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**Dates:** Received: 08 December, 2016; Accepted: 23 December, 2016; Published: 29 December, 2016

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## Research Article

# Adipogenic and Osteogenic Markers Characterization of Human Amniotic Fluid Stem Cells

## Abstract

**Objective:** Human amniotic fluid stem cells (HAFSCs) derived from human amniotic fluid during parturition are of good source in regenerative medicine for development to either adipocyte, chondrogenic or osteogenic cells.

**Methods and results:** The HAFSCs were obtained from amniotic fluid post-parturition. A written informed consent had been taken beforehand from mothers after parturition during admission in Mansoura University Hospital, Egypt. Cultured in adipogenic or osteogenic culture for 4,7,14 & 21 days. Flow cytometry of HAFSCs for Oct-4, CD13,, CD29 , CD 90 , CD34 and CD 14. Gene expression for adipogenic (PCR of leptin, peroxisome proliferator-activated receptor- $\gamma$  and lipoprotein lipase) and osteogenic (osteocalcin) cells were carried out. Biochemical assessments of adipogenic (lipoprotein lipase enzyme and glycerol-3-phosphate dehydrogenase) and osteogenic (alkaline phosphatase, B-galactosidase and calcium content) markers. HAFSCs were positive for Oct-4, CD13,, CD29 , CD 90 and negative CD34 and CD 14. Also, light and transmission electron microscopic investigation of adipocyte stem cell culture were investigated at 4,7,14 & 21 days in both two models. Adipocyte derived from HASCs displayed fibroblastic morphology and confluence at 7 days and flat-shape with positive oil red staining at 14 & 21 days. At ultrastructural level, the adipocyte derived from HASCs exhibited ideal structure. Also, it showed adipogenic gene expression and biochemical investigation. Similar was observed of its osteogenic affinity of bone cells derived from HFASCs.

**Conclusion:** The authors concluded that HAFSCs are capable of differentiating into adipocyte, osteoblast and chondroblast depending on the culture medium and are important in regenerating medicine.

## Abbreviations

Human amniotic fluid stem cells, characterization, Light and transmission electron microscopy, PCR and biochemical characterization, adipocyte & osteogenic stem cells.

## Introduction

Amniotic fluid is a protective, nourishing fluid that surrounds the fetus during intra-uterine life [1]. The amniotic fluid forms during first and mid-trimester with the aid of active transport of sodium and chloride. This facilitated permeability of water across the chorio-amniotic membrane [2]. Amniotic fluid stem cells are separated from discarded amniotic fluid at termination of gestation and represent a novel source of stem cells for modeling of human genetic diseases [3] and regenerative medicine [4]. Amniotic fluid represent a good source of fetal stem cells, which are derived from mesoderm,

ectoderm and definitive endoderm. The HAFSCs cells was carried out by c-Kit (CD117). The cell lines show self-renewal capacity reaching 2 fold increase at 36 hours, and, maintain its telomere length at 250 doublings [5,6].

The hAFSCs cells have immunosuppressive properties with a great ability to differentiate into adipogenic, osteogenic, myogenic, endothelial, pulmonary, neurogenic, hepatogenic, cardiac, and pancreatic lineages. These cells have the capability in treatment of diseases such as diabetes, bone defects, neural degeneration, blood disorders and heart and kidney disease [7] through its capabilities of differentiating into adipocytes, osteocytes, chondrocytes, myocytes, and neuronal cells [8]. Human amniotic mesenchymal stem cells were positive for CD73, CD90, CD105, and CD44 and negative for CD34, CD14, CD45, and HLA-DR. Also, they have the great potential to express stem cell markers such as Oct3/4, Sox2, Nanog, Klf4, SSEA4, c-myc, vimentin, and nestin [9]. There is a detected

calcitonin receptor in proliferating and osteo-differentiated hAFMSCs. *In vitro* osteogenic capacity of hAFMs revealed upregulation of Runx2, osterix, alkaline phosphatase, collagen I, and osteopontin, with a capacity of osteogenic differentiation [10].

The present study aimed to illustrate the separation, characterization and adipogenic and osteogenic differentiation of hAFMSCs via real time PCR, cytological and biochemical assays.

## Materials and Methods

### Collection of samples

Mesenchymal stem cell are collected from the human amniotic fluid (AF) of 15 women who underwent a cesarean delivery. A written informed consent had been taken beforehand from mothers after parturition during admission in Mansoura University Hospital, Egypt. During cesarean delivery, the amniotic fluid was aspirated after the uterine muscle was opened. Cells were isolated from the AF samples immediately after collection by centrifugation at 1100 rpm for 5 minutes. The cells were cultured in 35-mm petri dishes containing 30 mL low-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 100 U/mL of penicillin, 0.1mg/mL of streptomycin, 10 ng/mL of basic fibroblast growth factor, 10 ng/mL of epidermal growth factor (Peprotech, Rocky Hill, NJ, USA), and 20% of fetal bovine serum (Invitrogen). The media was renewed after incubation of the cells at 37 °C with 5% humidified carbon dioxide for 7 days and the non-adhering AF cells were removed.

The media was replaced twice weekly until the cells reached 70% confluence. MSCs were separated after adding 0.25% trypsin and 1 mM EDTA (Invitrogen) for 3 minutes to the media. The MSCs were released, collected and replanted in a split ratio of 3 flasks under the same culture conditions.

### Harvesting and culturing of human amniotic mesenchymal stem cells ( hAFMSCs)

The hAFMSCs are cultured in regular media (DMEM, 10% serum and 1% penicillin/streptomycin, Sigma) for 3 weeks according to De Coppi *et al.*, (2007). Flow cytometric analysis of CD markers and transcription factors characteristics of hAFMSCs was carried out.

### *In vitro* assessment of viability, colonization and cd markers of The hAFMSCs

**Viability test:** It was checked by 0.04% trypan blue exclusion according to Mclimans *et al.*[11]. The number of viable stem cells/ml was then calculated according to the following equation.

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

### Colony forming units-fibroblast (CFU-F) assay

Fibroblast like colony growth was evaluated at densities of  $25 \times 10^6$  cells/well. After 7 days, the capability of mesenchymal

stem cells to form fibroblast-like colonies was assessed by contrast-phase microscope.

### Flow cytometry for cell surface antigen expressions

The hAFMSCs were released by trypsinization and assessments of their specific CD markers by flow cytometry. The stem cells at the concentration of  $(1 \times 10^6 / \text{mL})$  in phosphate buffer. The monoclonal antibodies used in the different combinations of fluorochromes are fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-cyanine 5 (PeCY5). Different combinations of mAb were used against various antigens CD14, CD90, CD105, CD13 and CD34.

### Osteogenic differentiation

Differentiated hAFMSCs into osteocyte-like cells was carried out, at a density of  $1.8 \times 10^4$  cells /cm<sup>2</sup> were cultured in DMEM low-glucose medium containing 10% FBS, 1% penicillin/streptomycin (Sigma Chemical Comp. St. Louis, MO, USA), and osteogenic supplement such as dexamethasone (100 nM, Sigma),  $\beta$ -glycerophosphate (10 mM, Sigma), and ascorbic acid-2-phosphate (0.05 mM, Sigma) [12].

### Chondrogenic differentiation

Chondrogenic differentiation was induced by ascorbate, dexamethasone and transforming growth factor according to Zuk *et al.*[13] and Huang *et al* [14].

Reverse transcription polymerase chain reaction (RT-PCR):

Adipogenic (adipocyte, adiponectin, leptin, lipoprotein lipase & peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) and osteogenic (osteocalcin) expressed genes were examined differentiated hAFMSCs at 4,7,14 and 21 days. Total RNA was extracted from the cultured cells (QIAGEN Inc., Valencia, CA) and treated with deoxyribonuclease I to remove contaminating genomic DNA. The RT-PCR was carried out using the one step RT-PCR kit. It is started at 50°C for 30 min and 95°C for 15 min for reverse transcription, then followed by 35 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, elongation at 72 °C for 1 min, and the final extension at 72°C for 10 min. The amplified DNA fragments were visualized through 2% agarose gel electrophoreses and photographed under UV light [15]. Reverse transcription was carried out using 1  $\mu$ g RNA in a total volume of 20  $\mu$ L.

Real-time PCR was carried out using 25  $\mu$ L amplification reactions contained primers at 0.5  $\mu$ M, deoxynucleotide triphosphates (0.2 mM each) in PCR buffer, and 0.03 U Taq polymerase along with SYBR-green (Molecular Probes, Inc., Eugene, OR) at 1:150,000. Aliquots of cDNA were diluted 5- to 5000-fold to generate relative standard curves with cDNA samples [16]. Threshold cycle values of target genes were standardized against GAPDH expression. The primer sequences are illustrated in Tables 1,2). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard control.

### Biochemical assessments of adipogenic and osteogenic markers

Adipogenic differentiated stem cells were collected, centrifuged and pellet of cells were then disrupted by

sonication, and centrifuged at  $12,000 \times g$  for 20 min at  $4^\circ\text{C}$ . The supernatants were assayed for lipoprotein lipase (LPL) and glycerol-3-phosphate dehydrogenase (GPDH) activities (units/mg protein) using Cell Biolab Elisa Kit according to the manufacturer's protocol.

In osteogenic differentiated stem cells, alkaline phosphatase ( $\mu\text{moles/cell/hour}$ , catalogue number ABIN2067575) and B-galactosidase activities (Catalogue Number, E1091R) were determined by BALp Elisa kit, Germany. The assay was carried out according to manufacturer's protocol. Calcium content was determined by colorimetric assay kit of Biovision diagnostic company (USA, cat. Nu, K380-250).

### Light microscopic investigation

Adipogenic, osteogenic and chondrogenic stem cells derived from human amniotic mesenchymal stem cells were stained with either oil red O or alizarin red or safranin O respectively. The stained cell culture were examined under bright field inverted light microscope and photographed.

### Transmission electron microscopic investigation

The adipogenic and osteogenic cell cultured cells at 21 days were centrifuged at  $800 g$  for 10 min and immediately fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH7.4) for 15 min. These was followed by dehydration in ascending grades of ethyl alcohol, cleared in propylene oxide and embedding in epoxy-resin. Ultrathin sections were cut with a diamond knife on a LKB microtome and mounted on grids, stained with uranyl acetate and Lead citrate and examined at 10X Joel Transmission electron microscope.

### Statistical analysis

Data were tabulated as mean  $\pm$  SE. of different cells. Statements of significance are based on probability (p) levels of  $\leq 0.05$  was considered statistically significant. All statistical analyses were undertaken using SPSS program software (version anova).

## Results

### Flow cytometry characterization and identification of human amniotic fluid stem cells (hAFMSCs)

Human amniotic fluid-derived mesenchymal stem cells (hAFMSCs) have a characterized surface protein expression. Surface protein expression analysis, which allows for a rapid identification of a cell population following flow cytometric analysis of CD markers. Purified amniotic fluid stem cells express high level of Oct-4, CD13, CD29, CD105 and CD90 and these markers were maintained at high levels. Also it is negative for the endothelial and hematopoietic-specific markers CD34 and CD 14 (Figure 1).

### Adipogenic stem cells differentiation

Adipocytes stem cells differentiation from amniotic fluid stem cells firstly, appeared as dedifferentiated and spindle-shaped semi-like fibroblast cells. However, at 4 days, a

population of both fibroblastoid and non-fibroblastoid cell types were identified. By 7 days, there is a marked increase fibroblastic activity and increased in its confluency. At 14 and 21 days, the cells acquired flat-shapes with positive oil red staining (Figure 2A,D).

### Osteogenic and chondrogenic stem cell differentiation

For confirming osteogenesis, the *in vitro* cultures were stained with alizarin red and von kossa stain. Following investigating the alizarin-red staining of 4 days cultured osteogenic stem cells derived from hAFMSCs post-osteogenic induction revealed more expansion and dense reddish-brown staining of nodules in isolated regions. Dense mineralized dark-brown deposits was detected at 7 days of osteogenic induction. The peripheral mesenchymal cells of each nodule become spindle-shaped. There was a detected polygonal

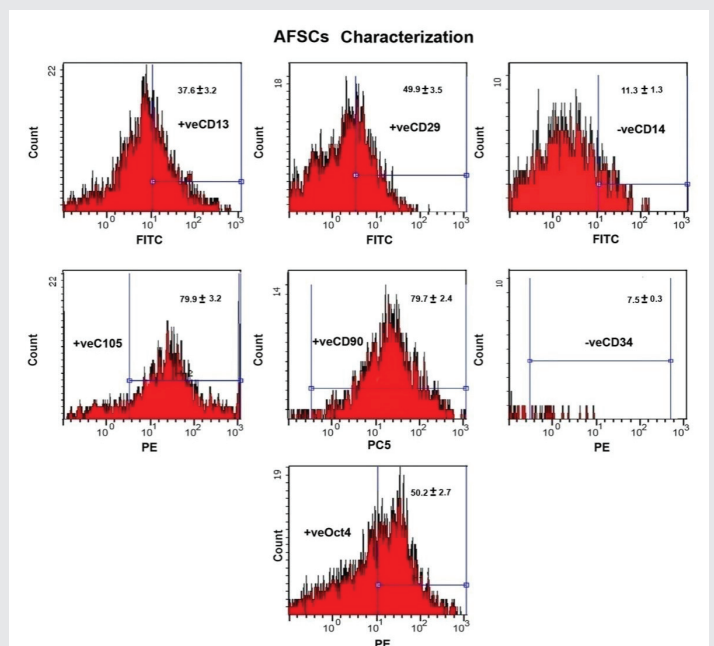
**Table 1:** Primers for RTQ-PCR and RT-PCR for adipogenic cells:

Gene	Forward Primer	Reverse Primer
Adipocyte	TACCTGGAACTTGTCTCCAGTGAA	CCATTCTGCACATGTACCAGGACA
Adiponectin	TCCTGCCAGTAACAGGGAAG	GGTTGGCGATTACCGTTTG
Leptin	ACCTCTGGTTCACAAAAAGG	TTGGCACAGGCACAAGACAT
Lipoprotein	TCATTCCCGGAGTAGCAGAGT	GGCCACAAGTTTTGGCACC
Lipase	TCTGAGTCTGTATGGAGTGACAT	CCAAGTCGTTACATCTAGTTCA
PPAR- $\gamma$	AAGGTGAAGGTCGGAGTCAAC	GGGGTCATTGATGGCAACAATA
GAPDH		

Abbreviations; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPAR- $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; RT-PCR, reverse transcriptase-PCR.

**Table 2:** Primers for RT-qPCR for osteogenic cells:

Gene	Forward Primer	Reverse Primer
Osteocalcin	ACACTCCTCGCCCTATTG	GATGTGGTCAGCCAATC



**Figure 1:** Flow cytometry of human amniotic fluid derived mesenchymal stem cells showing positive expression of surface protein markers of Oct-4 (50%), CD13 (38%), CD29 (50%), CD 90 (80%), and CD 105 (80%) and negative expression of CD14 (1%) and CD34 (8%).

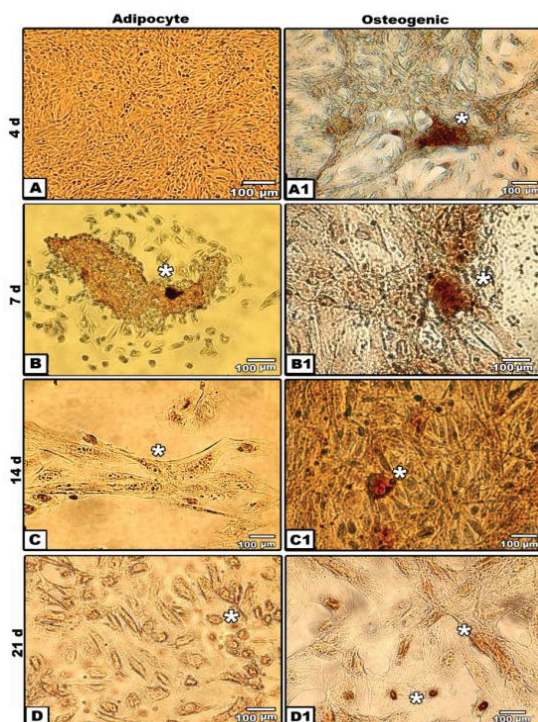
osteoblast-like phenotypes with mineralization of their extracellular matrix after 14 days of culture manifesting the early sign of bone formation. After 21 days of culture, there was a marked evidences of newly bone formation with dense calcium deposits and mineralization of the extracellular matrix (Figure 2A–B).

On the other hand, von kossa staining of *in vitro* culture revealed more expansion of oval- and spindle-shaped osteoblast cell with fine granulated mineralized calcium salts during early culturing materials at 4 days post-osteogenic induction. At 7 days, dark mineralization calcium staining was detected. At 14 and 21 days of culture, expansion was markedly increased and mineralization become more visible (Figure 2A1,D1).

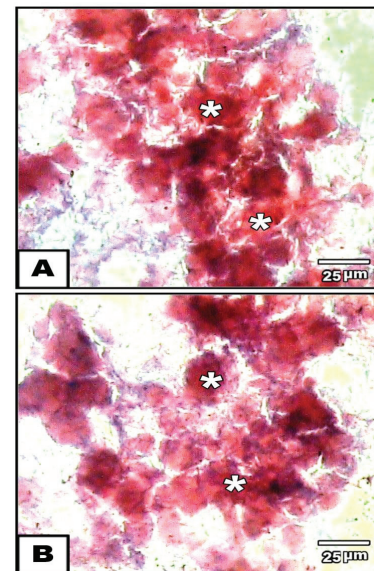
Concerning chondrification, the chondroprogenitor stem cells acquired oval-shaped structure at 21 days post – chondrogenic induction and stained intense red colouration with safranin o (Figure 3A,B).

### Transmission electron microscopy of adipogenic and osteogenic stem cells at 21 day culture

At ultrastructural level, the adipocyte stem cells exhibited eccentric nucleus, lipid droplet formation, abundant



**Figure 2:** Photomicrographs of human adipose tissue derived mesenchymal stem cell (hATMSCs) showing adipogenic (A-D) and osteogenic differentiation (A1-D1). A. 4 days showing confluent and early fibroblastic differentiation. B. 7 days showing adipocyte stem cell differentiation with cherry red spheres. C. 14 days showing adipocytes surrounded by networks of reticular fibers. D. 21 day showing oval and spindle-shaped adipocytes. \* indicate oil droplets. A1. 4 days showing osteogenic differentiation stained with alizarin red S. A2. 7 days showing calcified extracellular matrix with large sphere osteogenic oval-shaped cells. A3. 14 days showing oval-shaped cells with mineralized calcium deposition. A4. 21 days showing reddish osteogenic cells surrounded by faintly stained semi-like osteoclast cells. \* indicate calcium deposition.



**Figure 3:** Photomicrograph of safranin O stained of 21 day *in vitro* differentiation of chondrogenic cells derived from hAFMSCs showing red dense chondrogenic nodules. \*→ proteoglycans stained in red colors. \* indicates proteoglycans with red color characteristic of chondrogenesis.

ribosomes, rough endoplasmic reticulum with small cisternae and mitochondria (Figure 4A,D). Concerning the osteogenic cells possessed centrally-located nuclei with either oval or convoluted nuclear envelope. Its cytoplasm contain abundant number of mitochondria and rough endoplasmic reticulum with dilated cisternae. The plasma membrane showed well-developed microvilli. Oval-shaped mineralized calcium deposits become clearly evident. Osteoprogenitor cells revealed the presence of collagen fibrils (Figure 4A1,D1).

### PCR identifications of marker genes of adipocyte and osteogenic stem cells

PCR analysis showed expression of the adipocyte, adiponectin, leptin, lipoprotein lipase and peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) at 4,7,14 and 21 days of culturing the adipocyte derived stem cells. The gene expression of the assayed genes were 280, 100, 120, 150 and 130 bp respectively (Figure 4). Overexpression of leptin, PPAR- $\gamma$  and lipoprotein lipase in adipocyte stem cells and osteocalcin in osteogenic stem cells (Table 1). De novo expression of osteocalcin, was more detected at 4 days culture of osteogenic induction comparing with the other *in vitro* stages (Table 3, Figure 5).

### Biochemical confirmation of osteogenesis

To verify osteogenic differentiation of osteogenic stem cells from amniotic fluid-derived mesenchymal stem cells, biochemical determination of alkaline phosphatase and  $\beta$ -galactosidase in osteogenic tissue culture after 21 days of osteogenic induction. Comparing with normal rabbit tibial bone, the osteoblast stem cells reached to  $16.58 \pm 0.28$  comparing with tibia bone of  $11.28 \pm 0.22$ . Also, the osteoblast stem cells  $\beta$ -galactosidase reached to  $20.37 \pm 0.19$  comparing with control  $15.89 \pm 0.3$  (Tables 4,5).

## Discussion

From the present findings, hAFMSCs separated from human fluid was firstly appeared as dedifferentiated and spindle-shaped semi-like fibroblast cells and differentiated into both fibroblastoid and non-fibroblastoid cell types by 4 days. Following RT PCR analysis, the observed adipogenic potential of hAFMSCs were observed by increased expression of adiponectin, leptin, lipoprotein lipase and peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) *in vitro* culture cells at 4,7,14 and 21 days. Similar findings of gene expression

of adiponectin [17,18], leptin [19], lipoprotein lipase [20] and peroxisome proliferator-activated receptor- $\gamma$  [21] were reported in adipocyte differentiated from hAFMSCs.

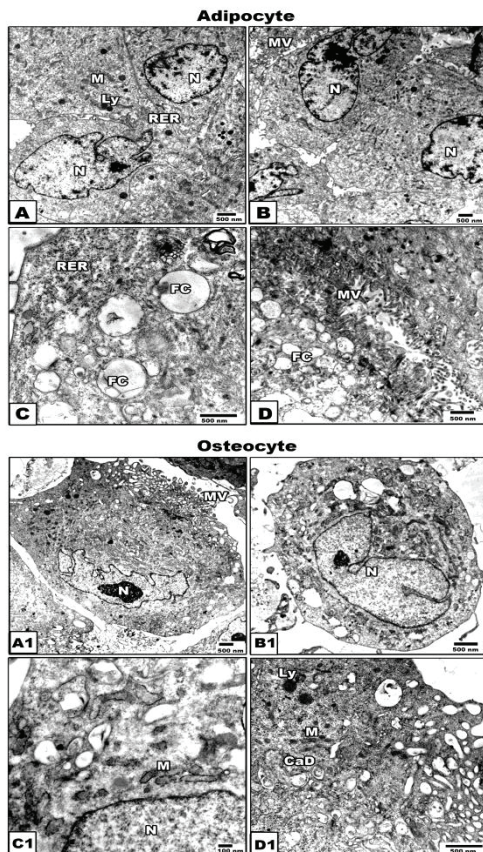
Cheong et al. [22] mentioned that cord lining mesenchymal cells differentiate into adipocyte following oil red O staining and presence of lipid droplets and expression of PPAR $\gamma$  and adipin. Leptin and adiponectin were extremely low.

Adiponectin is important factor in adipose tissue promoting its proliferation and differentiation from preadipocyte to adipocytes. It affects the expression of CCAAT/enhance binding protein  $\alpha$ ; peroxisome proliferator-activated receptor  $\gamma$  and adipocyte determining and differentiating factor 1/ sterol-regulatory element binding protein-1c [23]. Also it attain marked reduction in type 2 diabetic patients, obesity, or cardiovascular disease [24]. Hypertrophied adipocytes predicted the development of type 2 diabetes [25]. Whereas hyperplasia and hypertrophied of adipocytes associated increase burden of inflammatory cells were detected in obese patients.

Leptin is the obese gene, that plays a major role in the in the regulation of adipose homeostasis [26]. Adipocyte-specific cis-regulatory element and the transcription factor(s) responsible for leptin gene expression were recently identified in *in vitro* adipocyte cultures [27]. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a nuclear receptor that is a key regulator of adipogenesis and is present in two isoforms generated by alternative splicing, PPAR $\gamma$ 1 and PPAR $\gamma$  [28] and enhances lipogenesis, mitochondrial activity, and insulin sensitivity in adipocytes [29]. Lipoprotein lipase expression [20] and leptin expression was found to be increased intracellular lipids storage during adipocyte differentiation [28].

Adipocyte differentiation as confirmed by oil red O staining, gene expression was also confirmed by the bioassays of glycerol -3-phosphate dehydrogenase and lipoprotein lipase enzymes which highly expressed in adipocytes derived from hAFMSCs. The enzyme lipoprotein lipase activity was found to be increased during adipocyte differentiation [20]. Similar glycerol-3-phosphate dehydrogenase had been identified in the control of triglycerides synthesis and/or storage [30] which increased during differentiation of adipocytes [31,32].

Also, transmission electron microscopy of the 21 day of adipocyte cells derived from hAFMSCs showed apparent differentiation of cytoplasm lipid droplet and mitochondria.

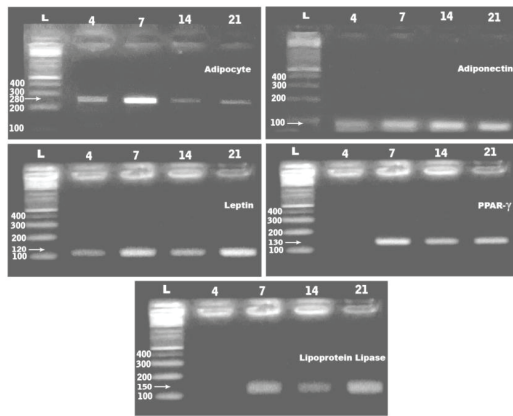


**Figure 4:** Transmission electron micrographs of *in vitro* differentiated adipogenic cells derived from ATMSCs at 21 days. Note adipocyte with eccentric nuclei and cytoplasm rich in mitochondria, lysosomes and rough endoplasmic reticulum (A&B) and presence of numerous fat vacuoles (A1,A2& B3). Abbreviations; AC, adipocyte;CaD, calcium deposits, FC, fat cell; Ly, lysosome; M, mitochondria; Mv, microvilli; N, nucleus; OsC, osteogenic cell; RER, rough endoplasmic reticulum.

**Table 3:** RT-qPCR of genes characteristic to adipogenic and osteogenic derived from hAFMSCs

	4		7		14		21 days	
	GAPDH	CT	GAPDH	CT	GAPDH	CT	GAPDH	CT
<b>Adipogenic cells</b>								
Leptin	35.4±0.9	36.5±1.2	32.7±	33.8±1.5	-	25.1±1.2	32.6±1.1	31.4±1.3
PPAR- $\gamma$	26.7±1.7	35.5±1.1	25.3±1.3	33.6±0.9	-	26.4±0.8	27.6±1.4	32.4±1.2
LPL	35.2±1.7	33.7±1.4	35.1±1.3	36.5±1.9	35.4±1.8	38.6±1.7	35.8±1.4	37.3±1.8
<b>Osteogenic cells</b>								
Osteocalcin	32.8±1.3	32.7±1.4	32.7±0.9	33.8±1.8	35.3±1.2	31.4±1.7	32.6±1.1	31.8±1.6

Each result represent the Mean  $\pm$ SE (n=10).



**Figure 5:** Agarose gel electrophoresis of gene expression of adipogenic-derived stem cell culture at 4, 7, 14 and 21 days. A. Showing adipocyte gene expression at 280 bp. B. Showing adiponectin gene expression at 100 bp. C. Showing leptin gene expression at 120 bp. D. Showing peroxisome proliferator-activated receptor-γ (PPAR-γ) at 130 bp. E. Showing lipoprotein lipase gene expression at 150 bp.

**Table 4:** Biochemical markers of adipogenic and osteogenic cells derived from human amniotic fluid.

Adipogenic markers		
	HAF	hAFMSCs
Lipoprotein lipase (Umole/mg)	11.6± 1.2	13.4 ±1.6
Glycerol-3-phosphate dehydrogenase (Umole/mg)	30.1±3.1	41.1± 3.8
Osteogenic markers		
	Tibia O Rabbit	hAFMSCs
Alkaline phosphatase (Umole/mg)	11.3±1.2	16.6±1.8
B-galactosidase (Umole/mg)	15.9±2.3	20.4± 1.9

Each result represent the mean±SE (n=5)

**Table 5:** Calcium content in osteogenic cells derived from human amniotic fluid mesenchymal stem cells in 14 & 21 days.

	14 day		21 day	
	+ve	-ve	+ve	-ve
Calcium content mg/dL	3.64 ±0.03	5.00±0.4	4.11±0.02	15.16±0.06

Each result represent the Mean±SE (n=10). "+ve" means that media contain DMEM + osteogenic supplement such as dexamethasone, β-glycerophosphate and ascorbic acid. only -ve " means that the media contain DMEM) Dulbecco's Modified Eagle's Medium).

Similar distribution of mitochondria with characterized cristae was reported in adipocyte of human brown adipose tissue [33].

Similar findings of adipogenic differentiation of amniotic fluid-derived stem cells expressing CD117 were reported [34].

Also, the osteogenic stem cells were early recognized at 4 days of *in vitro* cultures of hAFMSCs stained with alizarine red S. The stem cells form a nodules with peripheral spindle-shaped mesenchymal cells. By 7 days polygonal osteoblast-like phenotype with mineralize their extracellular matrix was observed. By 21 days, there was a detected mineralized calcium deposits in the extracellular matrix. Differentiation of newly formed bone material become more prominent.

Cells belonging to e.g. the amnion, skin, the urogenital, respiratory, and digestive systems have been reported to be detectable. hAFMSCs of CD markers identification, our findings revealed the expression of Octa-4 in human amniotic fluid which represent characteristic type of cells actively divide. These cells were identified by [35] and having the capacity to express cyclin A, a marker for cell cycle proliferation [36].

The observation of the major mineralization events after 21 days of culture is similar with previous studies for mesenchymal stem cells suggesting similar osteogenic potentials [37-39].

Moreover, it is critical to know that osteocalcin represents one of the important genes responsible for osteoblast and extracellular matrix differentiation. At 21 days of cultured osteogenic cells, osteocalcin, alkaline phosphatase (ALP) and β-galactosidase were remarked. ALP is osteogenic marker and expressed in the process of calcification [40-42]. The extracellular matrix developed vesicles which are believed to arise by budding from the plasma membrane of hard tissue forming cells (osteoblasts, chondrocytes, odontoblasts and cementoblasts) [43].

Similar osteogenesis of cultured hAFMSCs with osteoblast differentiation and overexpression of alkaline phosphatase and mineralized calcium was reported by after implanted the osteogenic materials into an immunodeficient mouse [44,45]. Peister et al. [46], reported osteogenic activity of amniotic fluid stem cells compared to bone marrow-derived stem cells.

Osteogenesis was associated with increased expression of cytoplasmic collagen, osteonectin and bone alkaline phosphatase [47, 468].

Also, osteogenesis differentiation of hAFMSCs into chondrocytes, and osteoblasts, attained much more advanced differentiated process with marked expression of osteogenic markers which need future studies to focus on the mechanical properties of these cells for application in regenerative medicine of healing bone defects. Transmission electron microscopy for the osteogenic cells at 21 days of *in vitro* culture showed cytoplasm rich in mitochondria, rough endoplasmic reticulum, oval-shaped mineralized calcium deposits and abundant collagen fibrils characteristic for osteoblast differentiated cells. Also, cytological pattern of chondrocytes were detected.

Similar findings of *in vitro* chondrification of mesenchymal stem cells can produce replacement constructs for cartilage defects in osteoarthritis [49] and mouse calvarial mesenchyme [50].

Finally the author concluded that the observations described suggest that hAFMSCs represent a good sources for the isolation of stem cells in regenerative medicine depending on the disease condition.

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