

Differentiation of mesenchymal stem cells from human amniotic fluid to cardiomyocyte-like cells

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Abstract. Ischemic heart disease (IHD) is a major factor influencing worldwide mortality rates. Furthermore, IHD has become a significant health problem among the Thai population. Stem cell therapy using mesenchymal stem cells (MSCs) is an alternative therapeutic method that has been applied to improve the quality of life of patients. Amniotic fluid (AF) contains a heterogeneous cell population, including MSCs, which are multipotent stem cells that have the capability to differentiate into mesenchymal lineages. The purpose of the present study was to evaluate the MSC characteristics of human (h)AF and determine its potency regarding cardiogenic differentiation. MSC characterization following flow cytometric analysis revealed that the cells expressed MSC markers, cluster of differentiation (CD)44, CD90, human leukocyte antigen-ABC and CD73. The results of the alamar blue assay demonstrated that cell proliferation rate continuously increased from the early cultivation phase up to 5-fold during days 1 to 5 of cell culturing. The highest rate of cell proliferation was observed on day 17 with a 30-fold increase compared with that on day 1. During the cardiogenic induction stage, morphological changes were observed between day 0 and day 21, and it was revealed that the hAF derived-MSCs in the cardiogenic-induced group exhibited myotube-like morphology after 7 days of cell culturing. Following cardiogenic induction, immunohistochemistry staining was performed on day 21, and reverse transcription-quantitative polymerase chain reaction on day 7 and 21. These steps were performed to detect the protein and gene expression levels of

cardiac specific proteins (GATA4, cardiac troponin T, Nkx2.5 and Connexin43). The results of the present study indicated that hAF-MSCs possess the potential to differentiate into cardiomyocyte-like cells. Thus, it was concluded that hAF may be a suitable source of MSCs for stem cell therapy and tissue engineering.

Introduction

Ischemic heart disease (IHD) is a major factor in terms of influencing worldwide mortality rates. IHD has also become a significant health problem among the Thai population and has led to a high mortality rate in the country (1). Moreover, this disease is an important cause of myocardial infarction (MI), which is related to atherosclerosis. When the mechanical change of myocardium occurs after the ischemic condition, cardiomyocytes die and progress to form the scar tissue that often leads to irreversible defects in cardiac functions (2). Naturally, a heart has a limitation for tissue regeneration due to the fact that cardiomyocytes cannot divide after birth (3-5). Treatment options for the ischemic condition involve taking medicine, surgical procedures and stem cell therapy using mesenchymal stem cell (MSCs) (3). MSCs are a type of adult stem cells that have the capability of being differentiated into cardiomyocytes when they were induced by certain factors such as 5-azacytidine (5-aza) (6-11). These cells can be isolated from bone marrow, adipose tissue, amniotic fluid, the umbilical cord, placenta and peripheral blood (12,13). They have displayed plastic adherent properties when maintained under standard culture conditions. They express specific cell surface markers such as CD29, CD44, CD73 and CD90 and can be differentiated into various mesenchymal lineages (14,15). The most common source of MSCs for clinical use is human adult bone marrow; however, the differentiating capacities are low with regard to the age of the donor and the isolation method is considered an invasive technique (8). Thus, different fetal tissues have been studied for their potential as an alternative source. Amniotic fluid (AF) is a new putative source of MSCs which contain a heterogeneous cell population derived from placental membranes and fetal origins (15).

Human amniotic fluid (hAF) is a clear and yellowish fluid that surrounds and protects the fetus in the uterus. It contains

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a variety of cells that have generated from fetal origins (15). Embryonic and fetal cells from all three germ layers have long been identified in the AF (16). Previous studies have isolated and characterized these cells based on their morphology and have reported that AF colonies consist of adherent 'spindle'-shaped fibroblast-like cells and 'round'-shaped epithelioid cells, but the epithelial cells disappear during the propagation of mixed primary cell cultures (15). The properties and types of AF cells vary for different donors and the time needed for gestation and cultivation. Moreover, these cells can be classified into three types based on their morphological and growth characteristics: Epithelioid type (E-type cells) which appear at the beginning of the cultivation process and decrease during the cultivation process, amniotic fluid-specific type (AF-type cells) which appear at the beginning of the cultivation process and persist during the cultivation process and fibroblast type (F-type cells), which are believed to have originated from mesenchymal tissues (17). AF cells and amniotic fluid derived-mesenchymal stem cells (AF-MSCs) are different because AF cells are non-adhering cells, while AF-MSCs are cells that are adhering cells and display dividing colonies (17). The AF-MSCs obtained from amniocentesis in the second-trimester (16th-22nd weeks) of pregnancy for prenatal diagnosis also express MSCs surface markers such as hyaluronan receptor (CD44), Thy-1 (CD90), transforming growth factor-beta receptor endoglin (CD105) and ES marker CD117 (14,18). Moreover, about 90% of the AF-MSCs express the octamer-binding transcription factor 4 (Oct-4) that is recognized as the transcription factor of embryonic stem cells and can express pluripotent stem cells markers; TERT (17). The AF-MSCs represent the intermediate stage between embryonic stem cells and adult stem cells (18,19). The AF-MSCs have a high renewal capacity and can be expanded for over 250 doublings without loss of chromosomal telomere length (17). The advantageous consequence of obtaining AF-MSCs is that it is easy, safe and presents a low risk of destroying embryos. Certainly, MSCs obtained from AF are recognized for presenting little to no ethical objections and are useful in cell transplantation in immunologically matched recipients (17,19).

Materials and methods

The cell samples and preparation. The hAF cell samples were received from the 16th-22nd weeks of gestation by amniocentesis for prenatal diagnosis from the Human Genetics Laboratory, Department of Anatomy, Faculty of Medicine, Chiang Mai University. The cell samples displayed a normal karyotype (46, XX/46, XY). Written informed consent was obtained after approval by the Ethics Committee from the Faculty of Medicine, Chiang Mai University, 21st April 2015 no. ANA-2558-2942.

In this study, the direct adherence method was used to separate human amniotic fluid derived-mesenchymal stem cells (hAF-MSCs) (20). Briefly, hAF cells were centrifuged at 2,035 g for 6 min and then, plated in 25 cm² flasks (Corning Incorporated, NY, USA) with expansion medium (BIOAMF-3™ Complete Medium) (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C, 5% CO₂ and 95% humidity until the colonies of the adherent cells appeared. After that, the basal growth medium, Dulbecco's Modified Eagle Medium (DMEM)-high glucose (Gibco, USA) was

replaced supplement with 10% fetal bovine serum (FBS) (Gibco, South America), gentamycin, Pen Strep (penicillin and streptomycin) (Gibco, USA) in order to remove non-adherent cells from the supernatant. The medium was changed every 3 days. After the cells reached 80% confluence, they were sub-cultured using 0.25% trypsin-EDTA (Gibco, USA) and re-expanded in a 25 cm² flask under similar conditions at a dilution of 1:2. The hAF cell samples at passage 3 were used in the analysis.

Cell cultivation. The hAF cell samples in passage 3 (n=5) were washed twice with sterile phosphate-buffered saline (PBS) (Amresco, Ohio, USA) and trypsinized with 0.25% trypsin-EDTA. Subsequently, hAF cells were suspended in the basal growth medium (DMEM-high glucose with 10% FBS) and centrifuged at 2,035 g, for 6 min. After that, the supernatant liquid was removed and the hAF cells were used in the experiments.

The hAF cell samples were seeded in 24-well culture plates (Corning Incorporated, NY, USA) at the density of 1x10⁵ cells. Cells were divided into two groups under different culture medium conditions. The control group was cultured with basal growth medium and the cardiogenic induced group was cultured with cardiogenic induced medium (DMEM-high glucose, 10% FBS, gentamycin, Pen Strep + 10 µmol/l 5-aza (Sigma-Aldrich, St. Louis, MO, USA)) (8). After 24 h, the cardiogenic induced medium was changed to basal growth medium and the culture medium was changed every 3 days for the duration of the 21 days of cell culturing.

Flow cytometry. The hAF cell samples in passage 3 (n=3) were used to determine MSCs markers expression. The cells were trypsinized with 0.25% trypsin-EDTA and centrifuged at 2,035 g for 6 min and then, they were incubated with monoclonal antibodies; fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34, CD90 (Biolegend, San Diego, USA), mouse anti-human HLA-ABC (Immuno Tools GmbH, Friesoythe, Germany) and mouse anti-human fibroblast (EDM, Millipore Corp, UK), as well as phycoerythrin (PE)-conjugated mouse anti-human CD31, CD117, HLA-DR (Immuno Tools GmbH, Friesoythe, Germany), mouse anti-human CD44, CD105 (Pierce Biotechnology, Rockford, USA), mouse anti-CD45 (Biolegend, San Diego, USA) and mouse anti-human CD73 (Life Technologies, California, USA) for 60 min at 4°C. FITC mouse isotype control and PE mouse isotype control (Biolegend, San Diego, USA) were used as negative controls. Cell fluorescence was evaluated using FACscan (Becton Dickinson, Lincoln Park, NJ) and analyzed using the CellQuest Pro 9.0 software (Becton Dickinson).

Alamar blue assay. This assay is a colorimetric indicator used to measure living cell proliferation and cytotoxicity evaluation from the oxidation-reduction of the living cell metabolism through the conversion of resazurin (oxidised form, blue color) to resorufin (reduced form, pink color) and to monitor the absorbance of alamar blue at 540-630 nm. In brief, the cells in passage 3 (n=3) were cultured in 24-well culture plates of 2x10³ cells/well with basal growth medium for 24 h. Thereafter, the basal growth medium was removed and 100 µl 10% alamar blue in DMEM was added, and then the samples

Table I. The primers for RT-qPCR and products size.

Gene (marker)	Primer sequence	Size (bp)
<i>GATA4</i>	F5'-CTGTGCCAACTGCCACACCA-3' R5'-GGCTGACCGAAGATGCGTAG-3'	437
<i>cTnT</i>	F5'-GGCAGCGGAAGAGGATGCTGAA-3' R5'-GAGGCACCAAGTTGGGCATGAACGA-3'	150
<i>Cx43</i>	F5'-GAATCCTGCTCCTGG-3' R5'-GATGCTGATGATGTAG-3'	380
<i>Nkx2.5</i>	F5'-CTGCCGCCGCCAACAAC-3' R5'-CGCGGGTCCCTTCCCTACCA-3'	136
<i>GAPDH</i>	F5'-ATGGGGAAG GTGAAGGTCG-3' R5'-TAAAAGCAGCCCTGGTGACC-3'	70

were incubated at 37°C, 5% CO₂ with 95% humidity for 4 h. After that, the supernatant liquid samples were evaluated using the colorimetric change from each well, while hAF cells were continuously cultivated under the same conditions. Absorbance measurement was done on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 of cell culturing using a spectrophotometer plate reader (Original Multiskan EK, ThermoScientific, UK) and a sample consisting of 10% alamar blue solution that was unexposed to the cells was used as a control.

Immunofluorescence analysis. After 21 days of cells cultivation, the cells were cultured on coverslips (Thermo Scientific, UK). The basal and cardiogenic induced groups (n=5) were used to evaluate the expression of cardiac specific proteins. After fixation for 30 min at 4°C with 4% paraformaldehyde, the cell membranes were permeabilized for 5 min with 0.2% triton X-100 (Amresco, Ohio, USA) in PBS and blocked in 10% AB-serum in 1% bovine serum albumin (BSA) in PBS for 30 min at 4°C. The cells were incubated with mouse monoclonal primary antibodies against human GATA4, cardiac troponin T (cTnT) and Nkx2.5 (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 4°C. After being washed with PBS, the cells were incubated with goat anti-mouse secondary antibody conjugated with FITC (Thermo Scientific, UK) for 60 min at 37°C. Subsequently, the cover-slips were mounted onto the microscopic slides using anti-fade reagent with 4'-6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA). The cells were visualized using a fluorescence microscope Olympus AX70. Photographs were taken with DP manager and DP controller (Olympus Life Science, USA).

Immunoenzymatic analysis. After 21 days of cell cultivation, the cells cultured on coverslips (Thermo Scientific, UK) from the basal and cardiogenic induced groups (n=5) were used to evaluate the expression of cardiac specific proteins. After fixation for 30 min at 4°C with 4% paraformaldehyde, the cells were blocked in 10% AB-serum in 1% BSA in PBS for 30 min at 4°C, and then incubated with rabbit monoclonal primary antibody against human connexin43 (Cx43) (Sigma-aldrich, St. Louis, MO, USA) for 2 h at 4°C. After being washed with PBS, the cells were incubated with mouse anti-rabbit peroxidase-conjugated secondary antibody (Immuno Tools

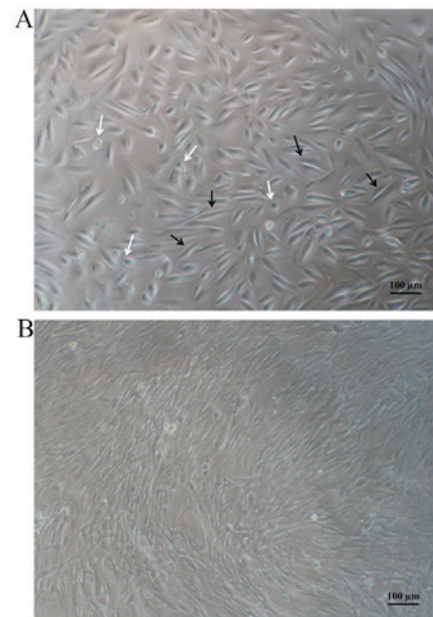


Figure 1. The morphology of hAF cells of the 1st passages; (A) showed a heterogeneous population consisting of fibroblast-like cells (black arrows) and epithelial-like cells (white arrows), (B) while the 3rd passages displayed the most of cells had fibroblast-like morphology.

GmbH, Friesoythe, Germany) for 60 min at 37°C. Finally, the immunoreaction was detected by using 3,3'-Diaminobenzidine (DAB) liquid substrate system (Sigma-aldrich, St. Louis, MO, USA) and analyzed under the Axiostar plus light microscope (Carl Zeiss, Germany). Photographs were taken with Canon pc 1049 PowershotG5 (Canon, USA) and were used to determine and normalize the average level of color intensity in both the basal and cardiogenic induced groups using iSolution FL Auto x64.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). After 7 and 21 days of cell cultivation, total ribonucleic acid (RNA) obtained from the basal and the cardiogenic induced groups (n=5) were prepared using an Illutra RNAspin Mini RNA Isolation kit (GE Healthcare, LittleChalfont, UK). Complementary DNA (cDNA) was

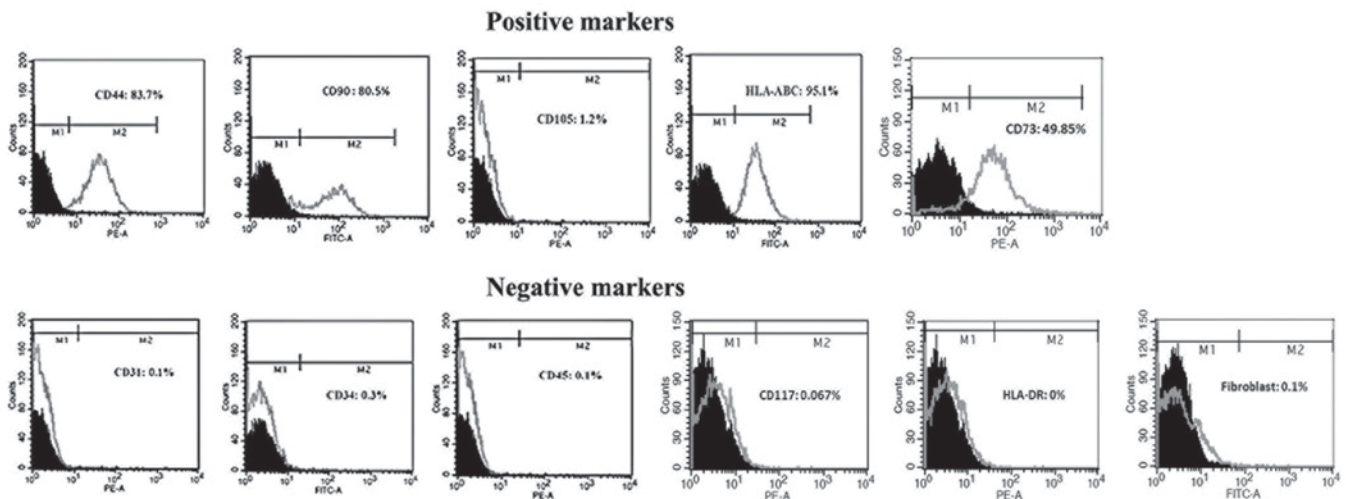


Figure 2. Flow cytometry analysis of hAF cells to determine the MSCs characteristics showed the positive for the MSCs markers CD44, CD90, HLA-ABC and CD73 while showing negative for CD105, platelet endothelial cell adhesion marker CD31, hematopoietic stem cell markers CD34 and CD45, amniotic fluid stem cells marker CD117, HLA-DR and fibroblast marker.

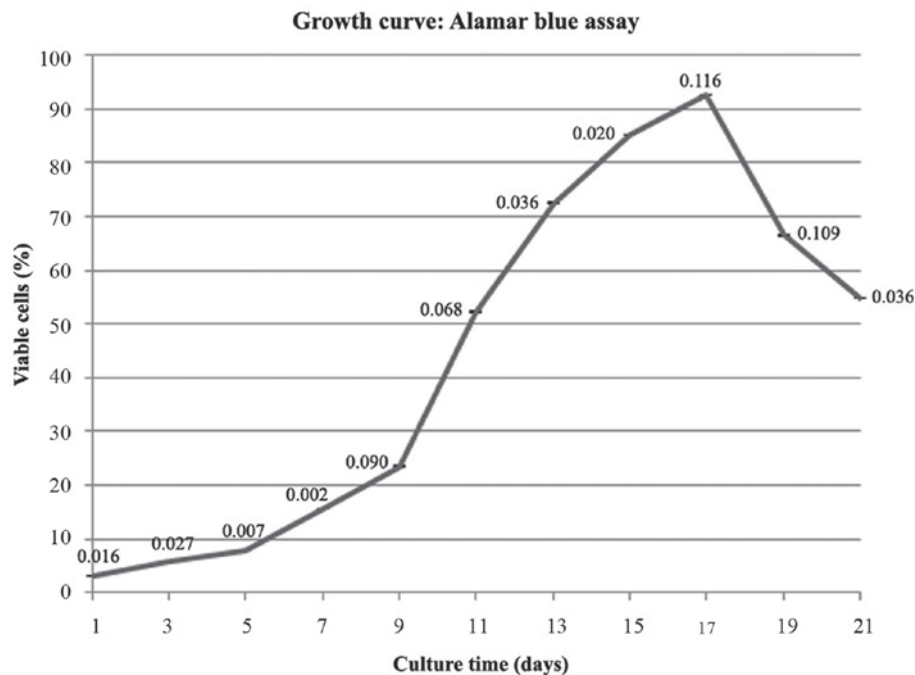


Figure 3. Results of alamar blue assay showed the percentage of the reduction and growth characteristics of hAF-MSCs after being cultured for 21 days. The error bars show the mean and standard deviations (SD) (n=3) on each day of cell cultivation.

synthesized using the iScript™ cDNA Synthesis kit (Bioline, USA) according to the manufacturer's instructions. The RT-qPCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad, Singapore) on a Chromo4™ Real-Time PCR Detector (Bio-Rad, USA) with gene specific primers (Table I). The PCR mix contained SsoFast™ EvaGreen® Supermix (Bio-Rad, Singapore), cDNA template, primers, and nuclease free water to reach a final volume of 20 μ l. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control gene for normalization. A melt curve analysis was performed at the end of the reaction and the profiles were obtained by plotting relative gene expression levels of the both basal and cardiogenic induced groups.

Statistical analysis. The data were analyzed using SPSS version 22 (SPSS, Inc., Chicago, IL). Data are expressed as the mean \pm SD. The significance was analyzed using the Mann-Whitney U test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Morphology. The hAF cell samples were cultured with expansion medium until the colonies of the adherent cells appeared. After being subcultured, the hAF cells were investigated using inverted microscopy. Inverted microscopy revealed that the hAF cell samples were adhered to culture flasks and showed a

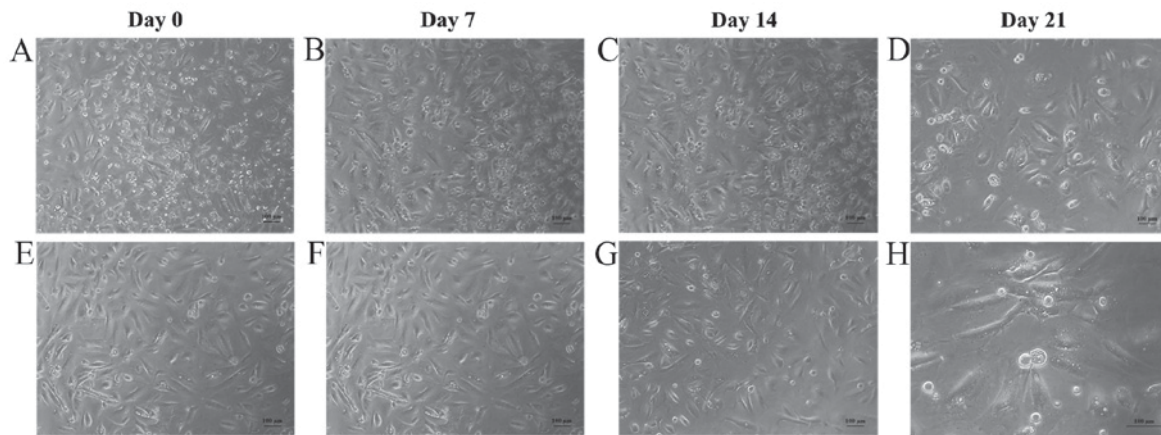


Figure 4. Morphology of hAF-MSCs after cardiogenic induction; (A-D) in basal group culture in the basal medium that maintained fibroblastic morphology from day 0 to day 21, (E-H) cardiogenic induced group showed elongated myotube-like morphology and form aggregations after 7 days and maintained this until 21 days of cell culturing. Scale bar in A-G represent 10x and in H 20x.

heterogeneous population. Moreover, the 3rd passages exhibited fibroblast-like morphology (Fig. 1).

Flow cytometry. The hAF cells were characterized for MSCs surface markers using flow cytometry analysis. The results showed that the hAF cells were stained positively for MSCs surface markers CD44 (83.7%), CD90 (80.5%), HLA-ABC (95.1%) and CD73 (49.85%). Interestingly, the hAF cells were not positively stained with anti-CD105 (1.2%). As expected, the hAF cells were negatively stained with platelet endothelial cell adhesion molecule CD31 (0.1%), hematopoietic cell surface marker CD34 (0.3%) and CD45 (0.1%), amniotic fluid stem cells CD117 (0.067%), HLA-DR (0%) and fibroblast (0.1%) (Fig. 2).

Alamar blue assay. The alamar blue assay was used to evaluate the ability of cell proliferation. The results showed that the hAF cell proliferation continuously increased from the early stage of cultivation (Day 5) following the logarithmic phase. After that, the cells began to proliferate swiftly and the cell population doubled over 12 days (day 5-day 17). This event showed an exponential phase of the cell cycle. The highest cell proliferation had been observed on day 17 with 30-fold relative to day 1. Afterward the cell numbers began to decrease (Fig. 3).

Cardiogenic differentiation potential. In the hAF-MSCs in both the basal and cardiogenic induced groups, morphological changes were observed using inverted microscopy beginning at day 0 until day 21. The cells in the cardiogenic induced group cultured with 5-aza showed elongated myotube-like morphology and formed aggregations after 7 days of culturing. The myotube-like morphology was maintained until 21 days of cell culture. In the basal group, culture in the basal growth medium was maintained their fibroblastic-like morphology (Fig. 4). Immunofluorescence staining, immunoenzymatic staining and RT-qPCR were used to determine the cardiogenic differentiation potential of hAF-MSCs. After 21 days of cell culturing, the cells that had been cultured on coverslips in both groups (basal and cardiogenic induced groups) were investigated for cardiogenic specific proteins (GATA4, cTnT, Nkx2.5

and Cx43). The results of the immunofluorescence staining and immunoenzymatic staining indicated that the cardiogenic induced group was strongly positive for the cardiac-specific markers GATA4, cTnT, Nkx2.5 and Cx43. Interestingly, the results of the immunofluorescence staining displayed positively stained GATA4, cTnT and Nkx2.5 in the cardiogenic induced group, but it was not positive in the basal group. The immunoenzymatic staining displayed positively stained Cx43 in the cardiogenic induced group (73.5%), but it was a very weak positive in the basal group (17.8%) (Fig. 5). Moreover, the cardiogenic differentiation capacity of these hAF-MSCs was confirmed by using RT-qPCR. After 7 and 21 days of cell culturing, the cells in both groups (basal and cardiogenic induced groups) were evaluated for cardiogenic specific genes (GATA4, cTnT, Nkx2.5 and Cx43). The results of the RT-qPCR indicated that the hAF-MSCs in the cardiogenic induced group could express the cardiogenic specific genes that were related to cardiogenesis. The cardiogenic induced group at days 7 and 21 had a significant expression of cardiac specific genes that was greater than the control group ($P < 0.05$) (Fig. 6).

Discussion

Previous studies have shown that MSCs from bone marrow can improve cardiac function in myocardial infarction (21,22) and cardiogenic differentiation from bone marrow MSCs has long been investigated (23,24). However, the process of using MSCs obtained from this source has displayed certain limitations including the invasive technique needed to obtain MSCs from bone marrow, the decreased capacity of these cells that correlate with the increased age of the donor (25) and the obstacle of cell transplantation therapy by immunological rejection (26). Thus, alternative sources of MSCs have been studied and AF has recently become a new and interesting source of MSCs. It contains a heterogeneous population of cells in the placental membrane as well in other areas stemming from its fetal origin including the fetal skin, the epithelial, digestive and respiratory tract, along with the urinary tract (15). AF is considered fairly easy to collect by amniocentesis using ultrasound-guided transabdominal puncture for prenatal diagnosis with a low risk of damaging

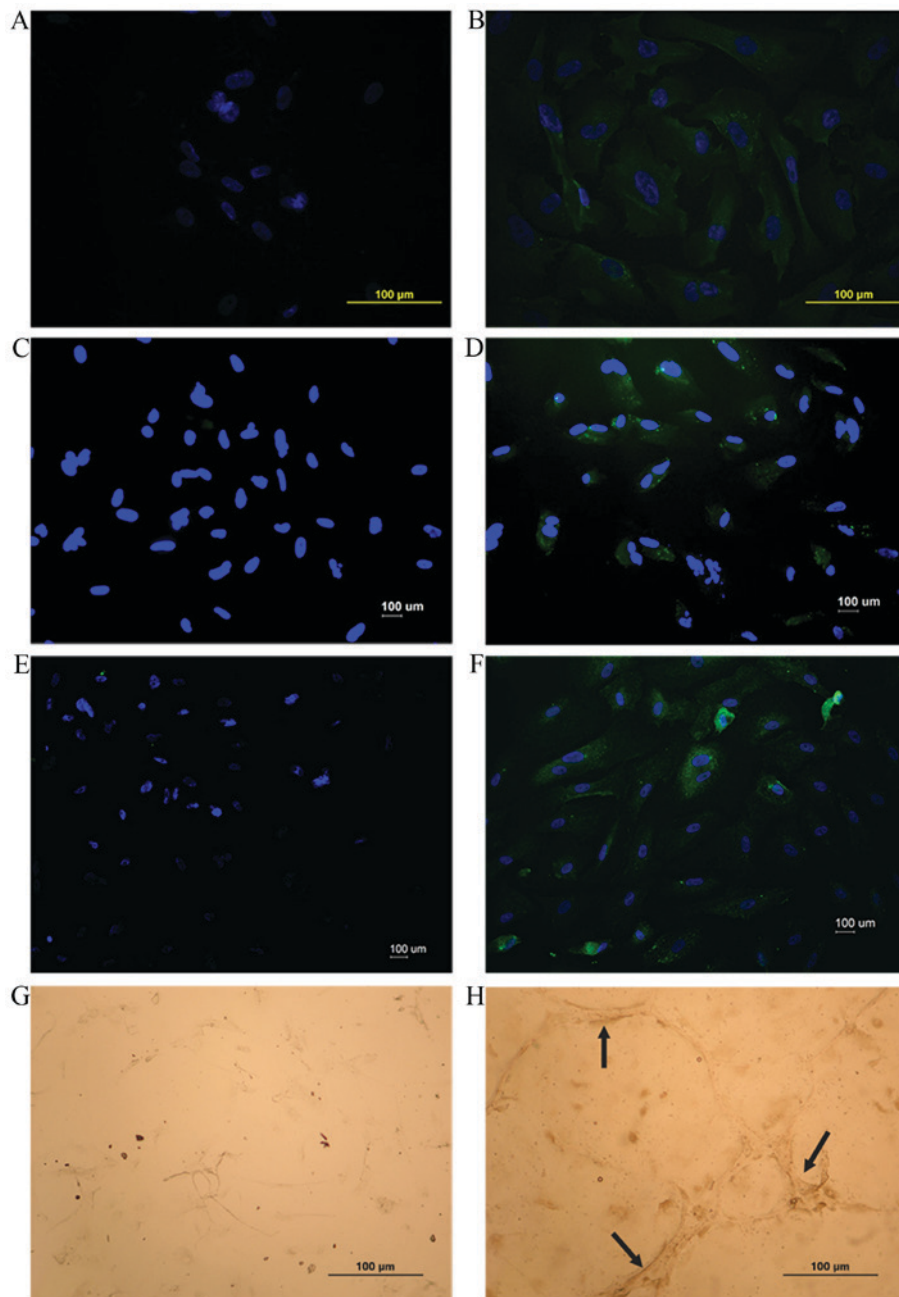


Figure 5. Detection of the cardiomyocyte specific proteins; fluorescence staining (A-F) and immunoenzymatic staining (G-H); (A) basal group, (B) induced group GATA4-positive cells (green fluorescence); (C) basal group, (D) induced group cTnT-positive cells (green fluorescence); (E) basal group, (F) induced group Nkx2.5-positive cells (green fluorescence); (G) basal group, (H) induced group Cx43-positive cells (pointed arrows indicate brown color).

the mother or the embryos and low ethical concerns when compared with other sources such as embryonic stem cells (17). Previous studies have demonstrated that AF-MSCs are multipotent stem cells, which are capable of self-renewal, high expansion rates, a high number of isolated cells (27), and a low risk of tumorigenicity (28). In addition, AF-MSCs are capable of chondrogenic, osteogenic, adipogenic and myogenic differentiation (14,29-31). Over the years, MSCs have been isolated from various tissues and organs including bone marrow, adipose tissue, skin, dental pulp, placenta and the umbilical cord (32). According to previous studies, it has been reported that mesenchymal amniocytes proliferate more rapidly *in vitro* when compared to fetal and adult stem cells (33,34). *In vitro*, MSCs usually grow as a

monolayer culture in a medium supplemented with FBS (35). Furthermore, the hAF-MSCs expressed both embryonic and adult stem cell markers. The ability of this cell has been acknowledged at the intermediate stage of embryonic stem cells and adult stem cells (18,19,36). These cells may present more advantages than adult stem cells and show a wide differentiation of properties that can induce mesenchymal lineages (14).

The hAF cells showed heterogenous population colonies and these cells exhibited fibroblast-like morphology in the 3rd passage. They adhered to the culture flask, a feature that resembled previous studies and it was concluded that hAF-MSCs could easily be expanded without feeder cells, exhibit fibroblastoid spindle shape morphology (29,31,32) and

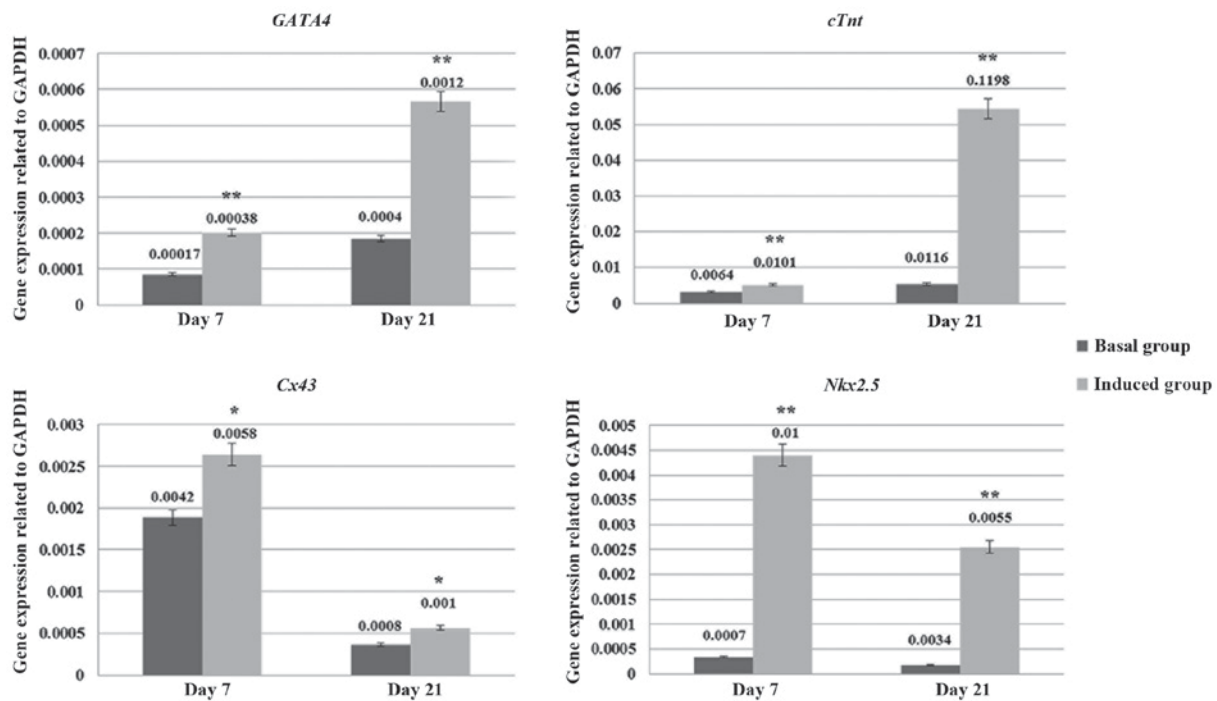


Figure 6. Results of cardiac specific gene expression (*GATA*, *cTnT*, *Cx43* and *Nkx2.5*) in the cardiogenic induced group had significant greater than the basal group on the day 7 and 21. * $P < 0.05$, ** $P < 0.01$ vs. basal group.

could expose plastic adherents when maintained under standard culture conditions (14).

The present study aimed to characterize hAF-MSCs and evaluate the cardiogenic differentiation capacity of the hAF-MSCs. Flow cytometry was used to determine the surface antigens of the MSCs (Cut). It has been previously reported that hAF-MSCs strongly expressed MSCs markers such as CD29, CD44, CD73, CD90, CD144, CD166, and HLA-ABC (37-40). Moreover, hAF-MSCs showed a positive co-expression of pluripotent markers such as *Otc-4* (19), embryonic antigens *Nanog* and (*SSEA*)-4 (12,32,38). The results of our experiments showed that the hAF cells in passage 3 were strongly positive for MSCs surface markers including the transmembrane glycoprotein CD44, the hyaluronan receptor which plays a role in MSCs migration, membrane glycoprotein CD73 that is often used for MSCs marker, membrane glycoprotein CD90 or *Thy-1* that is often used as a marker for several kinds of stem cells such as keratinocyte stem cells, endometrial stem cells and mesenchymal stromal cells (32,41,42) and the HLA-ABC (HLA class I) marker. On the other hand, these cells were negative for platelet endothelial cell adhesion marker CD31, hematopoietic cell surface markers including CD34 and CD45, CD117 or *c-kit* that found in amniotic fluid stem cells (43), HLA-DR and fibroblast marker. Furthermore, this investigation found that the MSCs surface antigen was different. The percentage of positive expression was between 80-95% in each marker and excluded CD105, transforming growth factor-beta receptor endoglin, or SH2 expression of 1.2%. In previous studies, it has been reported that the percentage of CD105 expression was varied among the different cell populations in the amniotic fluid (36). CD105 is a proliferation-associated and hypoxia-inducible protein that is abundantly expressed in the angiogenic endothelial cell, while

monocyte and immunohistochemistry studies have revealed that CD105 is strongly expressed in blood vessels of tumor tissues (44). Moreover, the expression of CD105 depends on the serum supplemented in the culture medium, cell passage, age of gestation, maternal age and source of MSCs (15,45,46). All factors influenced the different levels of surface antigen expression (47). The hAF cells were strongly positive for HLA-ABC (HLA class I) and negative for HLA-DR (HLA class II), which suggested that this cell has the potential for clinical applications. The previous study showed that cardiomyocytes differentiated from AF-MSCs transplantation and may be helpful in avoiding unexpected events such as calcification (48).

In this study, the determination of hAF-MSCs proliferation using alamar blue assay exposed the continuously increased from the early cultivation phase up to 5-fold during day 1 to 5 of cell culturing, following a logarithmic phase or the induction phase of the cell cycle. After that, the cells started to proliferate swiftly and the cell population doubled from day 5 to day 17. The highest cell proliferation had been observed on day 17 with 30-fold relative to day 1. This event showed an exponential phase of the cell cycle. Afterward, the cell numbers began to decrease. These results indicate that these cells had a proliferation capacity, which is related to MSCs growth and proliferation properties. In agreement with previous study, the hAF-MSCs presented a slow rate of proliferation in the first week of cell cultivation, after which the proliferation swiftly increased; this finding was different in AF cells that were derived from various gestational stages (16,33,47).

The MSCs revealed a multipotency capability differentiation with regard to the variety of cell types of all three embryonic germ layers (14,15) including cardiomyocytes (25). In this study, the hAF-MSCs were induced to cardiomyocyte-like

cells using 5-aza for 24 h. (Cut) The morphology was observed from day 0 until day 21 of cell culturing in both the basal and cardiogenic induced groups. From previous studies have been reported that, the MSCs morphology were changed to myotube-like structure after induced with 5-aza for 24 h and maintained in standard culture condition for 7-10 days (7,11). In this study the cells in the cardiogenic induced group showed the myotube-like morphology and formed aggregations after 7 days of culturing. The myotube-like morphology was maintained until day 21 and related to the cardiac specific gene expression on day 7 and day 21 of cell culturing. After day 21, cells in both the basal and cardiogenic induced groups were examined for cardiac specific proteins using immunofluorescence and immunoenzymatic staining. Expression of the cardiac specific genes was detected using RT-qPCR on days 7 and 21 of cell culturing. The results showed that the cardiogenic induced group strongly expressed the cardiac specific proteins (GATA4, cTnT, Nkx2.5 and Cx43) and genes (*GATA4*, *cTnT*, *Nkx2.5* and *Cx43*) ($P<0.05$). These were highly expressed in the cardiogenic induced group, which was different from the basal group. GATA4 is an important transcription factor in term of early myocardial development, which is expressed in the cytoplasm. Moreover, the expression of GATA4 increases the potential of MSCs to be differentiated into cardiomyocytes (49). The cTnT is a protein which functions as a part of the troponin complex of myofibrils only in the cardiac muscles and it is expressed in the cytoplasm (50,51). Nkx2.5 is a transcription factor for cardiac development, which play an important role in early cardiac development and express in the cytoplasm (51). Cx43 is one variety of the gap junction proteins, which found in the atrial and ventricular myocytes (52) and maintains electrical activity (9). GATA4, cTnT, Nkx2.5 and Cx43 promote the differentiation of MSCs into cardiomyocytes and are required for cardiogenic differentiation (53,54). Moreover, GATA4 and Nkx2.5 are the greatest transcription factors that have major regulatory roles in the cardiogenic differentiation of MSCs (9). All of the results in this study indicated a strong correlation with the early evaluation of cardiogenic differentiation of AF-MSCs and corresponded with previous studies, which reported that AF-MSCs can be expanded *in vitro* and can be differentiated into cardiomyocytes with a strong expression of cardiac specific proteins and genes (12,55,56).

5-aza is a DNA methylation inhibitor that is mainly used in the treatment of myelodysplastic syndrome (MDS) and it can induce MSCs into cardiomyocytes. In previous studies, it has been reported that 5-aza induced the up-regulation of phosphorylated cardiac specific genes through the ERK pathway and these results suggested that the sustained activation of ERK by 5-aza contributed to the induction of the differentiation of MSCs into cardiomyocytes *in vitro* (57). Additionally, 5-aza can change the morphology of hAF-MSCs from a spindle shape to myotube-like morphology at about 7 days of cell culturing and maintained this until day 21. The results of the morphological changes can confirm cardiogenic differentiation with cardiac specific proteins expression on day 21 and genes expression on day 7 and day 21. (Cut).

In conclusion, our study has demonstrated that AF is an important source of MSCs that can be obtained from the routine amniocentesis investigation. These cells are easily

isolated and can be grown *in vitro* without feeder layers. In addition, they show the characteristics of multipotency MSCs and demonstrate the ability of increased proliferation. Nevertheless, they exhibit the capacity and potential for cardiogenic differentiation. The data obtained suggests that they are worthy of further investigation in term of the study of the regenerative medicine in stem cell transplantation and tissue engineering.

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