that no single approach used to culture-expand and select for MSCs, is regarded as a standard in the field (Phinney and Prockop, 2007).

Thus, for therapeutic applications, enough numbers of homogenous MSCs are required. MSCs derived cells that were cultured for prolonged periods may maintain their multi-lineage differentiation or result in transformed cells (Rosland *et al*., 2009). In this study, the mesengenic multipotency of bone marrow derived human MSCs for chondrogenesis, osteogenesis and adipogenesis was examined in early and late passages. It is aimed at evaluation of multilineage activity of long-term hMSCs. We also investigated the Expression of oct 4, sox2 and Nanog genes in undifferentiated human BM-derived MSCs, since these three transcription factors have essential roles in early development and are required for the propagation of undifferentiated embryonic stem (ES) cells in culture (Boyer *et al*., 2005).

MATERIALS AND METHODS

Isolation and culture of bone marrow-derived hMSC: After obtaining written informed consent, Stromal cells were obtained from iliac crest marrow aspirates from healthy donors and cultured according to previously reported protocol by Wang *et al*. (2005). Briefly, mononucleated cells (MNC) were separated by Ficoll-Hypaque density gradient (1.0779/0.001 g mL^{-1}; Sigma-Aldrich Inc., USA) and centrifuged at 2000 rpm for 30 min. Low-density MNC from the gradient interface were collected and washed three times with PBS (GIBCO-BRL, USA) with 1% FBS (Equitech-Bio Inc., USA). MNC were seeded in T162 cm^2 cell culture flasks (Corning Inc., USA) at an initial density of 5×10^6 cells mL^{-1} in 50 mL alpha-modification of Eagle's medium (a-MEM; Mediatech Inc., USA) supplemented with 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin (GIBCO-BRL) (Pen/Strep) and 20% FBS and incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO 2. After 72 h the supernatant and nonadherent cells were removed and the adherent layer cultured until it reached approximately 90% confluence. Cells were then harvested with 0.05% trypsin-EDTA solution (GIBCO-BRL) for 5 min at 37°C and subsequently re-plated 1:3 as passage 1 (P1). The culture was passaged when it reached 60 to 80% confluency, as described above. The culture medium was changed every 3 days.

Morphological changes were examined microscopically every day. After 10 doublings, cells were harvested to examine their multi-lineage potential. Further, cells from 10th doublings were kept in culture to proliferate up to 30 doublings and then collected for histological examination. This was repeated in three different sets.

The population doubling of cultured MSCs was calculated at every passage according to the equation, $\log_2$ (the number of harvested cells/the number of seeded cells).

Expression study of Oct4, Sox2 and Nanog genes in undifferentiated BM-MSCs by reverse transcriptase PCR: Total RNA was isolated from cultured cells using the TRIsoln kit (GeNei). cDNA synthesis was done with a cDNA kit (GeNei) according to the manufacturer's protocol. Total RNA was extracted from undifferentiated bone marrow derived MSCs cell suspension (in early and late passages) using 1 mL of TRIsoln as the cell denaturing agent. cDNA was synthesized from 4 μg of RNA, 1 μL of oligo(dt) primers made up to 10 μL with nuclease free water and placed at 65°C for 10 min in dry bath and then at room temperature for 2 min to remove any secondary structures. Then 1 μL of RNase inhibitor, 1 μL of 100 Mm DTT, 5 μL of 5X M-MuLV RT buffer, 2 μL of 30 Mm dNTP mix, 1 μL Reverse Transcriptase and 1 μL of Nuclease free water were added and incubated at 37°C for 1 h.

The PCR reactions were performed in volumes of 50 μL containing 5 μL of cDNA, 5 μL of each of forward and reverse primers, 5 μL of 10X PCR Buffer (GeNei, Germany), 1 μL of 30 mM dNTP mix (GeNei, Germany), 1U Taq DNA polymerase (GeNei, Germany), Nucleases free water (GeNei, Germany) was used to bring the reaction mixture to 50 μL. Polymerase chain reactions were performed at 94°C for 2 min, 30 cycles 94°C for 45 s, 52°C for 30 s and 72°C for 1 min and 72°C for 5 min.

The PCR products were analyzed using 2% agarose gel electrophoresis and visualized using ethidium bromide staining. The following oligonucleotide sequences were used: Oct-4, forward primer 5'-TGTCCGCCCGCATACGAGTTC-3' and reverse primer 5'-CAGGGGCCGCAGCTTACACAT-3'; Sox2 forward primer 5'- CGAGATAAACATGGCAATCAAATG-3' and reverse primer 5'- AACGTTTGCCTTAAACAAGACCAC-3'; Nanog, forward primer 5'-ATGAAGTGCAAGCGGTGGCAGAAA-3' and reverse primer 5'- CCTGGTG GAGTCACAGAGTAGTTC-3'.

Multilineage differentiation potential of human BM-MSC: The potential of the isolated cells to differentiate into osteogenic, adipogenic and chondrogenic lineages was examined according to the manufacturer's instructions. Adipogenesis was induced by culturing in Adipogenic Differentiation Medium (GIBCO). Osteogenesis was induced by culturing in osteogenic differentiation medium DMEM with 10% FBS, 1% ABAM (Antibiotic and Antimycotic solution), 10 mM β-glycerphosphate, 0.25 mM absorbic acid and 100 nM dexamethasone. For chondrogenic differentiation, MSC cell pellets were cultured in chondrogenic differentiation medium, which consisted of high-glucose DMEM supplemented with 500 ngmL BMP-6 (RandD system); 10 ng mL^{-1} transforming growth factor h3; 10^{-7} mol L^{-1} dexamethasone; 50 μg mL^{-1} ascorbate 2-phosphate; 40 μg mL^{-1} proline; 100 μg mL^{-1} pyruvate; and 50 mg mL^{-1} insulin, transferrin and selenium +premix (Becton Dickinson; 6.25 Ag mL^{-1} insulin, 6.25 Ag mL^{-1} transferrin, 6.25 ng mL^{-1} selenous acid, 1.25 mg mL^{-1} bovine serum albumin and 5.35 mg mL^{-1} linoleic acid). The medium was replaced every 2 to 3 d for 21 days (Sekiya *et al*., 2001).

The extent of adipogenic induction was verified by fixation in 10% buffered formalin and staining of lipid vacuoles in 0.5% Oil-Red-O. Osteocyte induction was confirmed by 2% Alizarin Red S to demonstrate mineralized calcium deposit (Williams *et al*., 2008). chondrogenic induction was verified by staining with alcian blue to detect Proteoglycans (PGs) in the extracellular matrix.

RESULTS

Determination of Population Doubling Time (PDT): The culture was passaged when it reached 60 to 80% confluency. Bone marrow derived mesenchymal stem cells with spindle shaped morphology were observed. The population doubling time was measured at every passage, as

Table 1: Determination of the population doubling time at early and late passages

Initial No. of cells	Obtained cell No.	No. of days in culture	Population doublings	Passage No.	
Set 1					
0.1	1	10	3	1	Approximate doubling
1	8	10	3.33	2	2 million frozen
6	10	8	10.86	3	4 million frozen
		Total No. of doublings	17.19		
6	11	11	12.85	4	7 million frozen
4	12	16	10.09	5	8 million frozen
4	12	12	7.571	6	6 million frozen
		Total No. of doublings	30.511		
			48.411		
Set 2					
0.1	1	10	3	1	Approximate doubling
1	10	10	3.01	2	6 million frozen
4	12	9	5.679	3	6 million frozen
		Total No. of doublings	11.689		
6	12	8	8	4	8 million frozen
4	13	15	8.821	5	9 million sforzen
4	11	12	8.222	6	6 million frozen
		Total No. of doublings	25.043		
			36.732		
Set 3					
0.1	1	10	3	1	Approximate doubling
1	12	10	2.789	2	6 million frozen
6	12	4	4	3	6 million frozen
		Total No. of doublings	9.789		
6	11	6	6.861	4	7 million frozen
4	13	15	8.821	5	9 million sforzen
4	12	12	7.571	6	6 million frozen
		Total No. of doublings	23.253		
			33.042		

shown in Table 1. In healthy cells, generally, the population doubling increases over time due to the slower rate of cell growth, depicted in Fig. 1a-c.

Differentiation Capacity of BM-MSCs in early and late doubling: According to our findings in three sets, the early passage hMSCs treated with the differentiation agents demonstrate approximately 100, 48±10.33 and 28.6±6.62% osteocytes, chondrocytes and adipocytes, respectively as shown in Fig. 2. Whereas, the late passage hMSCs subjected to the differentiation agents only demonstrated the high degree of osteogenicity but they revealed neither chondrogenicity nor adipogenicity, illustrated in Fig. 3. Unlike other stem cell populations such as human embryonic stem cells, most human MSCs in culture display a limited expansion potential *in vitro* (i.e., 5-10 passages) and decreased differentiation potential with increased culture.

Examination of Oct4, Sox2 and Nanog expression in undifferentiated human BM-derived MSCs in early and late passage: Expression of oct4, sox2 and Nanog genes in

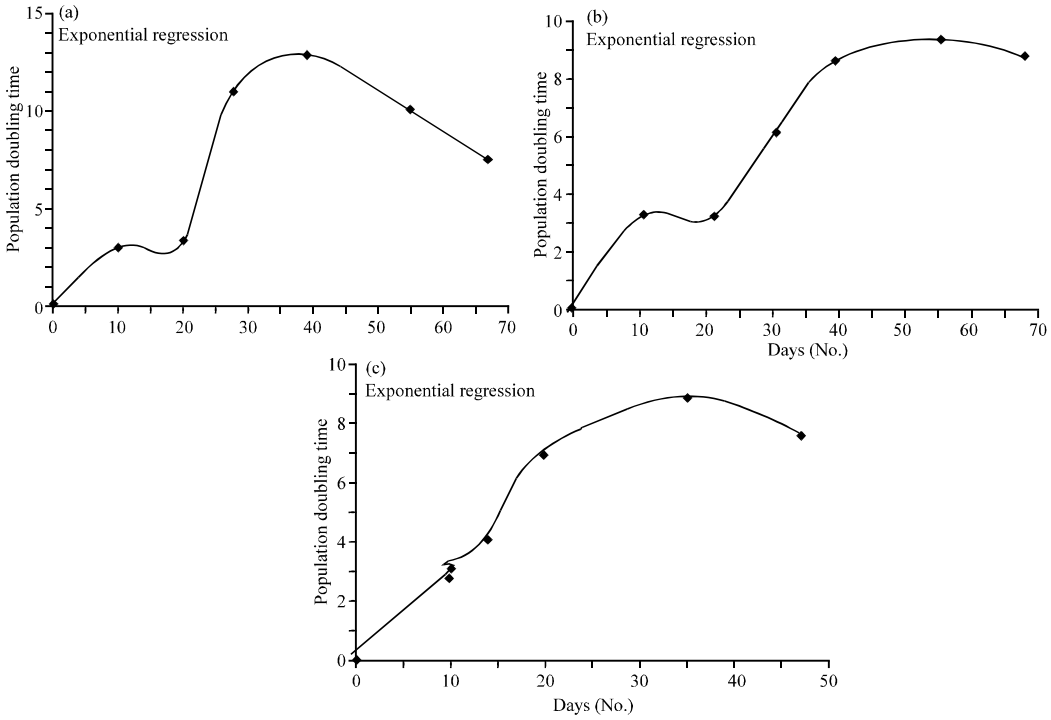

Fig. 1(a-c): *In vitro* population doubling time (PDT) of human bone marrow derived MSCs cultures in three sets. (a) Set 1 (b) Set 2 and (c) Set 3

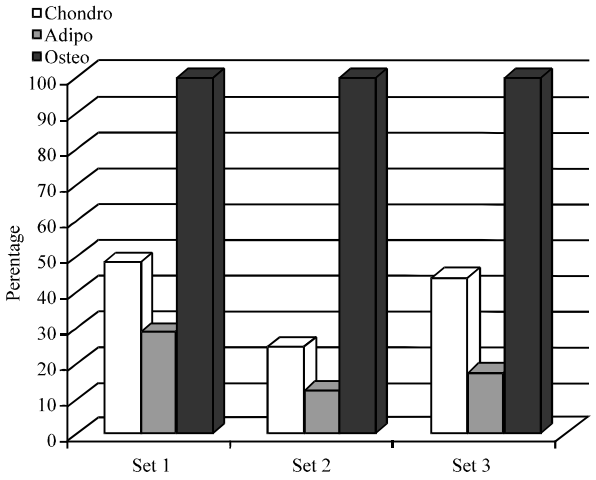

Fig. 2: Percentage of multilineage differentiation capacity of human bone marrow derived MSCs in each set. There is no considerable variation among the sets

bone marrow derived stromal cells was investigated by reverse transcription PCR. As, to Fig. 4, the Oct4 is expressed at very low levels in early passage MSCs and disappears at late passage whereas Nanog and Sox2 are almost undetected in MSCs even at early passage.

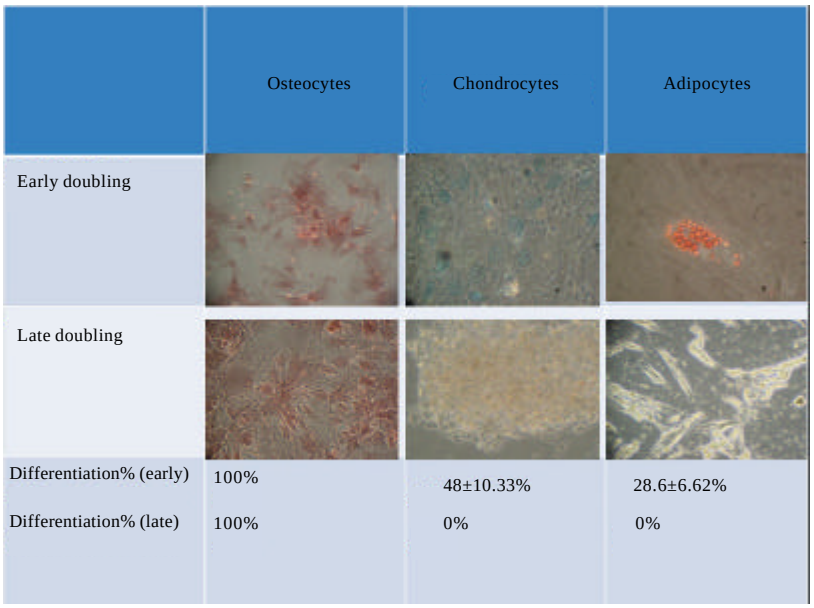

Fig. 3: Multi lineage differentiation of BM-derived MSCs into osteogenesis, chondrogenesis and adipogenesis in early and late doublings

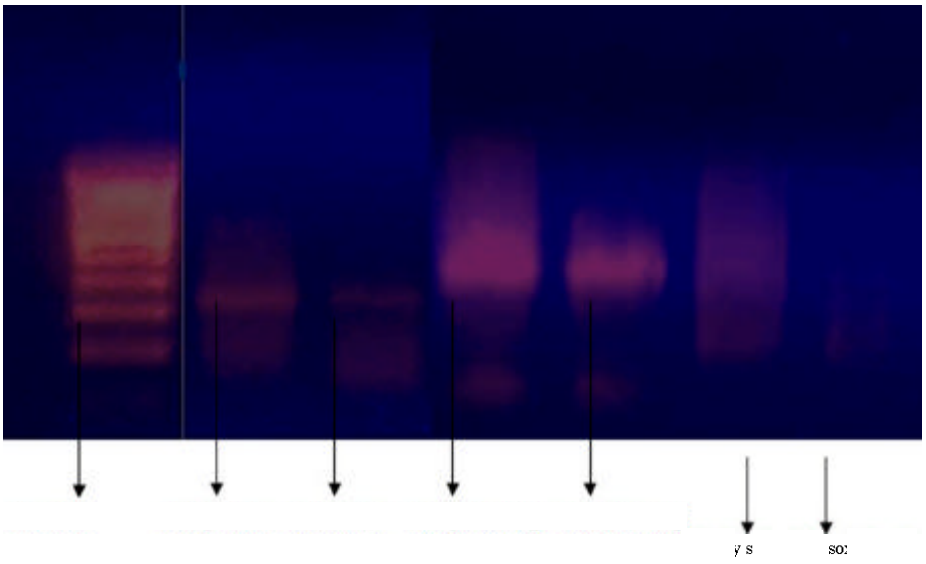

Fig. 4: Characterization of undifferentiated BM-derived MSCs based on the expression of Oct4, Nanog and Sox2 by reverse transcription PCR

DISCUSSION

Culture expansion and multipotential differentiation of MSCs: *In vitro*, human bone marrow derived MSCs can be expanded and be identified easily as they tend to adhere to culture plastic containers. They can rapidly proliferate and exhibit colony forming units. Expanded MSCs

could be guided to differentiate into multilineage pathways such as osteocytes, chondrocytes through specific media containing growth factors or other substances (Jones *et al*., 2002). Moreover, the differentiation of MSCs is chiefly directed by signals from culture conditions or the microenvironment *in vivo*. In most cases the signals that drive differentiation *in vivo* remain unidentified and therefore cannot be reproduced *in vitro*.

Furthermore, to collect adequate cell numbers for clinical approaches, extensive *ex-vivo* expansion is essential. However human MSCs lose their differentiation potential as they go through continuous passaging. This is virtually in agreement with our findings as also described by Digirolamo *et al*. (1999), Muraglia *et al*. (2000) and Pittenger *et al*. (2000). Nevertheless, Choi *et al*. (2008) showed that Periosteum-Derived Progenitor Cells (PDPCs) are able to propagate up to P15 and also maintain their differentiation capacity. Therefore, PDPCs can be used for autologous transplantation for patients older than 55.

Gregory *et al*. (2005) revealed that Adult bone Marrow Stem/progenitor Cells (MSCs) display varied gene expression patterns among different donors with applying the same isolation method. These variations may be extended through serial passages and ultimately resulting in senescence. Currently, there is a considerable controversy over the bio-safety of human MSCs. Several groups have reported no spontaneous transformation of hMSCs after long term *in vitro* culture (Bernardo *et al*., 2007; Meza-Zepeda *et al*., 2008), in contrast to other studies, indicating the spontaneous transformation of hMSC derived from adipose tissue (Rosland *et al*., 2009) or bone marrow (Wang *et al*., 2005). In order to clarify this argumentative issue, it is advised to verify all the involved cell lines using DNA fingerprinting due to the possibility of cross-contamination. Additionally, countless variables are likely to contribute to the dissimilarities in these observations. One is the differences in the properties of MSCs prepared in different laboratories, frequently with the investigators being unaware of the differences. Therefore, although Mesenchymal Adult Progenitor Cells (MAPCs) isolated form bone marrow and other MSC-derived cells that were cultured for protracted periods may display broad plasticity, it is not obvious whether their unique properties suggest that of stem/progenitor cells resident *in vivo*. Additionally, cells that acquire enhanced plasticity due to genetic alterations would be ruled out cell-based therapies.

Stemness of hBM-Derived MSCs: The OCT4, SOX2 and NANOG are considered core transcription factors in early embryo development and pluripotency maintenance in embryonic stem cells and they also display autologous feedback in a large regulatory circuit (Chen and Daley, 2008). There are conflicting reports concerning the role of these factors in adult stem cells. On one hand, for instance, Liu *et al*. (2009) stated that Nanog or Oct4 involved in cell proliferation and colony formation of MSCs, or Go *et al*. (2008) proved that forced expression of Sox2 or Nanog in human bone marrow derived mesenchymal stem cells sustains their expansion and differentiation capabilities but on the other, some scientists showed evidence to the contrary, for example, Lengner *et al*. (2008) reveals Oct4 is unnecessary for both self-renewal and maintenance of somatic stem cells in the adult mammal since the methods applied to detect oct4 and the interpretation of resulting data are possibly flawed. Moreover, Zangrossi *et al*. (2007) defy Oct-4 expression in various adult stem cells regarded as a real marker of stemness. Thus, the postnatal role of the transcription factors OCT4, SOX2 and NANOG is still not obvious and comprehensive investigations are required to precisely determine the stemness markers for MSCs.

CONCLUSION

The proliferation rates and multilineage differentiation capacity of BM-derived MSCs gradually change during expansion, hence, it is recommended to not culture-expand hMSCs beyond four or five passages for any stem cell based therapy.

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