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Characterization of Adipogenic Differentiation of Mesenchymal Stem Cell Derived from Mice Adipose Tissue

KEYWORDS: Adipose tissue derived stem cells (ATSCs), adipogenic differentiation, C57BL/6 mice

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ABSTRACT *Mesenchymal stem cells (MSCs) isolated from adipose tissue (AT) 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 adipose tissue. This is characterized by gene expression in different 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 adipose tissue is a good alternative source for mesenchymal stem cells (MSCs) and show that there are specific genes for adipogenic differentiation which play vital role in differentiation.*

Introduction:
Adipose tissue, because of its abundance and relatively less invasive harvest methods, represents a practical and appealing source of mesenchymal stem cells (MSCs) (Katz et al., 2005). It has been demonstrated by many groups that MSCs within the stromal-vascular fraction (SVF) of subcutaneous adipose tissue display multilineage plasticity in vitro and in vivo (Rigotti et al., 2009). Several terms have been used to name these pluripotent adult progenitor cells, such as adipocyte precursor cells (Dudas et al., 2008), preadipocytes (Taniguchi et al., 2008), adipose-derived adult stem (ADAS) cells (Griesche et al., 2010), adipose-derived stromal cells (Liu et al., 2009), adipose-derived adherent stromal cells (ADASC) (Katz et al., 2005), processed lipoaspirate cells (Conde-Green et al., 2010), and adipose-derived stem cells (ASCs). According to the consensus reached in the 2004 IFATS (Annual Inter- national Fat Applied Technology Society) meeting, more researchers have currently adopted the term, ASCs. Common method to obtain ASCs is to isolate the SVF from the adipose tissue by mechanical dissociation and enzymatic treatment, followed by fluorescence activated cell sorting (FACS) or culture selection (Gimble et al., 2007). However, because the adipose SVF portion is composed of heterogeneous groups of cells and there is short of specific markers for ASCs, it remained a challenge to isolate purified ASCs and proceed with further bio- engineering applications (Mitchell et al., 2006).

Materials and methods:
Isolation, culture, and expansion of mesenchymal stem cells
The inguinal fat pad was collected under sterile conditions from 6-weeks-old male C57BL/6 mice that were killed by cervical dislocation. Adipose tissue washed with phosphate-buffered saline (PBS) (Thermo scientific, USA). The washing step is repeated until all blood vessels and connective tissue disappeared.

Adipose tissue cut into fine pieces. Then digested with 1 mg/ml Collagenase 1A (sigma-Aldrich, USA) for 1 hour at 37o C with shaking speed of 135 rpm then The samples were taken out of the centrifuge and shake vigorously to complete the separation of the stromal cells from the primary adipocytes. The released cells were centrifuged for 15 min at 400Xg to remove them from the tissues. The cell pellet suspended with PBS was filtered through a 70µm mesh to remove debris and centrifuged for 5 min at 1500 rpm each time to wash the cells. The supernatant was removed and the cell pellet was resusbended in DMED (lonza, USA) containing 10% fetal bovine serum (FBS) (lonza, USA) and 100 U/ml penicillin/streptomycin solution in 75 cm2 tissue culture flask and maintained in an incubator supplied with humidified atmosphere of 5% CO2 at 37oC (Sung etal., 2008).

Cell culture
After 3 days, 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 × 103 cells were inoculated in 75 Cm2 culture flask that were incubated at 37oC and 5% CO2. Cell cultivation was maintained up to the 3rd passage.

Adipogenic differentiation
At passage 3 ADSCs were harvested by Trypsin digestion as described above, the cells were counted and seeded at a density of (5x104 /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(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 (reverse transcriptase) RT-PCR. Adipogenic differentiation was confirmed by the formation of neutral lipid-vacuoles stainable with Oil Red O.

RT-PCR
Total RNA of ATSCs 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

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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 3 days of culture ADSC. 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. ADSCs show the ability to form multilayer after confluent. (Figure 1).

Adipogenic differentiation

The morphology of ATSCs 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, B). No red staining was detected in control groups (Figure 3C). The expressions of PPARγ, C/EBPβ, LPL, FABP4 and β-actin were analyzed at 3, 8, 10 and 14 days after induction by RT-PCR. PPARγ, 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 3rd after induction while FABP4 reached a peak at 10th after induction. In order to quantify the ratio of lipogenic differentiation, additional slides were stained with Oil Red O 14 days after induction and prepared for densitometric analysis.

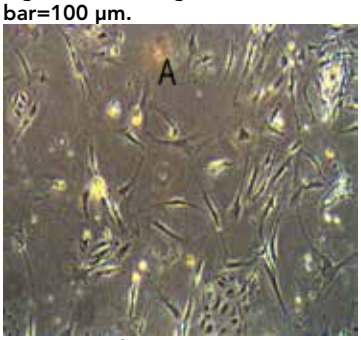
RT-PCR

The four genes were upregulated and down regulated during the differentiation process. PPARγ gene was initially detected in ATSCs and their expression level reached a peak at day 8 after induction and its level became upregulated during the adipogenic process. C/EBPγ gene was initially detected in ATSCs 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 ATSCs 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. γ-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).

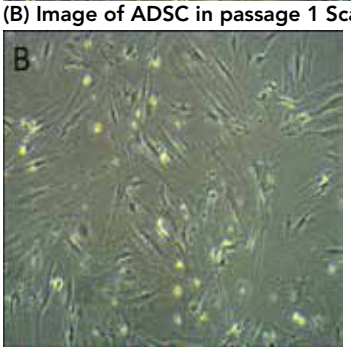
Detection of lipid droplets after adipogenic induction

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 ADSCs differentiated cells is 0.27±0.001. vs. control 0.15 (figure 7)

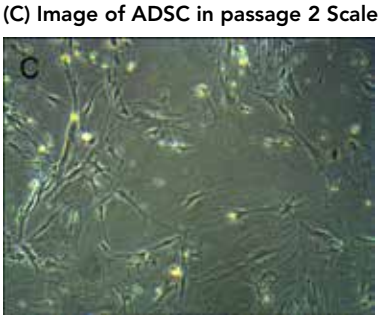
Figure 1. (A)Image of mice ADSC after isolation Scale bar=100 µm.



(B) Image of ADSC in passage 1 Scale bar=100 µm.



(C) Image of ADSC in passage 2 Scale bar=100 µm.



(D)Image of ADSC in passage 3 Scale bar=100 µm

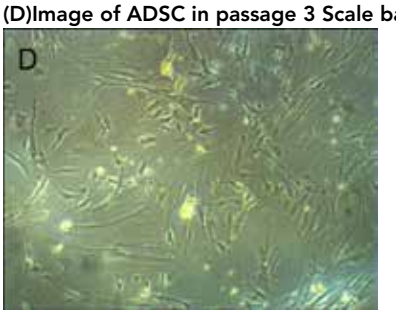
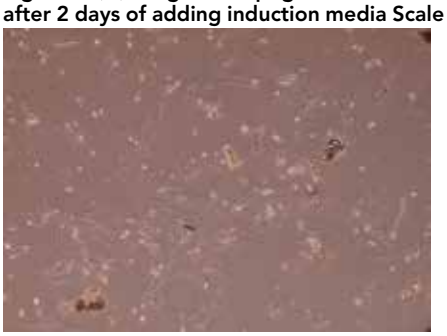


Figure 2. (A) Image of adipogenic differentiation stem cell after 2 days of adding induction media Scale bar=100 µm.



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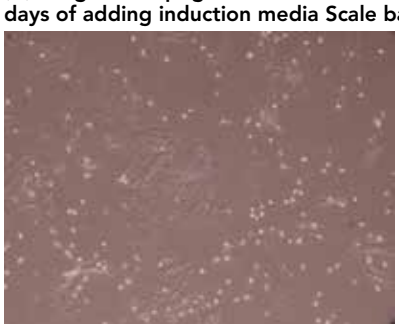
(B) Image of adipogenic differentiation stem cell after 8 days of adding induction media Scale bar=10µm.

(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=100µm.

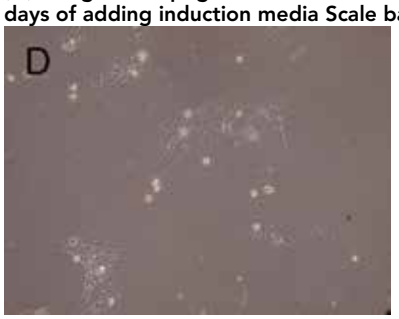
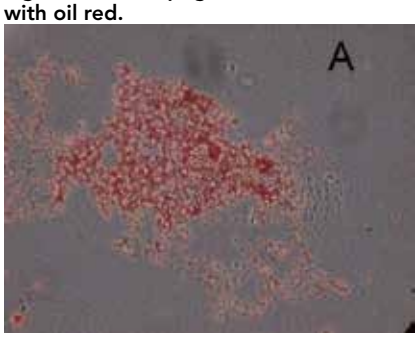
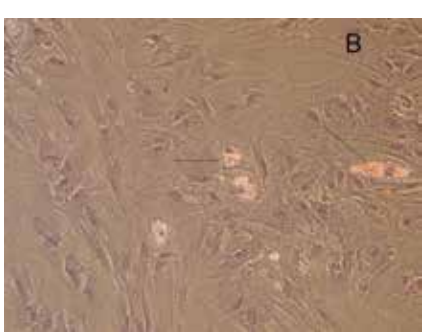


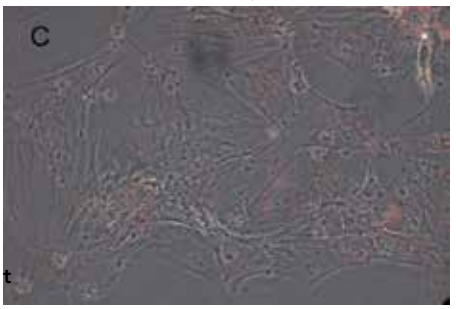
Figure 3. (A) Adipogenic Differentiation of ADSCs stained with oil red.



(B) Show lipid droplet stained with Oil Red O stain.



(C) Oil Red O stain of negative control ATSCs.



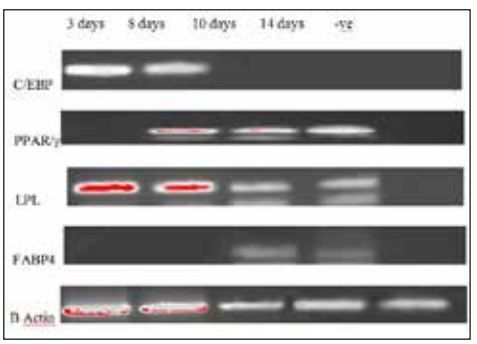
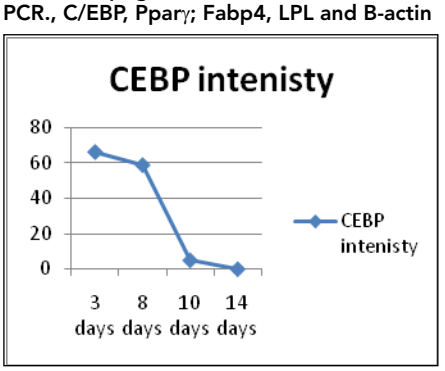
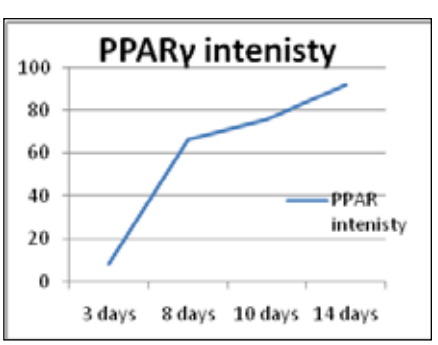
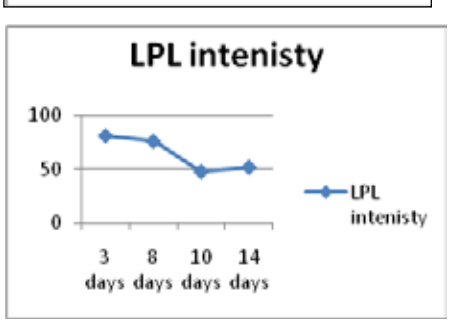


Figure 4. Analysis of mRNA expression by RT-PCR. The mRNA expression levels at 3, 8, 10 and 14 days of induction of adipogenic differenation were determined by RT-PCR., C/EBP, Pparγ; Fabp4, LPL and B-actin







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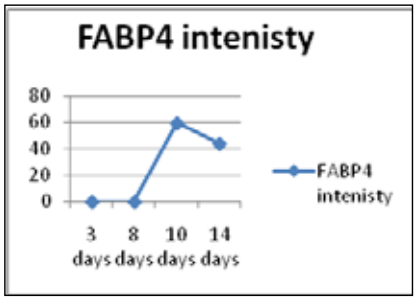

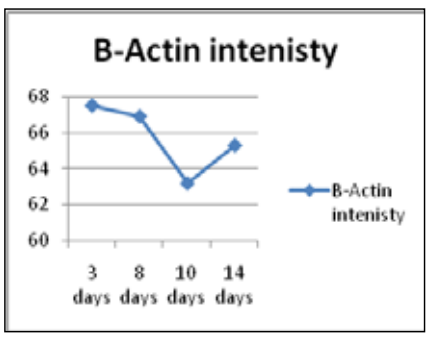

Figure 5. Intensity of each gene during the adipogenic process

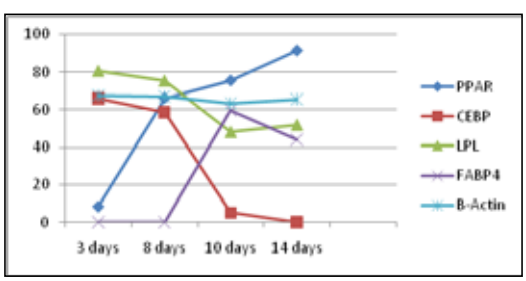

Figure 6. Show the level of all genes during adipogenic differentiation process

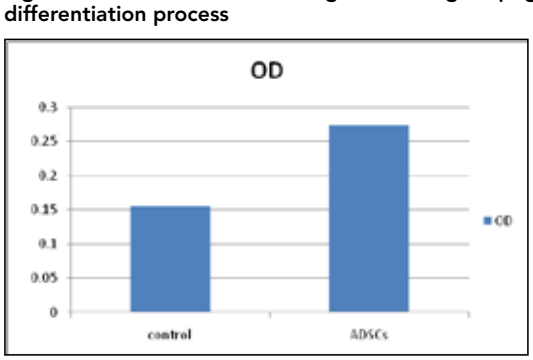

Figure 7. Spectrophotometric analysis of Adipogenic Differentiation of ADSCs

Discussion:

In the present study, adipose tissue harvested from inguinal fat pad from six-weeks-old C57BL/6 mice, then washed with (PBS) to ensure that any contamination have been removed. ADSCs isolated from adipose tissue by Collagenase type IA like (yamamoto et al; 2007) and this method gave high number of ADSCs. Mice ADSCs were cultured in DMED containing 10% FBS. In this condition, the cells adhered to the tissue culture flask and constituted a rapidy expanding population of polygonal or fibroblast-like cells. ADSCs have the ability to form multilayer after confluent and this due to low of contact inhibition and that are consistent with (Yanxia Zhu et al., 2008) who study the properties of human ADSCs. Others use Trypsin/EDTA for dissociation of adipose tissue and give good result from rat (salama etal., 2012)

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 a peak at day 3 after induction as showen in figure 5 (wang et al., 2006; musri et al., 2007) and then became lower in 8 days after induction due to it activate other gene which help in the enhancement of PPARγ gene which help in lipid accumulation and formation of mature adipocyte (LI et al., 2010). 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 peak at day 10 after induction (Qian et al., 2010).

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