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RESEARCH ARTICLE

Isolation, characterization, and *in vitro* differentiation of osteogenic human amniotic fluid-derived stem cells

Mohamed M. S. Farag[1*], Mohamed A. Sobh[2], Ahmed A. A. Shalaby[1], Emad Eldin A. Ewais[1]

1. Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo, Egypt

2. Experimental biology, Urology & Nephrology Center, Mansours University

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Corresponding Author

Mohamed M. S. Farag

Abstract

Stem cells (SCs) regenerative therapy represents an emerging strategy for the treatment of human diseases. Mesenchymal stem cells (MSCs) offer significant promise as a multipotent source for cell-based therapies and could form the basis for the differentiation and cultivation of tissue grafts to replace damaged tissue. The main goal of this study was to focus on MSCs and to analyze their differentiation capacity into osteogenic cells. To achieve this aim, MSCs were isolated from amniotic fluid-derived cells (AFSCs). The expression of CD29, CD90, CD105, CD13, CD34, CD14, and Oct-4 of MSCs were characterized by flow cytometry. The proliferation and differentiation of MSCs into osteogenic were examined *in vitro*. Osteogenic differentiation was determined by staining with Alizarin Red S and real time polymerase chain reaction (RT-PCR) analysis of genes markers expression after 4, 7, 14, and 21 days. Our results revealed that the patterns of CD expression markers were highly positive for CD29 (50%), CD 90 (79.1%), CD 105 (79.5%), CD13 (36.5%) and Oct-4 (31.4%) and negative for CD14 (11.3%) and CD34 (15%). MSCs showed proliferative potential and were capable of osteogenic differentiation characterized by Alizarin Red S staining and expression of molecular detection of genes. MSCs from amniotic fluid had the ability to differentiate *in vitro* into osteogenic under specific culture conditions. AFSCs may represent sources of characterized pluripotent SCs that can be easily collected and amplified *in vitro*. These MSCs may be used in preclinical studies on large animals to develop future human therapies.

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 non-hematopoietic stem cells, which widely exist in the matrix of various organs and tissues. They have a high degree of proliferation, self-renewal and multi-directional differentiation ability [4]. In recent years, the research progress of MSCs develops quickly due to the improvement of the MSCs' culture conditions and detection means. MSCs can be extracted from many sources such as bone marrow (BM), adipose tissue, umbilical cord, peripheral blood and placenta [5].

In the past, the human bone marrow-derived mesenchymal stem cells were the most widely researched, but their application was limited as they could only be obtained through bone marrow biopsy [6]. Amniotic fluid stem cells showed the characteristics of MSCs with the ability of highly proliferation, self-renewal and multiple differentiations potential. As the amniotic fluid can be gained through the amniotic cavity puncture and the operation has a little impact on fetal and maternal, it is not only with high security, but also avoids the ethical issues related to embryonic stem cells [7,8]. So, the amniotic fluid mesenchymal stem cells (AFMSCs) get high attention of scientists [9]. Recent studies showed that AFMSCs had gradually become the hot spot in the research of immune regulation, repair medicine and tumor targeted therapy.

Human amniotic fluid-derived stem (AFS) cells are isolated from amniotic fluid after routine amniocentesis [6,7,8,10-14]. The AFS cells express both embryonic and adult stem cell markers and are broadly multipotent; they can be induced to differentiate into cells representing all three embryonic lineages, such as cells of the osteogenic, adipogenic, chondrogenic, myogenic, endothelial, neuronal, and hepatic lineages [11,12,15-20]. Unlike embryonic stem cells, AFS cells are not tumorigenic and can expand extensively without the use of feeder layers or expensive defined media [11]. The AFS cell lines have been shown to expand over 250 population doublings and retain telomere length and have a normal chromosomal karyotype [11]. Therefore, AFS cells have great potential for cellular tissue engineering and osteogenic differentiation.

1. Materials and Methods

1.1. Isolation of mesenchymal stem cells from amniotic fluid (AF)

Mesenchymal stem cells were isolated from the human amniotic fluid (AF) of women (37- 39 weeks), who underwent a cesarean delivery for breech presentation. The mean volume of the AF samples was 11.2±4.7 mL. Amniotic fluid cells were cultured on three 35 mm diameter cell culture dishes using Dubelco modified Eagle's medium (DMEM) containing low-glucose (Invitrogen, Carlsbad, CA, USA) as the culture medium. For complete media, 100 U/mL of penicillin, 0.1mg/mL of streptomycin (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL of basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL of epidermal growth factor (Peprotech, Rocky Hill, NJ, USA), 10% fetal bovine serum (Invitrogen, USA), and 1% L-glutamine (Invitrogen, USA) were added. The medium was renewed after incubation of the cells at 37 °C with 5% Co_2 for 7 days and the non-adhering AF cells were discarded.

1.2. Cells cultivation

After 1 day, non-adherent 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 reached 70-80% confluence. Cell passaging was performed using trypsin- EDTA solution (0.25%, Sigma Aldrich, USA). The numbers of recovered cells were evaluated using hemocytometer and the cellular viability was quantified by the trypan blue exclusion test. Approximatly $250–300\times10^3$ cells were plated in 75 cm^2 tissue culture flasks and incubated in complete media at 37°C with 5% CO_2. Cell cultivation was performed up to the 3rd passage.

1.3. Flow cytometry analysis

MSCs cells were characterized using cell surface markers by fluorescence-activated cell sorting (FACS) analysis. The cells were stained with different fluorescently labeled monoclonal antibodies (mAb) (eBioscience company). In briefly, 5×10^5 of cells were added in FACS buffer (in 100 µl PBS/0.5% BSA/2 mmol/EDTA) and mixed with 10 µl of combination different fluorescently labeled mAbs (CD13-FITC, CD14-FITC, CD29-FITC, CD34-PE, CD105-PE, Oct4-PE, and CD 90-PCY5). After mixing, the cells were incubated in the dark at 2-8°C for 30 minutes. Washing with PBS containing 2% BSA was done twice and the pellet was re-suspended in PBS and analyzed immediately on EPICS-XL flow cytometer (Coulter, Miami, Fl, USA).

1.4. Osteogenic differentiation

For osteogenic differentiation, MSCs were harvested by trypsin and the cells were counted. Approximately, 5×10^4 of cells per well were plated in 6-well plate. After 80% of cell confluent, osteogenesis differentiation media consists of DMEM supplemented with 10% FBS, 0.1 µM dexamethasone (OS medium), 50 µM Ascorbic acid, 10 mM β-glycerol phosphate (Sigma-Aldrich, USA) was added. One 6-well plate more was left for control without osteogenesis differentiation media. The medium was changed twice per week for 2-3 weeks. The differentiation potential for osteogenesis was assessed by 40 mM Alizarin Red (pH 4.1), staining was done after fixation with 10%

neutral buffered formalin. The cells were usually stained at 4, 7, 14, 21 days after addition of OS medium using a commercially available alizarin red stain.

1.5. Gene expression by real time polymerase chain reaction (RT-PCR)

Total RNA was extracted from osteogenic differentiated MSCs at 4, 7, 14, and 21 days by using RNeasy Mini kit (Qiagen, Valencia, CA, USA), and treated with deoxyribonuclease to remove contaminating genomic DNA, following the manufacturer's instructions. Reverse transcription was performed with 1 µg RNA in a total volume of 20 µl per reaction. In order to osteogenic differentiation, the cells were assessed by RT-PCR using specific primers as follows: human osteocalcin (product size 279 bp) forward, 5'-ACACTCCTCGCCCTATTG-3' and reverse, 5'-GATGTGGTCAGCCAACTC-3'. Real-time PCR was performed with 25 µl amplification reactions contained primers at 0.5 µm, deoxynucleotide triphosphates (0.2 mm each) in PCR buffer, and 0.03 U Taq polymerase along with SYBR-green (Molecular Probes, Inc., Eugene, OR, USA) at 1:150,000. Aliquots of cDNA were diluted 5 to 5,000-fold to generate relative standard curves with which sample cDNA was compared. Standards and samples were run in triplicate, and use glyceraldehyde-3-phosphate dehydrogenase was also included as an internal control. Reactions were performed on a 7000 Real-Time PCR System (ABI PRISM, Applied Biosystem, Foster City, CA, USA). A model introduced by Pfaffl was used for calculation [21,22].

2. Result

2.1. Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) were harvested from different human amniotic fluid (AF) donors. Our results showed that MSCs were generated by stander procedures and grown for at least two passages in culture. Contaminating hematopoietic cells were depleted during passage 1 and MSCs were morphologically defined by a fibroblast-like appearance and by their adherence to plastic consisted of a heterogeneous cell population with a predominant spindle-shaped morphology and were able to form fibroblast like colonies (Figure 1).

2.2. Flow cytometry analysis for MSCs

To identify the differentiation of human amniotic fluid (AF) towards MSCs, flow cytometry analysis of cultivated cells was performed. Phenotype analyses of MSCs were identified by expression of the following CD markers: CD13, CD14, CD29, CD34, CD105, Octa4, and CD 90. Our results showed that our cells were negative for CD14 (11.3%) and CD34 (15%) (Figure 2). On the other side our cells were positive for CD29 (50%), CD 90 (79.1%), CD 105 (79.5%), CD13 (36.5%), and Oct4 (31.4%) (Figure 2). The expression markers for MSCs which isolated from human amniotic fluid were showed in figure 3**A**, 3**B**, and 3**C**.

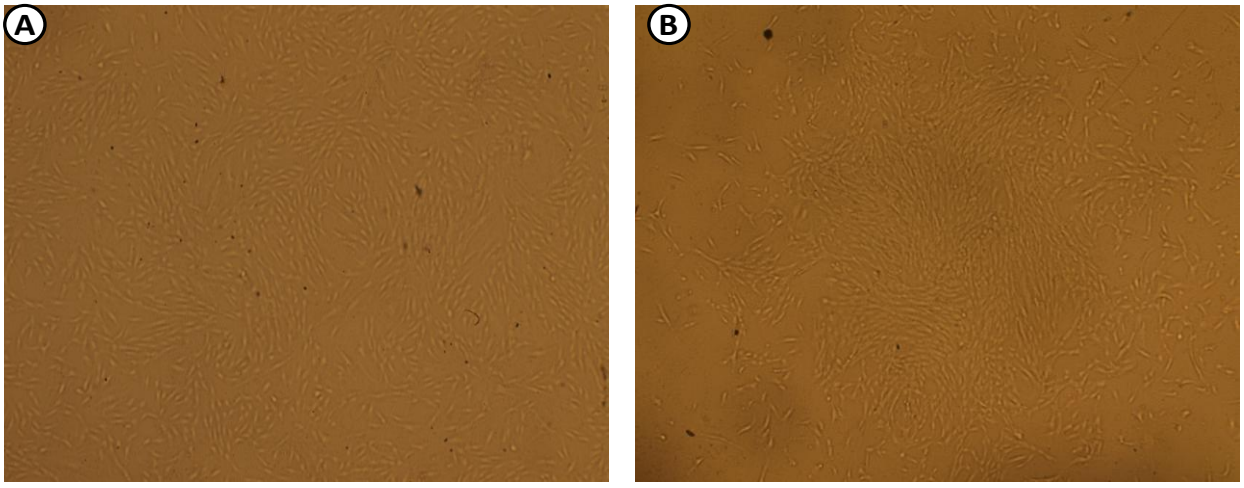

Figure 1: Under an inverted microscope, cultured human amniotic fluid (AF) mesenchymal stem cell (hMSCs) **A)** showed the morphologically of MSCs which defined by a fibroblast–like appearance **B)** Showed the heterogeneous small spindle-shaped fibroblastoid cells and more rounded cells (original magnification × 100).

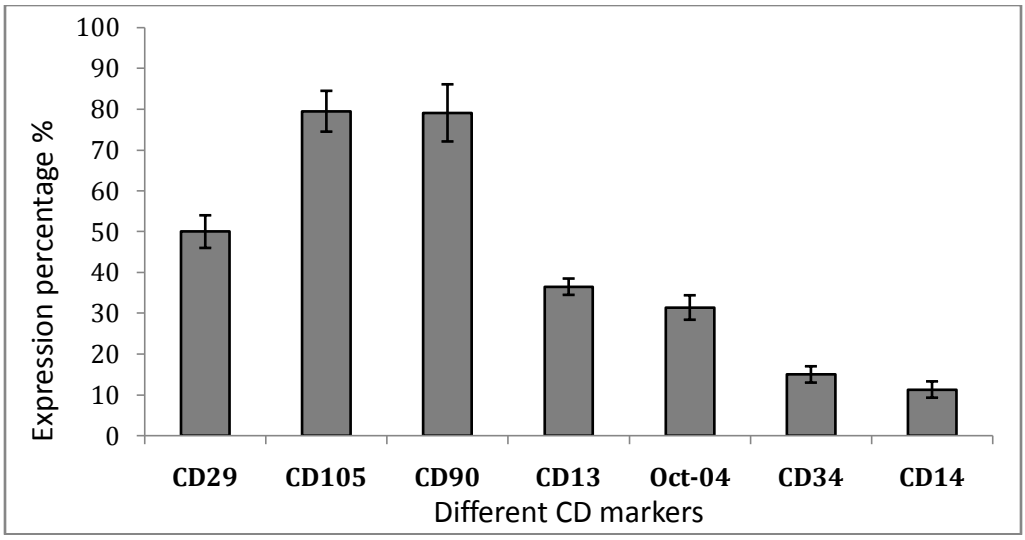

Figure 2: Showed the phenotype analyses of MSCs by expression of different CD markers: Our results showed that our cells were negative for CD14 (11.3%) and CD34 (15%), while it was positive for CD29 (50%), CD 90 (79.1%), CD 105 (79.5%), CD13 (36.5%), and Oct4 (31.4%).

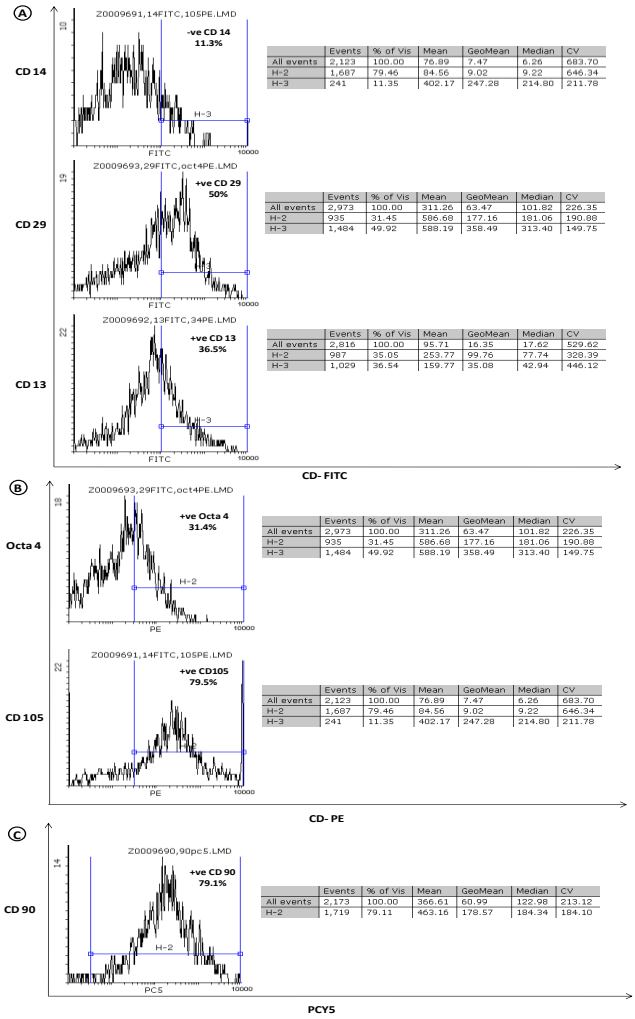

Figure 3: Showed the phenotype analyses of MSCs by expression of different CD markers: **A)** Showed mAbs-FITC including CD14, CD29, and CD13. **B)** Showed mAbs-PE including Octa 4, and CD105.**C)** Showed mAb-PCY5 including CD90. Flow cytometry analysis of human amniotic fluid mesenchymal stem cells revealed that their expression of surface antigens such as CD29, CD 90, CD 105, Oct4 and CD13 was strongly positive; but CD14 was negative.

2.3. Osteogenic differentiation assay

2.3.1. Light microscopy

To evaluate mineral deposition, osteogenic differentiation was performed in monolayer culture for 3 weeks. Cells were induced towards osteogenesis after reaching confluences. With the induction medium the cells changed from a fibroblastic appearance to a more polygonal appearance and formed nodules. After 3 weeks culture time, the induced cells were stained positive with Alizarin Red S stain for mineral deposition in their newly formed matrix. The differentiation of MSCs toward osteoblasts was indicated by the formation of calcium-rich hydroxyapatite which detected with Alizarin red and appears as irregular red –orange (Figure 4B and 4C). Control cultures, although becoming over-confluent after 3 weeks, retained their fibroblast like appearance, did not form cell aggregates and were stained negative for mineral deposition (Figure 4A).

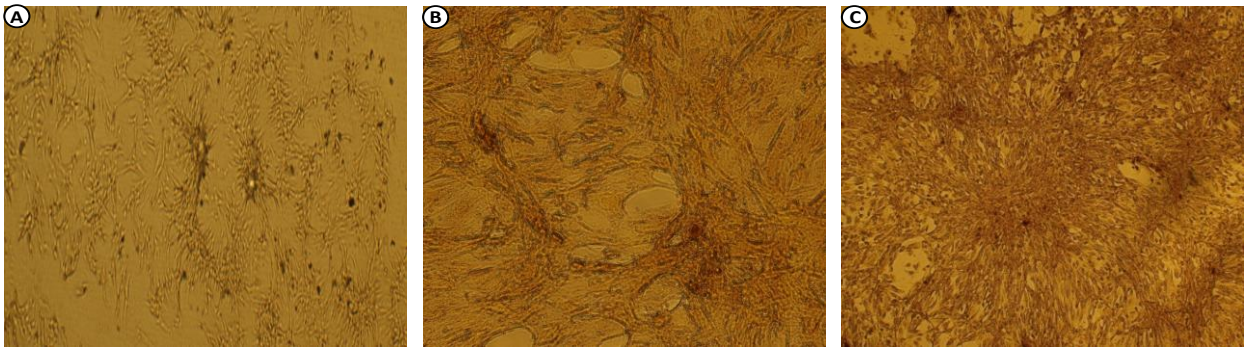

Figure 4: Photomicrographs of mesenchymal stem cells (MSC) derived from human amniotic fluid (AF) tissue showing differentiative potential towards osteogenic. **(B & C):** The differentiations of MSCs toward osteoblasts were indicated by the formation of calcium-rich hydroxyapatite which detected with Alizarin red and appear as irregular red –orange. **(A)**: Control cultures, although becoming over-confluent after three weeks culture time, retained their fibroblast like appearance, did not form cells aggregates and were stained negative for mineral deposition (original magnification × 200).

2.3.2. Real time polymerase chain reaction

Differentiation of human amniotic fluid (AF) tissue MSCs were further demonstrated by RT-PCR analysis of genes markers expression. Differentiation was further demonstrated by RT-PCR analysis of osteocyteic genes markers expression. Human amniotic fluid (AF) MSCs in osteiogensis culture conditions expressed osteocalcin at different time intervals, after 4 days, 7 days 14 days and 21 days and not for control (Table 1) (Figure 5).

Table 1: Osteocyteic genes markers expressions were detected by RT-PCR after 4 days, 7 days 14 days and 21 days.

Days	Osteocalcin gene CT		GAPDH CT
	Treated	Control	
4	32.73	Undetectable	32.85
7	33.85	Undetectable	32.73
14	31.38	Undetectable	35.32
21	31.79	Undetectable	32.59

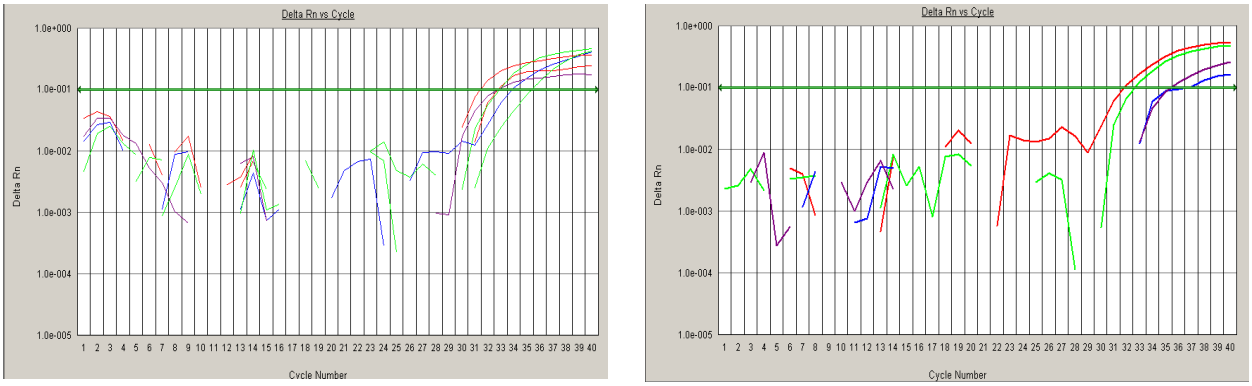

Figure 5: Showed RT- PCR gene expression for osteocalcin during osteiocytic of amniotic fluid MSCs after 7 and 14 days.

3. Discussion

The present study demonstrated that after placing human amniotic fluid (AF) in plastic culture dishes with different passages in culture media contains hematopoietic non-adherent cells (hematopoietic stem cells) along with a rare population of plastic-adherent cells (MSCs). After a few days, these adherent cells, of heterogeneous appearance, start to proliferate. The initial clones of adherent cells expanded into round-shaped colonies composed of fibroblastoid cells, thus the term of CFU-F. These results are consistent with another report [23].

In our study we were able to investigate cell-surface markers. Prusa *et al*., 2003 [6] demonstrated that human amniotic fluid cells expressed Oct4 mRNA, scored positive for mesenchymal markers such as CD90, CD105 (SH2), CD73 (SH3/4), CD166 but were negative for hematopoietic markers such as CD45, CD34, CD14, and this was in agreements with our results [14]. Another report showed that AFCs expressing Oct4, CD44 and CD105, but not expressing CD34 [7]. De Coppi *et al.*, in 2007 [11] isolated clonal cell lines from human Octa 4 positive AFCs positive for mesenchymal but negative for hematopoietic markers [11]. Our findings were in agreements with all previous authors [6,7,11,14]. Our data obtained from flow cytometry analysis revealed that human amniotic fluid cells expressed different surface antigens (CD markers) such as CD29, CD13, CD105, CD90 and Oct4 as markers for MSCs and lacking of the hematopoietic stem cell markers such as CD34 and CD14.

In the present study, osteogenic differentiation was performed in monolayer culture for 3 weeks. Cells were induced towards osteogenesis after reaching confluences. With the induction medium cells changed from a fibroblastic appearance to a more polygonal appearance and formed nodules. After 3 weeks culture time, the induced cells were stained positive with Alizarin Red S stain for mineral deposition in their newly formed matrix. Control cultures, although becoming over-confluent after 3 weeks, retained their fibroblast like appearance, did not form cell aggregates and were stained negative for mineral deposition. This data was corresponds to another study by [24], who reported the same results that suggested after 3 weeks of culture in osteogenic induction medium, showed amnion mesenchymal cells calcium deposition, which was visualized by positive staining for alizarin red.

The established real-time quantitative RT-PCR technology has made mRNA analysis more reproducible, precise, and sensitive than conventional RT-PCR, because it allows measuring the amount of amplified product (i) using a quantitative laser-based method and (ii) in the early exponential phase of the PCR reaction, when none of the reagents is rate limiting [25]. In this study, we developed real-time quantitative PCR assays for genes encoding the most typical osteoblast-related membrane and extracellular matrix molecules.

Osteocalcine gene found that mRNA levels for OC increased with time in culture [26], this finding supported our work and revealed that human amniotic fluid (AF) MSCs in osteiogensis culture conditions expressed osteocalcin at different time intervals, after 4, 7,14, and 21 days. Osteogenic differentiation was observed after induction with osteogenic medium for 2 to 3 weeks. In our study, we were able to measure the osteogenic differentiation by quantitative analysis of RT-PCR and not qualitative assay of PCR in contrast with [26], to confirm osteogenic differentiation.

In conclusion, our results demonstrated that human amnuotic fluid have the ability to survive, proliferate, and differentiate into osteogenic lineage when cultivated with the appropriate induction components, differentiate into highly specialized and functionally. The resulting differentiated cells display structural features similar to resident cells in the original tissues as determined by light microscopy and gene expression (RT-PCR) techniques. A deeper insight into the differentiation potential of MSCs may help to promote the future use of MSCs in regenerative medicine.

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