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Differentiation potential of human amniotic fluid-derived mesenchymal stem cells into hepatocyte-like cells

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ABSTRACT: Liver disease is recognized as a significant cause of death worldwide. Treatment methods involving tissue and/or organ transplantation utilizing stem cells have emerged as an attractive alternative. Among the candidate cells employed in regenerative medicine, mesenchymal stem cells (MSCs) have exhibited a promising potential as beneficial cell source. Herein, the purpose of this study was to characterize the attributes of human amniotic fluid-derived MSCs (hAF-MSCs) and induced hepatocyte-like cells. The results indicated that MSCs acquired from human amniotic fluid cells uncovered proliferative potency along with the expression of MSCs markers. After hepatogenic induction, the cells exhibited strong levels of expression of *ALB*, *AFP*, *HNF4*, and *CYP1B1* genes, as well as ALB and AFP proteins. In collaboration with the periodic staining associated with energy storage, the accumulation of glycogen within the cytoplasm was observed after 21 days of cell cultivation. Additionally, the cells were capable of internalizing indocyanine green into the cytoplasm. This reflected the potential detoxification of differentiated hepatocytes derived from hAF-MSCs, which could support the use of MSCs in cell-based therapy.

KEYWORDS: amniotic fluid, mesenchymal stem cell, hepatocyte, liver disease

INTRODUCTION

Liver conditions are considered a reflection of human health. Knowledge of liver conditions can play an important role in setting global health policy. Currently, 844 million people are inflicted with liver disease, while two million deaths per year result from complications of cirrhosis, viral hepatitis, and hepatocellular carcinoma. The current reported prevalence of cirrhosis is between 4.5% to 9% worldwide and in conjunction with a steadily rising mortality rate [1–3]. In reality, the figure could be higher because majority of patients do not exhibit clinical symptoms [2]. In addition, cirrhosis is the eleventh most common cause of death in adults worldwide. Accordingly, the health of approximately two billion people is negatively influenced by the consumption of alcohol, while more than 75 million people have currently been diagnosed with alcohol dependence. These two important factors have been recognized for their role in causing liver disease. Furthermore, people who are overweight and/or who have diabetes mellitus experience an even greater risk of suffering from liver disease [3]. Liver cirrhosis is the last stage of chronic liver disease, and it is very difficult to treat patients at this stage [4]. Nowadays, liver transplants are considered the most promising option for the effective treatment of chronic liver diseases. The demand of liver for organ transplants is the second largest in the world, with a current recorded supply of less than 10% of the current need. In addition, organ transplantation surgery is very costly, and patients might experience postoperative complications and/or the rejection of the newly transplanted organ [3, 4].

Stem cell therapy is a promising form of regenerative medicine that promotes the use of stem cells for applications in human development, restoration, and replenishment of tissues and organs due to their affirmed potential of self-renewal and related aspects of self-specialized differentiation [5]. The use of stem cells as an alternative method of treatment in regenerative medicine has revealed their potential to reduce invasive reactions in patients undergone cell and/or organ transplant surgery and suffering from post-surgery complications. Moreover, stem cell transplantation can also diminish the problems associated with donor-patient tissue rejections [6]. Stem cells, especially MSCs, exhibit a positive application potential in the field of regenerative medicine. The MSCs are known to express typical biomarkers such as CD44, CD73, CD90, and CD105; and they can be beneficially differentiated into cells belonging to various mesenchymal cell linages including adipocytes, myoblasts, cardiomyocytes, osteoblasts, and chondrocytes [7–9]. Normally, MSCs can be isolated from adipose tissue, tooth buds, bone marrow, blood, umbilical cord blood, Wharton's jelly, amniotic fluid, and placenta [10]. However, amniotic fluid (AF) is another more easily accessible source of MSCs. It can be obtained from wombs without causing any harm to the mother while having minimal detrimental effects to the fetus [7].

Human amniotic fluid-derived MSCs (hAF-MSCs) have been regarded as autologous therapeutic cells, and they can be derived from skin, urine, fetal membrane, and trophoblasts [9, 11, 12]. In previous studies, it has been reported that hAF-MSCs possess a significant ability to proliferate and profuse differentiation. Herein, the aim of this study was to induce the differentiation of hAF-MSCs into hepatocyte like-cells and to characterize the attributes of those hepatocyte-derived stem cells.

MATERIALS AND METHODS

Cell preparation

Human amniotic fluid (hAF) cells were isolated from the process of amniocentesis, involving 16 to 22 weeks of gestation during the course of prenatal diagnosis, with normal karyotypes (46, XX or 46, XY) obtained from the Human Genetic Laboratory, Department of Anatomy, Faculty of Medicine, Chiang Mai University and the Chromosome and Gene Clinic, Chiang Mai, Thailand. The process was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University (ANA-2562-06585) on the 11th of September 2019. Written informed consents were obtained from all participating patients.

In this study, hAF-MSCs were cultivated in a solution of basal growth medium, Dulbecco's Modified Eagle Medium (DMEM)—high glucose (Gibco, New York, USA) with 10% (v/v) fetal bovine serum (FBS) (Gibco, Brazil, South America), 40 mg/ml gentamycin, 10,000 U/ml penicillin, and streptomycin (Gibco, USA). The cells were then maintained in an incubator containing 5% CO_2 at 37 °C. The culture medium was changed every three days. After reaching 80% confluence of cell growth, a subculture was made using 0.25% trypsin-EDTA (Gibco® by Life technologies, New York, USA) in order to obtain an optimal quantity of cell numbers suitable for further experiments, in which a second passage of the cells through the culture media were used.

Flow cytometry analysis

To characterize the expression of MSCs markers for hAF-MSCs in the 2nd passage ($n = 3$), the cells were trypsinized with 0.25% trypsin-EDTA and centrifuged at 3,700 řpm for 6 min. They were then incubated for 60 min at 4 °C with monoclonal antibodies (mAbs): phycoerithin (PE) conjugated mouse anti-human CD31, CD45, CD73, CD117, and HLA-DR (Immuno Tools, Friesoythe, Germany); fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD 34, CD44, HLA-ABC, and fibroblast surface protein markers (Immuno Tools); as well as allophycocyanin (APC) conjugated mouse anti-human CD90 (Immuno Tools). PE, FITC, and APC mouse isotype controls (Biolegend, San Diego, USA) were used as negative controls. The expression of MSCs surface markers was measured by FACScan (Becton Dickinson, NJ, USA) and analyzed by CellQuest Pro 9.0 software (Becton Dickinson).

Alamar blue proliferation assay

Alamar blue proliferation assay is a technique used to establish quantitative proliferation within living cells based on the detection of the degree of oxidation–reduction (REDOX) of survival cells, which can be measured by the absorbance at 540–630 nm through the conversion of resazurin (oxidized form, blue color) to resorufin (reduced form, pink color). The hAF cells obtained from the 2nd passage ($n = 3$) were cultured in 24-well culture plates at a density of 2×10^3 cells/well with basal growth medium for 24 h. Consequently, the culture medium was discarded, and 10% (v/v) alamar blue in DMEM was added. The samples were then incubated at 37 °C, 5% CO_2 with 95% humidity for 4 h. The degree of absorbance of resorufin was continually determined using a micro-plate reader (Original Multiskan EK, ThermoSCIENTIFIC, UK) every other day for 21 days.

Hepatogenic induction

The 2nd passage cells were divided into two groups under differing culture medium conditions: control group and hepatogenic-induced group. Firstly, cell samples were seeded into 24-well-plates at a density of 3×10^4 cells/well with 500 µl of hepatogenic-induced medium (DMEM-high glucose supplemented with antibiotics including Pen Strep and gentamycin, l0% FBS, 20 ng/ml hepatocyte growth factor, 10 ng/ml fibroblast growth factor, and 10^{-7} M dexamethasone) in the hepatogenic-induced group; and 500 µl basal growth medium (DMEM-high glucose supplemented with antibiotics, Pen Strep and gentamycin, and 10% FBS) in the control group. Next, both groups were cultured in a 5% CO_2 incubator at 37 °C for 24 h. During the course of cell cultivation, the culture medium was changed every three days over a period of 21 days.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

This method was used to determine the expression of hepatogenic-related genes, which were *Albumin* (*ALB*), *Alpha-Fetoprotein* (*AFP*), *Hepatocyte Nuclear Factor-4* (*HNF4*), and *Cytochrome P450 Family 1 Subfamily B Member 1* (*CYP1B1*). *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) was applied for gene internal control after hepatogenic induction. The sequence of the primers was shown in Table S1.

Immunofluorescent analysis

After the culture period, the expression of hepatogenic specific proteins including albumin and alpha-fetoprotein were detected. Briefly, after fixing with

4% paraformaldehyde at 4 °C for 4 h; cell membranes were permeabilized with 0.2% triton X-100 (Amresco, Ohio, USA); and, then, non-specific binding was blocked with 10% AB-serum in 1% BSA-PBS for 30 min at 4 °C. The primary antibodies, mouse anti human albumin (1:100) and alpha-fetoprotein (1:100) (Sigma-Aldrich, Missouri, USA), were subsequently used for treating cells by incubation for 1 h at 37 °C. After the incubation, cells were washed twice with PBS and incubated again with goat anti-mouse conjugated with FITC (1:100) (Thermo Scientific, Loughborough, UK) for 1 h at 37 °C. Subsequently, the nuclei were stained, and the cover-slips were mounted onto microscopic slides using anti-fade reagent with 4′-6′-diamidino-2-phenylindole (DAPI) (Invitrogen, Massachusetts, USA). Finally, the cells were visualized using a fluorescent microscope Olympus AX70. Photographs were taken with DP manager and DP controller (Olympus Life Science, USA).

Histological analysis

Staining with Periodic acid schiff stain (PAS) was performed in order to determine the accumulation of glycogen within the cytosol. In brief, the hepatogenic induced medium was removed, and the cells were washed three times with PBS. Subsequently, they were fixed with 4% paraformaldehyde at room temperature for 30 min. Next, the cells were rinsed three times with distilled water. The hAF cells were then treated with Schiff's reagent for 10 min and rinsed with distilled water for 10 min. The samples were counterstained with Mayer's hematoxylin for 30 s and run through an alcohol series of 70%, 80%, 90%, 95%, and 100%. They were then mounted with cover slips and observed under an Olympus IX71 light microscope. Photographs were taken with a DP73 manager and controller (Olympus Life Science).

Functional analysis by indocyanine green (ICG) uptake

In this study, ICG was used to identify the characteristic features of the hepatogenic-induced cells. ICG (100 mg; Sigma-Aldrich) was dissolved in 5 ml of DI water in a sterile vial and added into DMEM containing 10% FBS to obtain an ICG solution with a final concentration of 1 mg/ml. Exploratory experiments indicated that there were no adverse effects on cell viability at this concentration. The hAF-MSCs ($n = 5$) were seeded into 24 well-culture plates at a density of 3×10^4 cells/well and divided into two groups: control group and hepatogenic-induced group. Both groups were cultured for 21 days. The ICG solution was added to the cell-culture plates and incubated at 37 °C for 1 h. Subsequently, the plates were rinsed three times with phosphate-buffered saline (PBS). The cellular uptake of ICG was examined with the use of a light microscope. After examination, the plates were refilled with DMEM containing 10% FBS. ICG was finally excluded from the cells after 6 h, and they were then examined under an Olympus IX71 light microscope. Photographs were taken with a DP73 manager and controller (Olympus Life Science).

Statistics

The data were analyzed for the purposes of descriptive analysis by employing Kruskal Wallis Test using SPSS version 22.0 software. A p-value of less than 0.05 was considered significantly different.

RESULTS

Human amniotic fluid-derived mesenchymal stem cell isolation

Firstly, after isolation, the isolated cells exhibited a typical level of ability in terms of cell adhesive plasticity in the plastic culture flasks. Cells were observed under a light microscope after being sub-cultured at the 2nd passage. Cells resembling typical epithelioid cells were investigated in terms of their polygonal-shaped cell morphology (Fig. 1, blue arrows). Irregular cell morphology was also determined by the presence of the expanded and cylindrical-elongated cytoplasm of the cells (Fig. 1, yellow arrows). However, the elongated and spindle-shaped cell morphology resembling a fibroblast-like characteristic was insignificantly observed among the isolated cells (Fig. 1, black arrows).

Cell proliferation analysis by alamar blue assay

The results of ability of cell replication by alamar blue assay indicated that the isolated cells began replicating after being cultured for one day by increasing their population to 12.48 ± 2.50 (mean ± SEM), which resembled the exponential phase of other typical MSCs. The population were continuously and swiftly increased reaching the highest level of cell prolificacy on day 11, wherein the percentage of viable cells was significantly increased by 2.17-fold compared with the cell number of day 1 cultivation. This degree of prolificacy was consistently observed until day 15 of cell cultivation, when the number was gradually decreased. By the end of the cultivation, isolated cells showed attenuated replication by exhibiting a percentage of proliferation of 13.18 ± 4.90 (Fig. 2a).

Cell characterization by flow cytometry analysis

The hAF cells derived from the 2nd passage exhibited a strong positive expression of typical surface markers of the MSCs (Fig. 2b), which included CD44 ($93.93 \pm 1.25\%$), CD73 ($91.85 \pm 2.15\%$), CD90 ($95.79 \pm 0.80\%$), and HLA-ABC ($96.83 \pm 0.83\%$). Nevertheless, they exhibited a low level of expression of hematopoietic stem cells including CD31 ($0.2 \pm 0.02\%$), CD34 ($0.44 \pm 0.04\%$), and CD45 ($0.14 \pm 0.02\%$). Moreover, the expression of the amniotic fluid cell biomarker c-Kit tyrosine

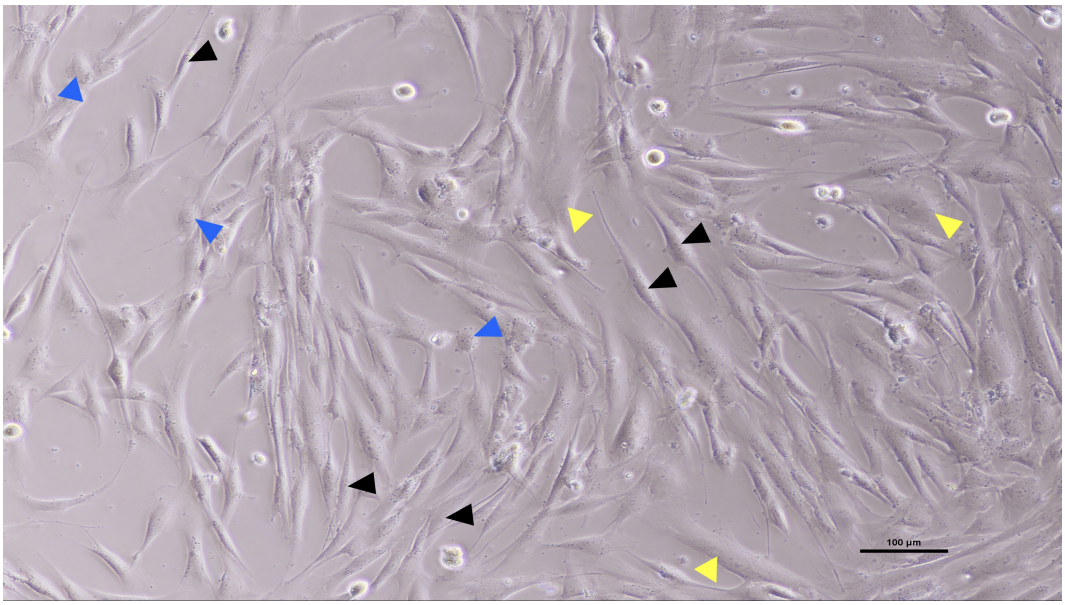

Fig. 1 Morphological observation after cell isolation demonstrated that the cells in the 2nd passage were allowed to adhere to plastic culture flasks without the support of feeder cells. Heterogeneous populations, some irregular-tubular shaped cells (yellow arrows), and polygonal shaped cells (blue arrows) were also observed. MSCs presented the typical attributes of spindle-elongated fibroblast-like morphology (black arrows).

kinase receptor, commonly known as CD117, was also under detected ($0.22 \pm 0.10\%$). Similarly, low levels of expression were also observed for HLA-DR ($0.3 \pm 0.04\%$) and the fibroblast marker ($0.21 \pm 0.01\%$), which suggested that these cells have the potential to be employed in various clinical applications.

Histological analysis by periodic acid schiff stain (PAS)

The hepatocyte is well known as a liver cell, and it functions as reaction to an accumulation of glycogen within the cell's cytoplasm. Due to this capability, glycogen storage has become a remarkable indicator of *in vitro* activities of the hepatocyte, which can be determined by staining the cells with PAS. After 21 days of cultivation, cells in both the control and the hepatogenic-induced groups were stained with PAS. The results indicated significantly intensive staining in the hepatogenic-induced group (Fig. 3b) compared with the control group (Fig. 3a). Similarly, when compared with the positive control using the hepatogenic cell line, HepG2 (Fig. 3c), these hepatogenic-induced cells also exhibited a potential for glycogenic hepatopathy as determined by the presence of a pink color within their cytoplasm.

Immunofluorescent analysis of hepatogenic markers

The expression of hepatogenic specific proteins, including albumin (ALB) and alpha-fetoprotein (AFP), was determined to investigate the ability of hepatogenic differentiation of hAF-MSCs. After hepatogenic induction under monolayer culture circumstances for 21 days, the results of immunofluorescent staining ($n = 5$) illustrated an intensive degree of expression of ALB (Fig. 4d–4f) and AFP (Fig. 4j–4l) in the hepatogenic-induced group. When compared with the control group, the fluorescent signal of the protein markers was shown at significantly low levels of expression (Fig. 4a–4c; ALB and Fig. 4g–4i; AFP). Additionally, when compared with the positive control using HepG2 cells, the expression signal of the protein markers in the hepatogenic-induced group also similarly reflected what observed in the HepG2 cells (Fig. 5).

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

The expression of the hepatogenic-related gene biomarkers of hAF-MSCs (including *ALB*, *AFP*, *HNF4*, and *CYP1B1*) that had been cultured in both the control group (basal growth medium) and the hepatogenic-induced medium for 21 days ($n = 5$) was investigated. On day 14 and day 21, the *ALB* gene expression was significantly higher in the hepatogenic-induced group compared with the control group by 4.08-fold and 2.87-fold, respectively ($p < 0.05$) (Fig. 6a). The expression level of the *ALB* gene among the hepatogenic-induced group was observed at a lower level on day 1. When compared with day 7, day 14, and day 21, the results indicated significantly increased levels of *ALB* gene expression by 3.68-fold, 5.16-fold, and 3.63-

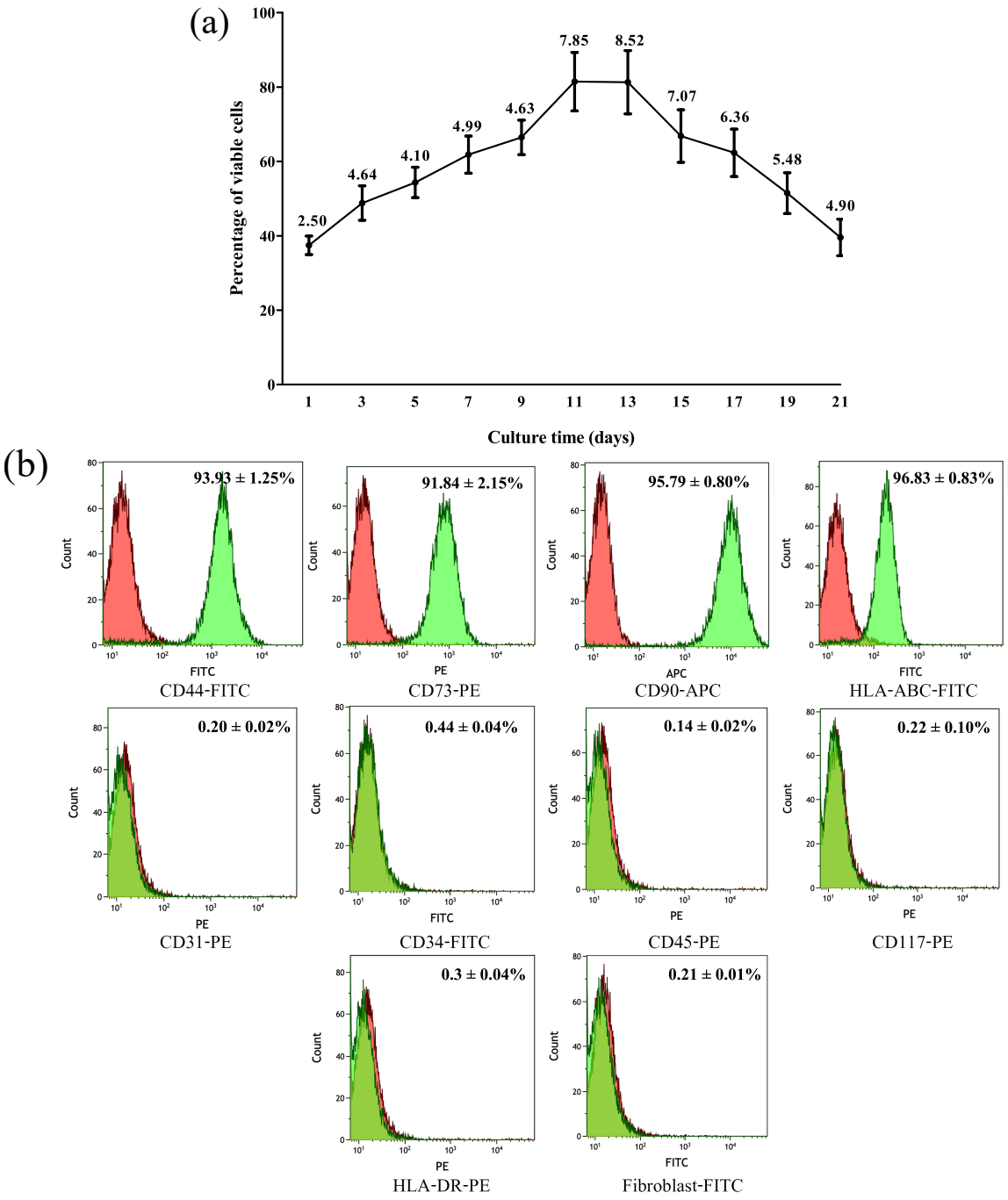

Fig. 2 Expression of biological characteristics demonstrated in the ability of cell replication after isolation (a) and the expression of immunophenotypic characteristics of MSCs (b). In (a), the results indicated that the isolated hAF cells presented the capability of replicating themselves as observed by the potency of the stem cell during the cultivation period. The data were presented as mean ± SD values of all three samples on each day. In (b), the results illustrated the expression of CD44, CD73, and CD90 biomarkers while they were being under detected in the expression levels of the typical hematopoietic biomarkers, CD31, CD34, and CD45, fibroblast and CD117 (c-kit), and the transmembrane tyrosine kinase receptor. In accordance with these results, the antigenic biomarker of the immune-privileged attributes was also observed by manifesting HLA-ABC, whereas the HLA-DR was under noticed. All data were presented as a histogram with the percentage of the expression markers ± SD.

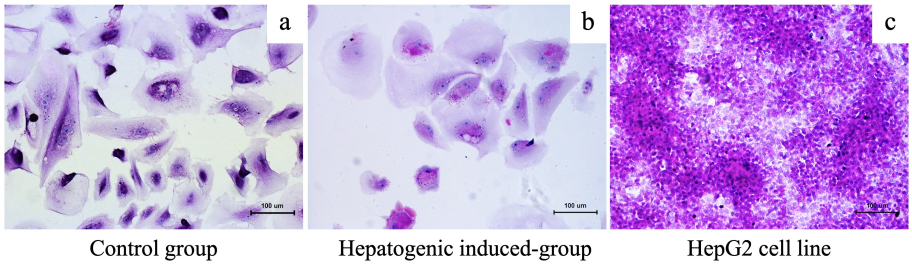

Fig. 3 Histological morphology of induced hepatocytes and hepatogenic cell line (HepG2) after staining with Periodic acid schiff staining (PAS) revealed the ability of glycogen accumulation inside the cytosol of the hepatogenic-induced group, (b), indicated by a pink color compared with the control (a). This hepatogenic potential of the hepatogenic-induced group also resembled to the potency of glycogenic hepatopathy of HepG2 cell line (c).

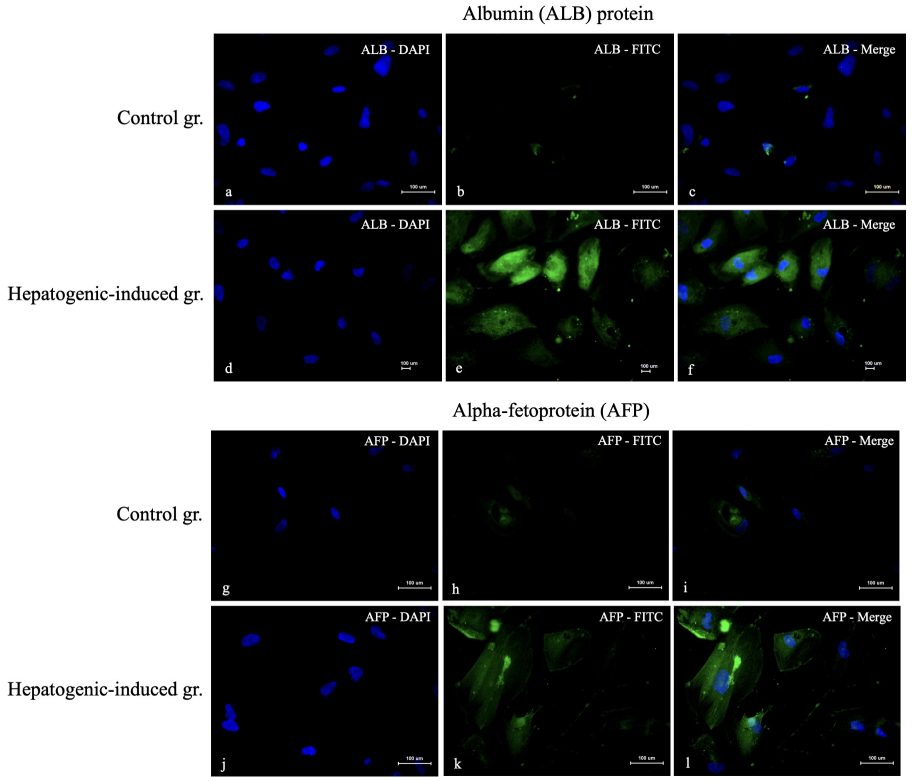

Fig. 4 Expression of hepatogenic specific proteins of hepatogenic-induced cells revealed that the immunofluorescent staining of MSCs after being cultured under hepatogenic-induced circumstances remarkably uncovered the hepatogenic specific protein biomarkers among cells of the hepatogenic-induced group including albumin protein (ALB) (d, e, and f) and alpha-fetoprotein (AFP) (j, k, and l). Conversely, when compared with the control, the cells cultured under these conditions obviously exhibited less expression of both protein markers (ALB: a, b, and c; and AFP: g, h, and i).

fold, respectively ($p < 0.05$) (Fig. 6a). Next, when compared with the control group, the expression level of the *AFP* gene was obviously higher on day 1, day 7, and day 14 by 1.55-fold, 4.85-fold, and 5.34-fold, respectively. However, the results indicated a dramatically significant reduction in *AFP* gene expression levels on day 21 compared with both the control group and the hepatogenic-induced group on different days ($p < 0.05$) (Fig. 6b). Additionally, the results also revealed remarkably higher expression levels of the *HNF4* gene at the early stage of cell cultivation by 3.37-fold on day 1 (Fig. 6c). Subsequently, on day 7, day 14, and day 21, there were significantly higher levels of expression of the *HNF4* gene in the cultivated cells of the hepatogenic-induced group compared with the control group by 5.48-fold, 5.19-fold, and 3.83-

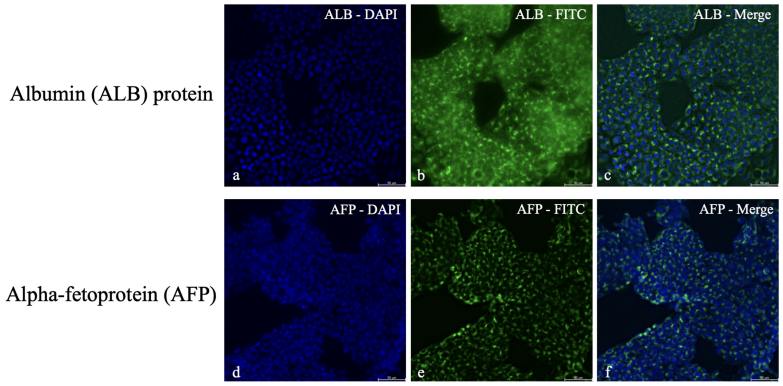

Fig. 5 Expression of hepatogenic specific proteins of HepG2 cell line uncovered that used as a positive control, immunofluorescent microscopy of the HepG2 cell line manifested the hepatogenic specific proteins consisting of the albumin protein (ALB) (a, b, and c) and the alpha-fetoprotein (AFP) (d, e, and f).

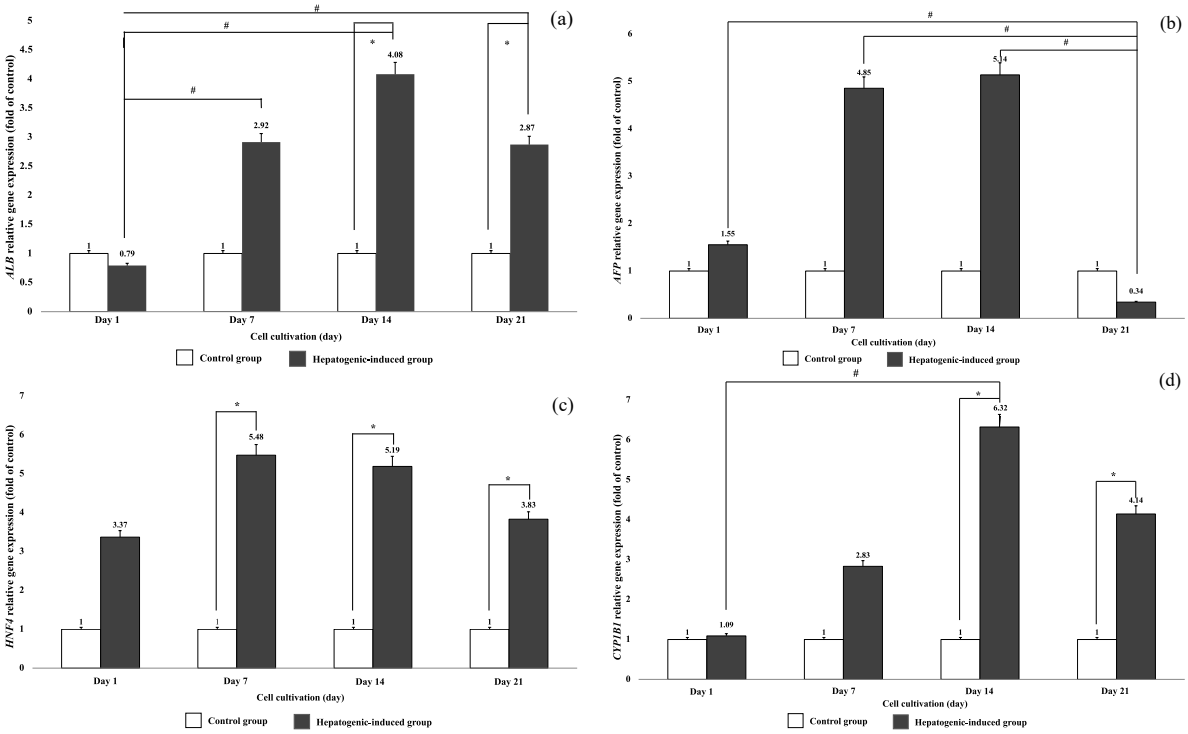

Fig. 6 Expression of hepatogenic-specific genes after 21 days of cell cultivation exhibited that the hepatogenic-related genes, *ALB* (a), *AFP* (b), *HNF4* (c), *CYP1B1* (d), of the hepatogenic-induced MSCs were accessed by RT-qPCR after being cultured under hepatogenic-induced circumstances for 1, 7, 14, and 21 days ($n = 5$). Under different circumstances, the basal growth medium (control group, white column) and the hepatogenic-induced medium (hepatogenic-induced group, black column), presented in each bar chart indicated the relative gene expression by which each gene was normalized to the control using the Kruskal–Wallis test ($p < 0.05$).

fold, respectively ($p < 0.05$) (Fig. 6c). Similarly, when compared with the control group, the expression levels of the *CYP1B1* gene were significantly higher in the hepatogenic-induced group on day 14 and day 21 by up to 6.32-fold and 4.34-fold, respectively ($p < 0.05$) (Fig. 6d). Normalized to day 1 of the hepatogenic-induced group, the expression level of the *CYP1B1* gene was significantly higher by 5.79-fold ($p < 0.05$) (Fig. 6d).

Functional analysis by indocyanine green (ICG) uptake

The biological function of differentiated hepatocytes was detected by ICG uptake, which can be commonly

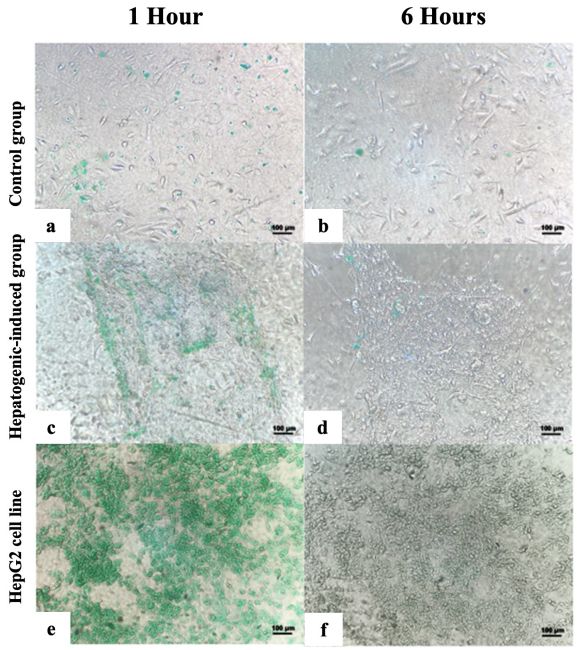

Fig. 7 Potential function of indocyanine green (ICG) uptake of hepatogenic-induced cells (hepatogenic-induced group: c and d) was accessed by ICG uptake and compared with the cells cultured in basal growth medium (control: a and b) and the HepG2 cell line (positive control: e and f). After being incubated for one and six hours, all experimental groups exhibited the functional ability to uptake ICG and excrete it via the cell cytosol, respectively.

applied in functional investigations for the perioperative dynamic assessment of liver function without resulting in toxicity to living cells. The results indicated that there was both an uptake and an exclusion of ICG among the cells of the hepatogenic-induced group, the control group, and the HepG2 cell line. The cells from all three groups were able to take up ICG into the cytosol, which was indicated by the presence of a green color after incubation with ICG for one hour (Fig. 7a, 7c, and 7d). Subsequently, after 6 h of incubation, the ICG was discharged from the cells (Fig. 7b, 7d, and 7f). In the control group, the cells exhibited a relatively low level of ability for ICG uptake. After 6 h of incubation, the ICG staining inside the cells was not observed to have changed overtime. In contrast, cells in the hepatogenic-induced group and the HepG2 cell line exhibited the ability to uptake ICG to the cytosol and were able to effectively eliminate the up-taken ICG after 6 h of cell incubation.

DISCUSSION

Liver disease can occur in people throughout the world irrespective of age, gender, region, race, gender, ethnicity, and socioeconomic strata. The prevalence of liver disease accounts for approximately two million deaths per year worldwide. Currently, it is the 11th most common cause of death globally, while liver cancer is recognized as the 16th leading cause of death worldwide. According to the World Health Organization (WHO), the rate of liver disease has steadily increased over the years [2, 3]. Although there has been a significant evolution in the development of vaccines and anti-viral agents, the global burden of liver disease is poised to surge even further due to certain health-modulating factors such as the extension of life expectations, increasingly inactive lifestyles, and over-nutrition or obesity, all of which have major implications on the health of people throughout the world [3, 13].

Currently, stem cell therapy has been studied extensively for its promising potential in the field of alternative cell regeneration. The cells acquired from various sources, namely adipose tissue, tooth buds, bone marrow, blood, umbilical cord blood, Wharton's jelly, amniotic fluid, and the placenta, have been investigated for their regenerative ability and their potential for transformation into different types of cells [9–11].

In this investigation, cell samples were derived from amniotic fluid. The samples were acquired by amniocentesis using ultrasound-guided transabdominal punctures during prenatal diagnosis. The method had fewer ethical risks compared with others associated with the acquisition of embryonic stem cells. Previous studies have reported that AF-MSCs are multipotent stem cells that exhibit high proliferation and a low risk of tumorigenicity [12, 14]. In the present study, hAF cells exhibited fibroblastoid characteristics by presenting spindle-shaped cell morphology and displaying adherent plasticity in the culture flasks. This outcome resembled the findings of several previous studies [15–17]. Additionally, according to our preceding findings, the results uncovered that the isolated stem cells also exhibited a differentiation potential into several cell lineages, for example, chondrogenic [18], cardiomyogenic, osteogenic, and vascular endothelial cell lineages [19]. Thus, the results provided affirmative attributes of isolated cells to give rise into distinct cell types. Furthermore, these acquired cells also exhibited many of the antigenic properties of typical MSCs through the expression of CD44, CD73, CD90, and HLA-ABC, while exhibiting no degree of expression of CD31, CD34, CD45, CD117, HLA-DR, and fibroblast markers. Correlatively, the preceding documentation has reported that these MSCs expressed certain typical markers, such as CD44, CD73, CD90, and CD105, which certified the potential characteristics of the MSCs acquired from amniotic fluid [9–11, 16**?**]. These findings suggest that these cells have potential to be utilized in a variety of clinical applications. To confirm the attributes associated with the potential growth of the isolated cells, alamar blue cell perforation assay was performed. The results indicated that hAF-MSCs

began proliferating on day 1 which resembled the exponential phase of the other typical MSCs. After that, they continuously and swiftly increased reaching the highest level of cell prolificacy on day 11. After day 15, the cells gradually decreased in numbers. In agreement with the outcomes of previous studies, hAF cells also presented the ability of cell replication by increasing their number during the first week of the experiment and slowly decreasing after the stationary phase [16–19].

Moreover, the hAF-MSCs exhibited the ability of cell differentiation into various cell types that were derived from all three embryonic germ layers. The results in the current study clearly indicated that hAF-MSCs induced hepatogenic cells expressed certain beneficial characteristics of hepatocyte-like cells, which are known to be able to gather glycogen to the cytosol. These findings were in accordance with those of previously published reports documenting the PAS staining that uncovered the hepatogenic glycogen accumulation within the cytosol. Moreover, it was reported that glycogen could be stored in the liver up to 6–8% of the liver wet weight [20–22].

Additionally, the hepatogenic-induced cells also strongly expressed hepatogenic-specific biomarkers, such as ALB and AFP, in both gene and protein levels. ALB is produced by liver cells and is normally used to surmise the well-being or conditional state of human body. Among its biological attributes, ALB plays a crucial role in maintaining intravascular oncotic pressure, facilitating the transportation of substances, and acting as a free-radical scavenger [23, 24]. Furthermore, AFP is one of specific proteins in embryos, a dominant serum protein, and an early human embryonic protein. It is first synthesized by the yolk sac and is subsequently predominated in the liver for 1–2 months [25–27]. The expression of AFP decreases when the embryo develops to maturity. The function of AFP in the fetus is unclear. However, it is believed that AFP was associated with liver regeneration and certain restorative processes in the adult liver [25, 27, 28]. Moreover, HNF 4 is a transcription factor or positive transcriptional regulator of hepatogenic genes, and it is normally detected in the primitive endoderm and plays a vital role in the regulation of liver genes involved in the differentiation of hepatocytes, hepatic development, and the maintenance of liver-specific functions. It is also a significant factor in establishing the differentiation of hepatogenic cell lineage [29, 30]. Additionally, CYP1B1 is a member of the cytochrome P450 family of enzymes observed in the primary cultures of hepatocytes. CYP1B1 involves the reactions that break down drugs, produce fatty acids, and initiate metabolic activation of the cells [31–33]. However, the results of this current study indicated the fluctuating patterns of hepatogenic related gene expression on different days of cell cultivation. According to these findings, the alternating expression levels of hepatogenic genes could be caused by the stage of differentiation of the hepatocyte [34–36]. All of the results in this study indicated a strong interaction with early evaluation of hepatogenic differentiation by reflecting the possibility of differentiation into hepatocyte-like cells including the expression of hepatogenic specific proteins and genes [16, 37, 38].

As mentioned previously, hepatocytes can both take up and secrete ICG, which is indicative of the function of hepatogenic cell differentiation. The ICG is commonly applied in functional investigations for the perioperative dynamic assessment of liver function. It has also been found to be very rarely toxic to living cells [39, 40]. Hepatocyte in the human liver is an essential metabolic unit that functions to store the polymeric form of glucose known as glycogen. The stored glucose can apply a negligible degree of osmotic pressure and can be degraded upon demand for the benefit of other tissues, especially those of the brain and erythrocytes [20, 22]. Liver metabolic function is controlled by insulin and other metabolic hormones. Glucose is transformed into pyruvate through glycolysis in the cytoplasm, and then pyruvate is subsequently oxidized in the mitochondria to generate adenosine triphosphate (ATP) through the tricarboxylic acid cycle (TCA) or the Krebs cycle [22]. Relatively, ICG is related with hepatic ATP during cell detoxification. Thus, cells with impaired function will reduce the amounts of ATP, which results in the limitation of ICG excretion [39, 40]. Similarly, in the current study, the hepatogenic-induced group was correlated with ICG excretion to the same degree as that of the positive control group using the HepG2 cell line. However, further investigations involving hepatogenic cell function and the signaling pathway should be conducted.

However, these current investigations of hepatogenic cell differentiation's attributes were performed through two-dimension cell culture, which resulted in the confinement of exploration in three-dimension cell morphology after treating with hepatogenic-induced medium. Additionally, this experiment was accomplished based on *in vitro* study in which the differentiation potency of hAF-MSCs and characteristic potential of hepatocyte-derived stem cells in the *in vivo* model were suggested for further investigation.

CONCLUSION

MSCs acquired from human amniotic fluid were determined to be an attractive potential cell source in alternative treatments. These cells were able to be obtained routinely after chromosomal diagnosis with less ethical concerns. Moreover, these cells remarkably illustrated typical stem cell plasticity of MSCs including the adherent attributes and the strong manifestation of the considerable biomarkers of the MSCs such as CD44, CD90, and HLA-ABC (MHC-class I). In contrast,

the hematopoietic biomarkers and c-Kit tyrosine kinase receptor have been under investigated, namely CD31, CD34, CD45, and CD117. Moreover, when cultured under appropriate circumstances, these cells also exhibited the differentiated capacity of transforming into hepatocyte-like cells by expressing the hepatogenic specific gene and certain protein markers consisting of *ALB*, *AFP*, *HNF4*, *CYP1B1* genes, and ALB and AFP proteins, respectively. These current results suggest that hAF-MSCs could be applied in therapeutic regenerative medicinal approaches involving cell-tissue engineering.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.058.

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Appendix A. Supplementary data

Table S1 Primer sequence of RT-qPCR.

Genes	Primer sequence	Reference
ALB	F5′-GAGACCAGAGGTTGATGTGATG-3′ R5′-AGGCAGGCAGCTTTATCAGCA-3′	Ghaderi Gandomani M et al, 2017 [16]
AFP	F5′-CATGAGCACTGTTGCAGAGGAGA-3′ R5′-CGTGGTCAGTTTGCAGCATTCTG-3′	Ghaderi Gandomani M et al, 2017 [16]
HNF4	F5′-CCAAGTACATCCCAGCTTTC-3′ R5′-TTGGCATCTGGGTCAAAG-3′	Nijimi M et al, 2007 [37]
CYP1B1	F5′-GAGAACGTACCGGCCACTATCACT-3′ R5′-GTTAGGCCACTTCAGTGGGTCATGAT-3′	Nijimi M et al, 2007 [37]
GAPDH	F5′-ATGGGGAAGGTGAAGGTCG-3′ R5′-TAAAAGCAGCCCTGGTGACC-3′	Markmee R et al, 2019 [19]