Research Paper

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Characterization of Adipogenic Differentiation of Mesenchymal Stem Cell Derived from Mice Bone Marrow

Biochemistry

KEYWORDS : bone marrow derived stem cells (BMSCs), adipogenic, differentiation, PPAR-γ, C57BL/6 mice

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ABSTRACT

Mesenchymal stem cells (MSCs) isolated from bone marrow (BM) possess the capacity for self-renewal and the potential for multilineage differentiation, in the present study we characterized the adipogenic differentiation of mesenchymal stem cells derived from C57BL/6 mice bone marrow. This is characterized by gene expression in different days (3, 8, 10, 14 days) during the process (C/EBP, PPAR-γ, LPL, FABP4, and B-Actin) which showed different stages of differentiation, although by stained the cells with Oil Red O stain which is specific for adipocyte and read absorbance for stained cells, so these results support that bone marrow from C57BL/6 give high yield of MSCs and show that there are specific genes for adipogenic differentiation which play vital role in differentiation.

Introduction

Reports of successful use of bone marrow derived mesenchymal stem cells (BMSC) in tissue engineering applications and disease treatments (Slynarski etal, 2006), in addition to concerns about the use of embryonic stem cells, have stimulated increased interest in the use of adult stem cells for therapeutic purposes. Interestingly, among adult stem cells, the mesenchymal stem cells (MSC) are featured with several therapeutic properties, which make them excellent candidates for tissue replacement therapies. The MSC are able to differentiate into multiple cell lineages (Kode etal, 2009), secrete several factors (growth factors and cytokines) with important functions in tissue regeneration, are immune privileged (Le Blanc *etal*, 2003), and secrete immunomodulatory factors (Tse *etal*, 2003).

Materials and methods

Isolation, culture, and expansion of mesenchymal stem cells

Bone marrow was collected from 6-week-old C57BL/6 mice that were killed by cervical dislocation. Their femurs and tibiae were carefully cleaned of adherent soft tissue and bone marrow harvested by flushing with DMED supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin solution. The BM extracted was placed in 75 Cm^2 tissue culture flasks (Mcfarlin *etal*., 2006).

Cell culture

After 1 day, nonadherent cells were removed by two to three washes with PBS and adherent cells further cultured in complete medium. The medium was changed every 3 days until the monolayer of adherent cells reach 70-80% confluence. Trypsinization was made for cell splitting by Trypsin-EDTA solution (0. 25%, lonza, USA) for passage 1 .Number of cells was evaluated by Homocytometer and cellular viability by the Trypan Blue exclusion test. Each 250-300 × 10^3 cells were inoculated in 75 Cm^2 culture flask that were incubated at 37°C and 5% CO^2. Cell cultivation was maintained up to the 3rd passage (Mcfarlin *etal*., 2006).

Adipogenic differentiation

At passage 3 BMSCs were harvested by Trypsin digestion as described above, cells were counted and seeded at a density of ($5x10^4$/well) in a 6-well culture plate then at 80-90% confluent differentiation media was added: StemXVivo Osteogenic/Adipogenic Base Media (R&D Systems, Catalog # CCM007) and 1% Adipogenic Supplement (R&D Systems, Catalog # CCM011) and 1% Penicillin-Streptomycin. Other wells were taken for negative control and cultured in complete media; the media was changed twice a week for two weeks. The expressions of peroxisome proliferator activated receptor-γ (PPAR-γ), CCAAT/enhancer binding protein β(C/EBPβ), fatty acid binding protein 4 (FABP4), lipoprotein lipase (LPL) and Beta actin (β-actin) were analyzed at 3, 8, 10 and 14 days after induction by RT-PCR. Adipogenic differentiation was confirmed by the formation of neutral lipid-vacuoles stainable with Oil Red O.

RT-PCR

Total RNA of BMSCs was extracted with GF-1 total RNA extraction kit (Vivantis, Malaysia). cDNA synthesis was performed using total RNA (1 µg) as a template using maxima first strand cDNA synthesis kit (Thermo scientific, USA). PCR amplification was performed using Ex Taq master mix (Takara Bio). The primer sets used for RT-PCR were the same as those used for real-time RT-PCR. PCR amplification was performed with an initial incubation at 94°C for 5 min, followed by 40 cycles at 94°C for 45 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 7 min. The products were electrophoresed on 2% (w/v) agarose gels and stained with ethidium bromide. PCR primers were as follows (5'-3'): PPAR-γ: FW ACATAAAGTCCTTCCCGCTG, REV GGAATTCATGTCGTAGATGACAAA; C/EBPβ: FW TTCCTCTCCGACCTCTTCG, REV GGCCGAGGCTCACGTAAC; FABP4: FW GCGTGGAATTCGATGAAATCA, REV CCCGCCATCTAGGGTTATGA; LPL: FW GTGGCCGAGAGCGAGAAC, REV AAGAAGGAGTAGGTTTTATTTGTGGAA; B-actin: FW CAGGATTCCATACCCAAGAAG, REV AACCCTAAGGCCAACCGTG (Nakamura *etal*. 2003).

Visualization of Oil Red O stained adipocytes and quantification of Oil Red O

Cells were washed three times with PBS and then fixed for15 min with 10% formaldehyde. Oil Red O (300 mg in 100 ml isopropanol) was diluted with water (3:2) filtered through filter paper and incubated with the fixed cell for 5 min at room temperature. Cells were washed with water and the stained fat droplets in the cells were visualized by light microscopy and photographed. Dye was extracted by isopropanol incubation for 15 min at room temperature. Quantitative assessment was obtained by absorbance of the extracted dye at 500 nm (Kim *etal*., 2009).

Results

Cell culture

Attachment of spindle-shaped cells to tissue culture flask was observed after 1 day of culture BMSCs. After 7 days, spindle-shaped cells reached 80% confluency. Morphology of cells changed gradually with passage number. Cells become more flat-shape with increasing in passage number. BMSCs show ability to form multilayer after confluent. (Fig. 1).

Adipogenic differentiation

The morphology of BMSCs began to change after addition of differentiation media, (**Figure 2)**. Adipogenic differentiation was

demonstrated by the accumulation of neutral lipid vacuoles indicated by the Oil Red O stain (**Figure 3A**). No red staining was detected in control groups (**figure 3B**). The expressions of PPAR-γ, C/EBPβ, LPL, FABP4 and β-actin were analyzed at 3, 8, 10 and 14 days after induction by RT-PCR. PPAR-γ, a lipocyte-specific transcription factor, and LPL, a lipid exchange enzyme, were upregulated during adipogenesis. The basic expressions of C/EBPβ and LPL were initially detected in AT-MSCs and their expression levels reached a peak at day 3 after induction while FABP4 reached a peak at days 10 after induction. In order to quantify the ratio of lipogenic differentiation, additional slides stained with Oil Red O 14 days after induction were prepared for densitometric analysis.

Reverse Transcriptase-PCR

The four genes were upregulated and down regulated during the differentiation process. PPAR-γ gene was initially detected in BMSCs and their expression level reached a peak at day 8 after induction and its level became upregulated during the adipogenic process. C/EBPβ gene was initially detected in BMSCs and their expression level reached a peak at day 3 after induction and began to disappear at day 8. LPL gene was initially detected in BMSCs and their expression level reached a peak at day 3 after induction and its level remain in the same level during the whole process. FABP4 gene was initially detected in ATSCs and their expression level reached a peak at day 10 after induction. B-actin gene as a reference gene detected during all stages. The gel electrophoresis showed differences in gene expression (**figure 4**), the intensity of band detected by gel documentation system (biorad) (**figure 5, 6**).

Detection of lipid droplets after adipogenic induction

Adipogenic differentiation was characterized by the accumulation of neutral lipid vacuoles indicated by the Oil Red O stain, while no red staining was detected in control group. Optical density for extracted dye from BMSCs differentiated cells is 0.23±0.001. vs. control 0.1 (**figure 7**)

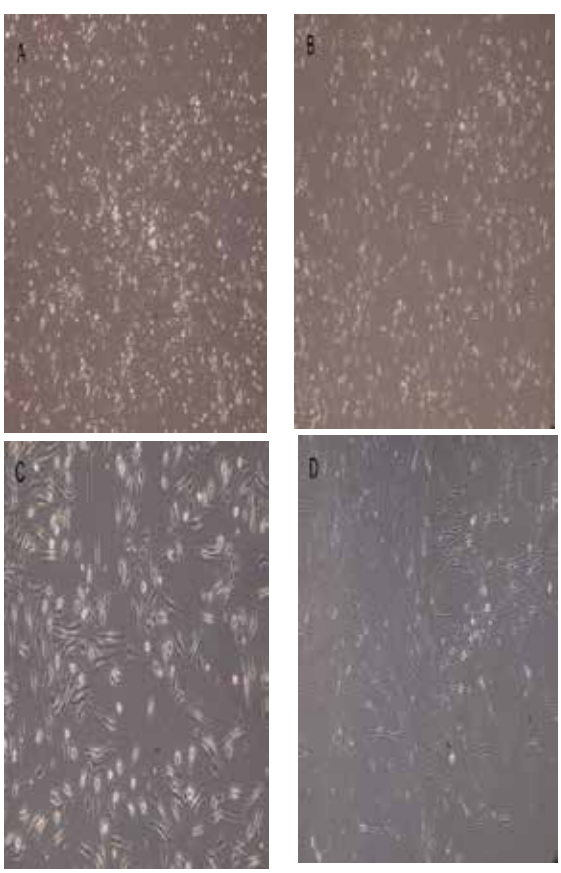


Figure 1. (A)Image of mice BMSC after isolation Scale bar=10 µm.(B) Image of BMSC in passage 1 Scale bar=20 µm.(C) Image of BMSC in passage 2 Scale bar=100 µm. (D) Image of BMSC in passage 3 Scale bar=100 µm

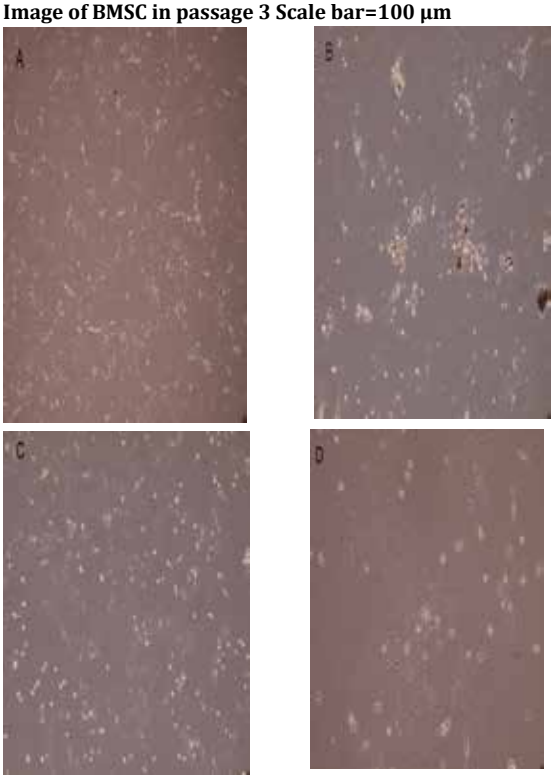


Figure 2. (A) Image of adipogenic differentiation stem cell after 2 days of adding induction media Scale bar=100 µm. (B) Image of adipogenic differentiation stem cell after 8 days of adding induction media Scale bar=10µm. (C) Image of adipogenic differentiation stem cell after 10 days of adding induction media Scale bar=100µm. (D) Image of adipogenic differentiation stem cell after 14 days of adding induction media Scale bar=200µm.

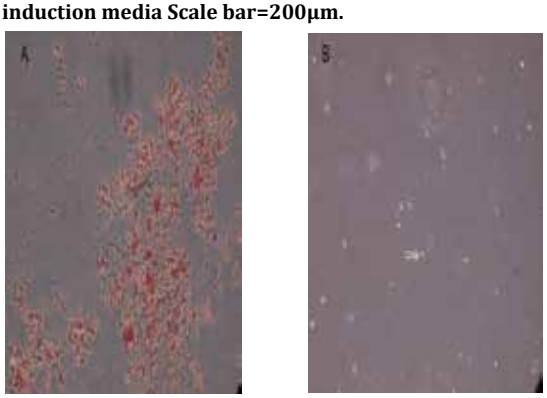
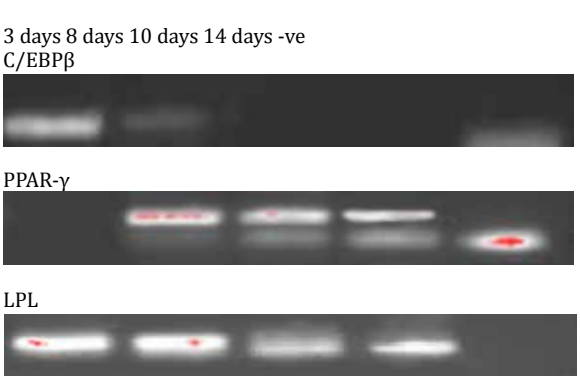


Figure 3. (A) Adipogenic Differentiation of BMSCs stained with oil red. (B) Oil Red O stain of negative control BMSCs.



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FABP4

B-Actin

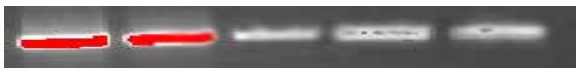
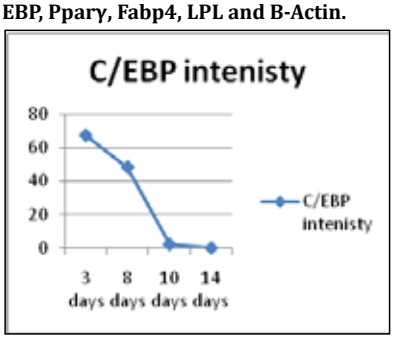
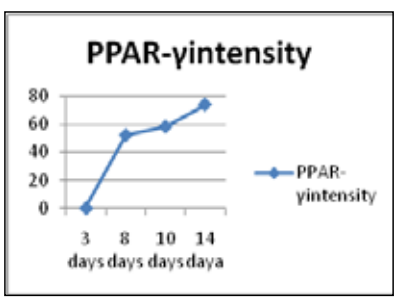
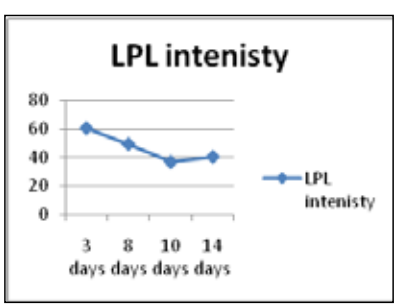
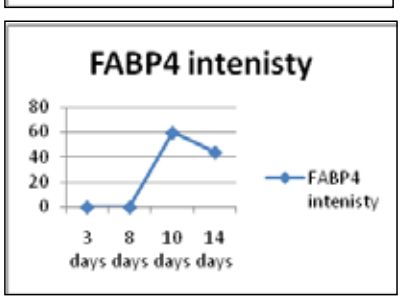


Figure 4. Analysis of mRNA expression by RT-PCR. The mRNA expression levels at 3, 8, 10 and 14 days of induction of adipogenic differenation were determined by RT-PCR. C/EBP, Pparγ, Fabp4, LPL and B-Actin.









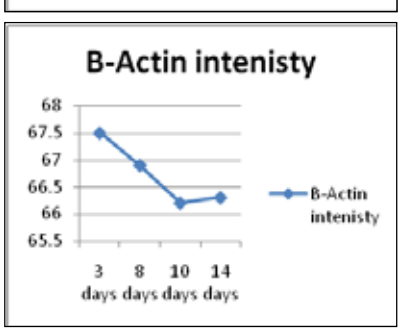


Figure 5. Intensity of each gene during the adipogenic process

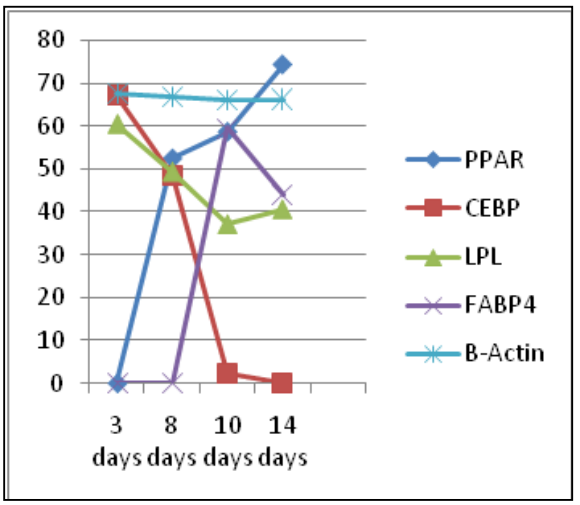


Figure 6. Show the level of all genes during adipogenic differentiation process

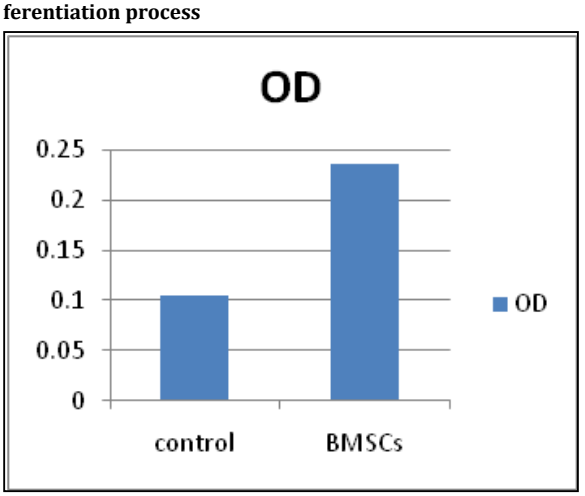


Figure 7. Spectrophotometric analysis of Adipogenic Differentiation of BMSCs

Discussion

In the present study, bone marrow was isolated from six-weeks-old C57BL/6 mice from femurs and tibiae by flushing with DMED containing 10%FBS and 1% penicillin/ streptomycin, then the product was cultured for 24 hour after that wash with PBS to remove non adherent cells. In this condition, the cells adhered to the tissue culture flask and constituted a rapidy expanding into spindle shaped cells and fibroblast like cells, and this in contrast to (sung et al., 2008) who excluded C57BL/6 mice BMSCs from his comparative study owing to their low growth rate. The low growth rate may be due to the unsuitable method used in the isolation.

Adipose cell differentiation is a multistep process characterized by a sequence of events during which preadipocytes divide until confluence. When being differentiated into adipocytes, fibroblastic MSCs are converted to a spherical shape and this was done by using adipogenic differentiation media containing adipogenic supplement and base media. Finally, differentiation of MSCs into adipocytes leads to accumulation of intracellular lipid-rich vacuoles that can be stained positively by oil red O. Quantitative analyses can be performed either by staining the cells with oil red O and extracting the dye from the cells with isopropanol and the spectrophotometric analysis of the dye at 500nm show high absorbance in the sample and low absorbance in the control.

The expressions of PPARγ, C/EBP, LPL, FABP4 and B-Actin were analyzed at 3, 8, 10 and 14 days after induction by RT-PCR. C/EBP gene expression level reached optimum peak at day 3 after induction (wang et al., 2006; musri et al., 2007) and then became lower in 8 days after induction due to activation of other gene which help in the enhancement of PPARγ gene which help in lipid accumulation and formation of mature adipocyte (huang and Donald, 2007). PPARγ gene expression level reached a peak at days 8 to the end of the process. In contrast with (Clarke et al. 1997, Wu et al. 1995) who said that the expression of PPARγ reached maximum at days 3 after induction. Lipoprotein lipase (LPL) was initially detected at day 3 after induction (peng et al., 2008) and expressed to the end of the process. FABP4 expression reached a high peak at day 10 after induction (Qian et al., 2010).

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