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In Vitro Comparison between Adipogenic Differentiation of Mesenchymal Stem Cells Derived from Human Adipose Tissue and Amniotic Fluid

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ABSTRACT---- ***Mesenchymal stem cells (MSCs) represent an archetype of multipotent somatic stem cells that hold promise for application in regenerative medicine. The present study aimed to isolate MSCs from adipose tissue and amniotic fluid (hAFSCs) and re-differentiation into either adipose. Adult human adipose tissue (hATSCs) contains a population of mesenchymal stem cells, which can be harvested readily, safely, and in relative abundance by modern liposuction techniques. MSCs were separated from adipose tissue liposuction and amniotic fluid, cultured in dullbecus modified egal media (DMEM) for two weeks for proliferation of MSCs, which recollected and regrowing in specific media for differentiation of adipocyte cells (ACs). ACs were determined by staining with oil-red O and RT PCR assessments of adipocyte and adiponectin genes.Our findings revealed that the MSCs derived from amniotic fluid cells showed high capacity of differentiation into adipocytes comparing with that derived from adipose tissue. The ACs derived from hAFSCs were more prominent and characterized by reddish brown-droplets following staining with oil red O. Both types of adipocyte stem cells derived from either hATSCs or hAFSCs showed similar expression of molecular bands of adponectin and adipocyte gene. The authors concluded that adipocytes derived from MSCs of hAFSCs were markedly growing and expanded comparing with that hATSCs for application in regenerative medicine.***

Keywords---- Human adipose tissue, Amniotic fluid cells, Mesenchymal stem cells, Adipocyte stem cells, adipocyte gene, adiponectin gene

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1. INTRODUCTION

Stem cells are defined as undifferentiated cells from the embryo, fetus or adult that have the unique potential to generate various differentiated tissue cells under appropriate biochemical, hormonal and mechanical stimuli *in vitro* and *in vivo* [1]. Adult stem cells in particular, represent a promising model for regenerative medicine and tissue engineering because the use of embryonic and fetal stem cells is limited by ethical considerations [2]. In contrast to embryonic and fetal stem cells, which are pluripotent, adult stem cells are multipotent, unspecialized cells that have been identified in various tissues and organs. They can serve as a multipotent reservoir to replenish specific tissue cells when they die [3]. Mesenchymal stem cells (MSCs) are adult multipotent progenitor cells having the capacity to differentiate into cells of mesenchymal lineage, including bone, fat, and cartilage (4). In 2003, Prusa et al. reported the discovery of OCT-4 positive cells in amniotic fluid, which is a pluri-potent characteristic (5). Adipose tissue, like bone marrow, is derived from the mesenchyme and contains a supportive stroma that is easily isolated. Based on this, adipose tissue may represent a source of stem cells that could have far-reaching effects on several fields. We have previously identified a putative stem cell population within human lipoaspirates (6). There are no mesenchymal stem cell (MSC)-specific cellular markers, therefore their identification is achieved through their ability to adhere to plastic in vitro, through their multilineage differentiation potential in vitro and through a combination of positive expression or distinct lack of defined cell surface markers [7]. These markers include CD105+, CD73+ and CD90+, whereas MSCs should lack CD45, CD34, and several other hematopoetic stem cell markers [7]. MSC are defined as adherent colony-forming unit fibroblasts (CFU-F) and self-renewing progenitor cells with a multilineage potential [8]. Therefore, characterization of genes associated with adipocyte development is the key to understanding the pathogenesis of obesity and developing treatments for this disorder [9]. Northern blotting and gene expression profiling results showed that adipocyte-specific genes and lipogenesis-related genes are highly induced in PPARα−/− livers with PPARγ1 overexpression. These include among of

them adipsin, adiponectin and malic enzyme among others, implying adipogenic transformation of hepatocytes [10]. MSCs cultured with dexamethasone, insulin, isobutyl methyl xanthine, and indomethacin will differentiate down an adipogenic lineage [11]. We aimed to describe the differentiation capacity of Mesenchymal stem cells derived from human adipose tissue and human amniotic fluid.

2. MATERIALS AND METHODS

Cell Culture

Human adipose Tissue

Human adipose tissue (fat) was separated during plastic surgery of seven cases of breast reduction. The fat tissue was already dissociated, by placed in a sterile specimen container at the time of the harvest. The obtained sample was stored at ambient temperature and processed within 8 h of operation. Refrigeration was avoided if possible because the lipid hardens at low temperatures. Fifty grams of adipose tissue was processed using eight 15-ml sterile, disposable conical centrifuge tubes.

Isolation of MSCs from adipose tissue

Processed lipoaspirate (PLA) cells were obtained from raw human lipoaspirates and cultured as described by Zuk *et al.*[6]. Briefly, raw lipoaspirates were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Washed aspirates were treated with 0.075% type IA collagenase (C- 2674; Sigma-Aldrich, St. Louis, MO) in PBS for 30 min at 37°C with gentle agitation. The digested adipose tissue was centrifuged at 1,200 *g* for 5 min to obtain a cell pellet. Supernatant was decanted, pipet or other sterile tool was used to dislodge the oily layer. Each pellet was resuspended in 160 mM NH_4Cl and was incubated at room temperature (RT) for 10 min to lyse contaminating red blood cells. Samples were pooled for convenient handling. The MSC-rich dense cell fraction was collected by centrifugation, as detailed above. The supernatant was removed and pelleted cells were resuspended in HMSC complete growth medium. Cell suspension was filtered through a 100-µm cell strainer to remove cellular debris and incubated overnight at 37°C in a humidified incubator with 5% CO_2 .Following incubation, the Flasks were washed twice with PBS to remove residual non adherent cells and fresh growth media was added. Cells were fed every 3–4 days until cells reached 70–80% confluency.

Human amniotic fluid mesenchymal stem cells (hAFMSCs)

Third trimester AF samples were harvested during 40 deliveries at a mean gestational age of 34+2 (SD ± 1) weeks involving women who underwent elective caesarean section for breech presentation. The AF samples were collected by puncture through the membranes after opening the uterine wall during caesarean section Cells were isolated from the AF samples no more than 4 hours prior to use.

The AF samples were centrifuged at 1100 rpm for 5 minutes and all the cells isolated from sample were plated in six 35-mm Petri dishes containing L-glucose Dubelco modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin, 0.1mg/ml of streptomycin (Invitrogen), 10 ng/ml basic fibroblast growth factor, 10 ng/ml of epidermal growth factor (Peprotech, Rocky Hill, NJ, USA) and 20% of fetal bovine serum (FBS; Invitrogen). The cells were incubated at 37 °C with 5% humidified CO_2 for MSCs culture. At day 7 after the initiation of culture, the medium was replaced with fresh medium and non-adhering cells were removed; the medium was replaced twice weekly.

When the cells reached about 70% confluence, they were treated with 0.25% trypsin (Invitrogen) and 1 Mm ethylenediaminetetra-acetic acid (EDTA; Invitrogen) for 3 min. The released MSCs were collected and replated 1:3 under the same culture conditions. The cell concentration was 4×10^4 cells/ml and they were labelled as cell passage 1 .

The MSCs at passages 2, 5 and 8 were trypsinized and inoculated into 24-well Petri dishes, 0.5 ml/well (cell concentration 1×10^4/ml). The cells were incubated at 37 °C with 5% humidified CO_2 and the medium was subsequently replaced twice a week.

Harvesting and Culturing of MSCs

Medium from primary culture was removed with a sterile Pasteur pipet, HBSS without Ca^{2+} and Mg^{2+} used to Wash adhering cell monolayer once or twice .Enough 1x trypsin was 37°C trypsin/EDTA solution to culture to cover adhering cell layer. The plate was placed on a 37°C 1 to 2 min. The bottom of plate was tapped on the countertop to dislodge cells. Incubation continued until in large sheets start to come off.

Complete media (DME + 10% FBS) added and cell suspension drawed into a Pasteur pipet and cell layer rinsed two or three times to dissociate cells and to dislodge any remaining adherent cells. Equal volume of cell suspension was added to fresh plates or flasks that have been appropriately labeled

In vitro assessment of viability and colony forming units-fibroblast (CFU-F)

The viability of stem cells was checked by Trypan blue according to the method of Maclimans *et al.* [12]. Equal volume of both solution 0.04% trypan blue and stem cells were mixed and incubated for 10 minutes at 37oC then the number of viable cells (unstained) was counted using a haemocytometer by light microscope. The number of viable stem cells/ml was then calculated according to the following equation.

$$\text{Viable cells (\%)} = \frac{\text{Number of viable cells / ml}}{\text{Total number of cells / ml}} \times 100$$

Concerning *colony forming units-fibroblast,* the growth was evaluated on 75ml flask containing DMEM media. After 7 days, the capability of mesenchymal stem cells to form fibroblast-like colonies was investigated by contrast-phase microscope.

Flow cytometry for cell surface antigen expressions

The mesenchymal stem cells at passage 3 were released by trypsinization. The cells were centrifuged at 1,200 rpm for 5 minutes then solved in phosphate buffered saline (PBS) at the concentration of (1×10^6/ml). The cells were stained with different fluorescently labeled monoclonal antibodies (mAb). In brief, 100 µl of cell suspension was mixed with 10 µl of the fluorescently labeled mAb and incubated in the dark at room temperature for 30 minutes. Washing with PBS containing 2% bovine serum albumin was done twice and the pellet was resuspended in PBS and analyzed immediately on flow cytometry. The mAbs were used in different combinations of fluorochromes; namely fluorescein isothiocyanate, phycoerythrin and phycoerythrin-cyanine 5. Different combinations of mAb were used against various antigens . The immunophenotyping was performed on EPICS-XL flow cytometry (Coulter, Miami, FL, USA). The cells were analyzed with the most appropriate gate FBS using the combination of forward and side scatters Cells were incubated for 3 weeks with adipogenesis induction media and adipogenesis maintenance media using the protocol described by (Pittenger et al., *1999*). Adipogenic differentiation was visualized in phase-contrast microscopy by the presence of highly refractive intracellular lipid vacuoles. Oil Red O (Sigma) staining was used to assess the accumulation of lipid droplets in these vacuoles. Samples were taken from adipogenic differentiated stem cell at different time intervals, after 4, 7, 14, and 21 days for light microscopy and reverse transcription polymerase chain reaction (RT-PCR) study.

Adipogenesis assay

Cells were incubated for 3 weeks with adipogenesis induction media and adipogenesis maintenance media using the protocol described by (Pittenger et al., *1999*). Adipogenic differentiation was visualized in phase-contrast microscopy by the presence of highly refractive intracellular lipid vacuoles. Oil Red O (Sigma) staining was used to assess the accumulation of lipid droplets in these vacuoles. The cell suspension plated in mesenchymal stem cell expansion medium at a density of 300,000 cells per 6 well plate with 2 ml volume per well.The cells incubated at 37℃ in a 5% CO2 humidified incubator for 3-7 days.The cells should be 100%confluent before initiating adipocyte differentiation.

When the cells are 100% confluent, the medium was aspirated from each well and 2-3 ml adipogenesis induction medium was added. This medium change corresponds to differentiation day 1. The fresh Adipogenesis Induction or Maintenance Medium was replaced according to the differentiation schedule. Every 2-3 days for 21 days. Lipid droplets can be detected by microscopic examination as early as 5 days into the differentiation period. After 21 days of differentiation, adipocytes can be fixed and the lipid droplets stained with Oil Red O Solution.

Oil-red O staining

The culture media was aspirated and each well washed 2x with PBS. The cells Fixed for 15-30 min in 10% formalin then Washed with 2x deionized water.2-3 ml 60% isopropyl alcohol added, mixed to ensure water is dispersed, then cells incubated at R.T for 5 min.(1-2ml) oil red O stain added for 3 min. Washing done very gently with water until stain is removed (3-4 washes). A dark reddish precipitate may form is normal. Under phase microscopy, the lipid droplets appear as cherry red spheres within the cells, also a dark reddish precipitate may be present in the wells.

3. RESULTS

Adipogenic cells isolated from mesenchymal stem cells of either human adipose tissue or amniotic fluid were shown to be able to proliferate in culture. During propagation, the cell morphology changed markedly, from spindle-shaped cells to flat ones. This morphological change was accompanied by a change in cell proliferation ability. Lipid vesicles could be observed. Adipocyte differentiation from human adipose tissue was markedly flourished at 14 days post *In vitro* culturing (Fig. 1(B). However these cells derived from human amniotic fluid cells showed highest proliferation Fig. 1(A). The MSCs derived earlier from either both types of human tissues were firstly appeared dedifferentiated and spindle-shaped semi-like fibroblast cells. However, at 4 days, where adipocyte was still less differentiated, a population of both fibroblastoid and non-fibroblastoid cell types were identified. Fibroblastoid cell population was still existed after enzymatic digestion.

Oil red O staining of adipocyte revealed discrete spots of cytoplasmic oil droplet within adipocytes derived from human amniotic fluid cells comparing with human adipose tissue (Fig. 2 & 4). RT PCR analysis showed similar expression of adipocyte gene and adiponectin in *In vitro* culture cells forming adipocytes at 4,7,14 and 21 days from either human adipose tissue or amniotic fluid cells.

flowcytometry Figures(4 and 5) shows the cell surface antigenic characteristics of the cultured MSCs derived from human adipose tissue and amniotic fluid at passage 4: 8 by flow cytometry. AT-MSCs and AF-MSCs expressed the typical MSCs marker proteins CD105, CD90. MSCs derived from both sources did not display expression of hematopoietic antigens (CD34 and CD14) (Figure 4 and 5). Oct4which is specific for AF-MSCs is positively expressed .The analyses revealed that the expression of surface antigens of MSCs derived from human adipose tissue were positive for CD13 (89%), CD 105 (92%), CD29 (94%) and CD 90 (92%), while they were negative for CD14 (4%) and CD34 (6%) at Passage 4:8(figure 4).

The analyses revealed that the expression of surface antigens of MSCs derived from human amniotic fluid were positive for CD 105 (79.5%), CD 90 (79.1%), CD29 (50%), CD13 (36.5%) and Oct4 (31.4%), while they were negative for CD14 (11.3%) and CD34 (15%) at Passage 4:8(figure5)

Figure 1(A,B) under an inverse microscopy culture mesenchymal stem cell (MSCs) derived from human adipose tissue(A) ,Amniotic fluid (B) at passage three (P3) were morphologically defined by the fibroblast–like appearance.

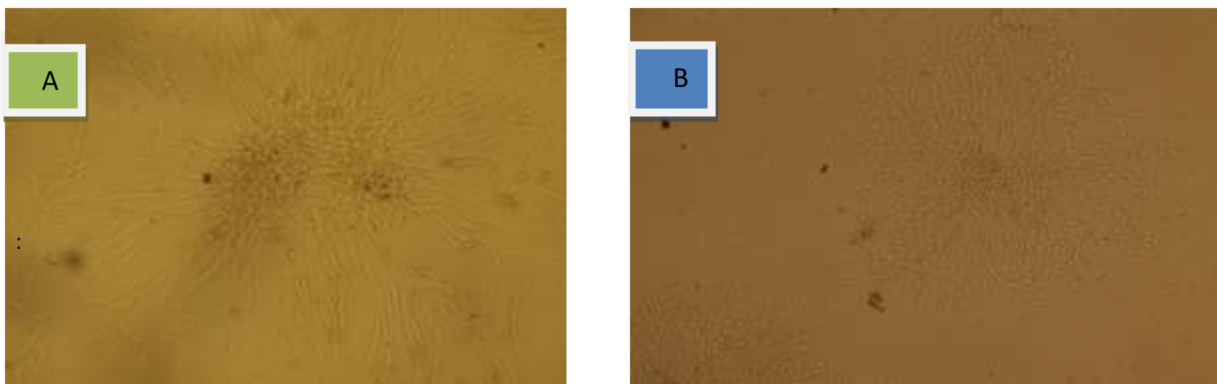


Figure 2 Photomicrograph of mesenchymal stem cells (MSC) derived from human adipose tissue showing differentiative potential. Adipocyte differentiation is visualized by highly refractive intracellular lipid vacuoles and droplets appear as cherry red spheres within the cells and by Oil Red O staining{magnification × 200}.

Figure 3 Photomicrographs of mesenchymal stem cells (MSC) derived from human amniotic fluid showing differentiative potential. Adipocyte differentiation is visualized by highly refractive intracellular lipid vacuoles and droplets appear as cherry red spheres within the cells and by Oil Red O staining {magnification × 200}.

Fig4 The flow cytometry analysis revealed that the expression of surface antigens of MSCs derived from human adipose tissue were positive for CD13 (89%), CD 105 (92%), CD29 (94%) and CD 90 (92%), while they were negative for CD14 (4%) and CD34 (6%).

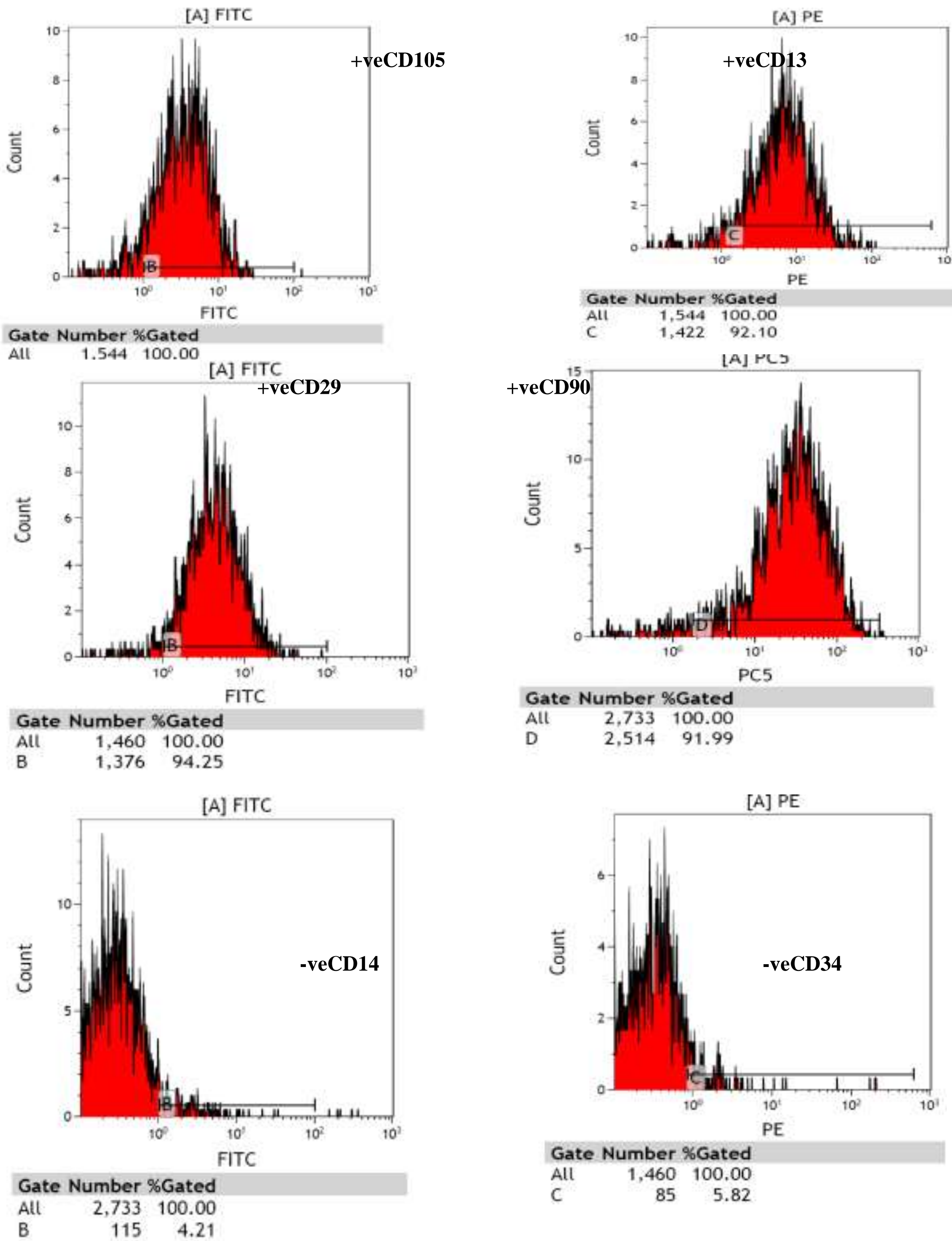
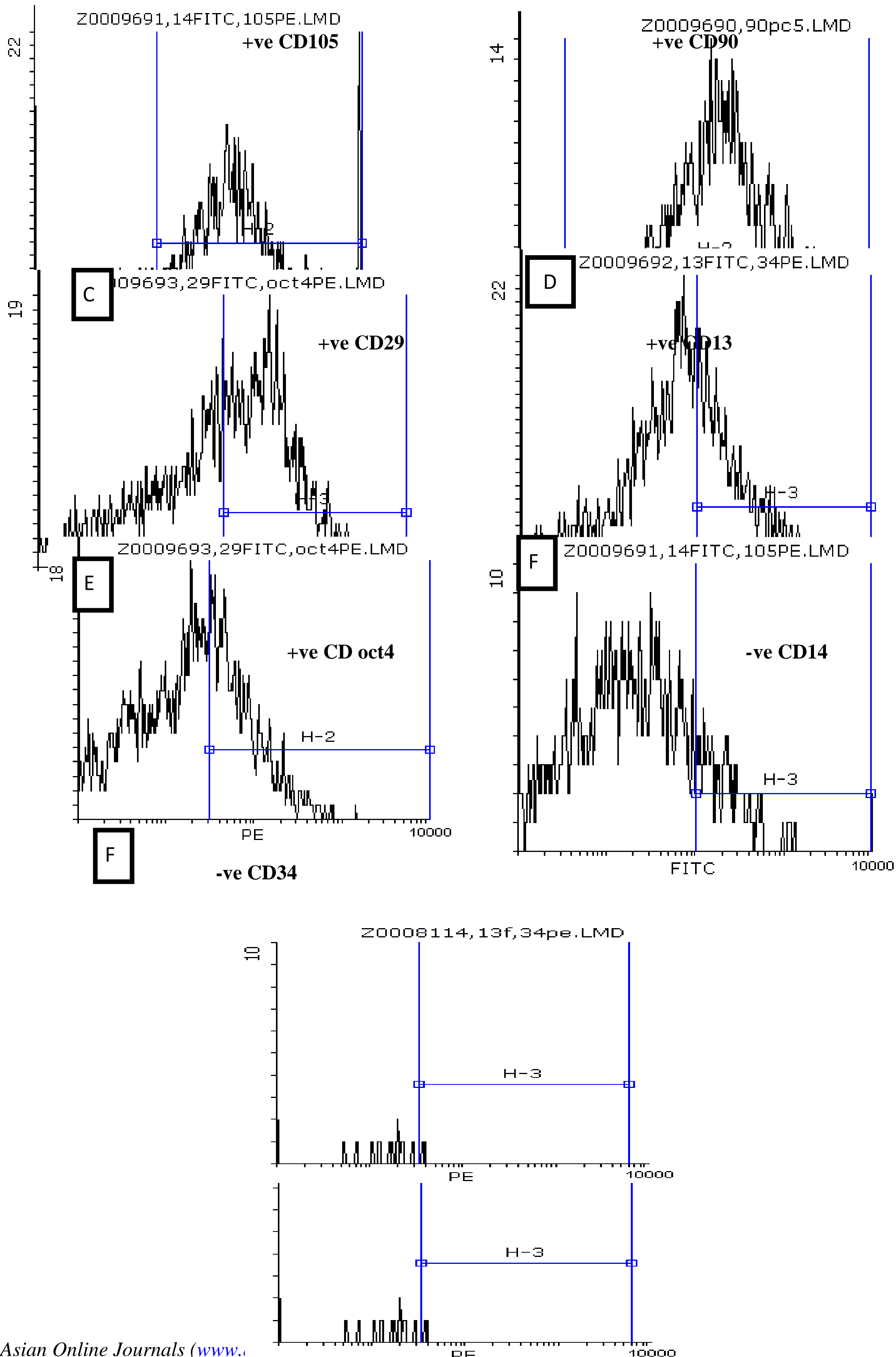


Figure 5 Flow cytometry analysis of human amniotic fluid mesenchymal stem cells revealed that their expression of surface antigens such as CD 105 (79.5%), CD 90 (79.1%), CD29 (50%), CD13 (36.5%) and Oct4 (31.4%) (Passage 4:8) was strongly positive; but CD14 and CD34 were negative.



4. DISCUSSION

Stem cells are defined as a population of undifferentiated cells with the capacity to divide for indefinite periods, to self-renew and to generate a functional progeny of highly specialized cells *(*13).

In the present study, the human amniotic epithelial cells and adipose tissue claimed that they possess stem cell potential capable of differentiating into adipocyte cells. These represent the major source of multilineage of cells (14). We confirm the multi-lineage capacity of a population of stem cells, termed PLA cells, isolated from human lipoaspirates. Preliminary studies characterized the heterogeneity and growth kinetics of this cell population and revealed that PLA cells may have multi-lineage potential [6]. our study revealed that the human amniotic epithelial cells and adipose tissue claimed that they possess stem cell potential, capable of differentiating into adipocyte cells. These represent the major source of multiline age of cells [15].

One of the criteria to identify MSC is that the population must express CD105 and CD90 (Dominici et al., 2006) which is compatible with our result. Phermthai et al received weaker expression of CD90 in flowcytometry analysis of MSC derived from amniotic fluid (16), although the reason for this is unknown. The heterogeneity of the cells analyzed may be one explanation.The analysis of flow cytometry show that the MSC derived from adipose tissue and the MSC derived from amniotic fluid stem cells show to have CD13, CD105, CD29, CD90 (which is compatible with result from (11) and lack CD14 and CD34 which are specific for hematopoietic markers which confirm that the purified stem cells are mesenchymal stem cells not hematopoietic stem cells.

Schaffler et al. defined the surface marker set for AT-MSC (ASC) as positive CD9, CD29, CD44, CD54, CD73 (SH3), CD90, CD105 (SH2), CD106, CD146, CD166 and HLA I expression and negative CD14, CD31, CD34, CD45, CD133, CD144, HLA-DR, STRO-1 and HLA II expression [17]. Our findings supported the work done by Schaffler et al and Dominici et al.The discrepancies in the expression of CD34 of AF-MSC and AT-MSC in the different studies may be caused by different isolation methods or different media compositions used which can result in a different expression of surface molecules.
Flow cytometry shows that cells from AF express the mesenchymal markers CD90, CD73, CD105, CD13, CD29, CD44, CD146, CD54, and CD71 (weak expression) and do not express CD106, CD34, or CD45 (Fig. 8). These data suggest that fetal MSCs are present in our cultures. Judging by flow cytometry, the absence of CD34 and CD45 expression shows that there are no hemopoietic stem/progenitor cells in the culture.To conclude, AF and AT MSC were successfully isolated and cultured for several passages. The cells had characteristics similar to MSC isolated from both sources.

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