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Original Article

# Development of Simple Protocol for Generation of Functionally Active Hepatocyte-like Cells from Human Adipose Tissue-derived Stem Cells

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## A B S T R A C T

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**Background and Aims:** Human adipose tissue-derived stem cells (hASCs) are considered as an attractive source of regenerative stem cells, mainly because of their higher proliferation rate, more accessibility and hepatocyte like properties as compared to mesenchymal stem cells isolated from other tissues. Numerous studies have described the beneficial use of adipose tissue-derived stem cells for generating hepatocyte-like cells. However, due to the lack of appropriate culture conditions, most of the produced cells exhibit poor functionality. The aim of the present study was to establish a new protocol for the efficient hepatic differentiation of hASCs.

**Materials and Methods:** hASCs were cultured in hepatic differentiation medium containing fibroblast growth factor 4, hepatocyte growth factor, dexamethasone and oncostatin M using a three-step protocol up to 21 days. Then, the functionality of the treated cells was evaluated by analyzing specific hepatocyte genes and biochemical markers at various time points.

**Results:** A significant upregulation in albumin, alpha-fetoprotein, cytokeratin 18 and hepatocyte nuclear factor-4α expressions was observed in differentiated cells relative to day 1 of differentiation protocol. Moreover, the finding of glycogen deposits increased urea production and positive immunofluorescence staining for albumin and alpha-fetoprotein in hepatocyte-like cells suggesting that most of the cells differentiate into hepatocyte-like cells.

**Conclusions:** Our report has provided a simple protocol for differentiation of hASCs into more functional hepatocyte-like cells.

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## Introduction

The liver is a significant organ in the human body performing vital functions including detoxification, metabolism, and hormone balance. In addition to these roles, liver is known to possess a remarkable regenerative capacity. However, this ability is impaired following numerous liver failures such as cirrhosis for which liver transplantation is the only available clinical option [1]. Indeed, due to discrepancies associated with liver transplantation, it is of utmost importance to find alternative strategies for the treatment of end-stage liver disorders [2].

In this context, cell-based therapy has become the focus of intense investigation in recent years [3]. Therefore, various kinds of stem cells have been studied to examine their clinical applications in regenerative medicine [4]. To date, there are numerous studies describing the beneficial use of mesenchymal stem cells (MSCs) for liver cell therapy, mainly because of their multipotent potential, lack of ethical concerns, and risk of rejection [5, 6]. MSCs can be isolated from different kinds of tissues including bone marrow (the first known tissue as a source of MSCs) [7], umbilical cord blood, amniotic fluid as well as adipose tissue [8]. Of particular interest to researchers are the human adipose tissue-derived stem cells (hASCs) which for the first time were isolated and described by Zuk et al. In addition to their multilineage potential [9], hASCs have been reported to exhibit higher proliferation rate, more accessibility and hepatocyte like properties [10] as compared to MSCs isolated from other tissues.

There are an increasing number of studies offering ASCs as an attractive candidate for generating hepatocyte-like cells. These studies have provided support for the use of a combination of growth factors and inductive agents to induce ASCs toward hepatic lineage [11-15]. However, most of the produced hepatocyte-like cells exhibit poor functionality, mainly due to the lack of appropriate induction conditions. Developmental studies have uncovered a number of transcription factors and regulators as being implicated in hepatocyte differentiation [16]. In this regard, fibroblast growth factors (FGFs) have been identified as being involved in early development and organogenesis [17, 18]. Of particular importance is FGF4 because of its involvement in hepatic induction [16, 19] as well as its role in liver regeneration [20]. In addition, it has been reported that MSCs pretreated with FGF4 in combination with hepatocyte growth factor (HGF) is able to reduce liver fibrosis and improve liver function in CCl4 induced mice [21]. Similar results have also been described upon using of FGF4 transduced bone marrow MSCs (BM-MSCs) in the cirrhotic liver model [22].

Recently, using a combination of inductive agents including HGF, oncostatin M, and dexamethasone, we have differentiated hASCs into hepatocyte-like cells [23]. Based on the mentioned results vividly showing

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the important role of FGF4 in hepatic differentiation, the current study was designed to examine a new protocol for generation of functionally active hepatocyte-like cells from hASCs.

## Materials and Methods

**Isolation and culture of hASCs**

hASCs were isolated from human lipoaspirates as described previously [23]. Briefly, lipoaspirates were digested using 0.075% collagenase I (Sigma-Aldrich, USA) at 37ºC for 30 min. Finally, the extracted cells were suspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA), and 1% penicillin/streptomycin (Sigma-Aldrich, USA) and incubated in a humidified atmosphere at 37º C with 5% CO2.

***In vitro* hepatic differentiation of hASCs**

To induce hepatic differentiation of hADSCs, a three-step procedure was used (Fig. 1A]. At passage 3, the cells ($1\times10^3$ cells/cm$^2$) were cultured in DMEM supplemented with 10 % FBS, 20 ng/ml HGF (Biolegend, USA), $10^{-7}$ mol/l dexamethasone (Sigma-Aldrich, USA) together with 10 ng/ml FGF4 (Biolegend, USA]. After 3 days, the cells were treated with the inductive agents as step one in the absence of FGF4, followed by the addition of oncostatin M (Biolegend, USA) to the medium at concentration of 10 ng/ml for the next 2 weeks. The culture medium was renewed every 3 days and hepatic differentiation assays were carried out at 1, 7, 14 and 21 days after hepatic induction. Three independent experiments were conducted in this study.

**RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR) analysis**

Total RNA was extracted using TRI reagent (Sigma-Aldrich, USA) on days 1, 7, 14, and 21 of differentiation protocol. After DNase (Fermentas, USA) treatment, Reverse transcription and cDNA amplification was performed with cDNA synthesis kit (Thermo Ficher, USA) and SYBR Premix Ex Taq II (Takara, Japan) respectively. Using specific primers (Table 1), qRT-PCR was conducted with a three-step procedure as follows: denaturation at 95°C for 5 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, for a total of 40 cycles. As a reference gene, *β-actin* expression was used for normalization and RNA levels were evaluated in triplicate. Finally, $2^{-\Delta\Delta Ct}$ method was applied to calculate relative quantification of gene expression.

**Immunofluorescence Staining**

After fixation with 4% paraformaldehyde solution for 10 min at room temperature, the cells were permeabilized using 0.2% Triton X-100 for 30 min. A solution containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS was used for blocking, followed by washing the cells with phosphate buffer solution (PBS) and incubation with 1:200 monoclonal anti-human albumin (Abcam, USA) and 1:200 monoclonal anti-human alpha 1 fetoprotein (Abcam, USA) overnight at 4ºC.

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**Table 1.** Primer sequences for qRT-PCR analysis.

| Genes | Primer | Sequences |
|---|---|---|
| **Albumin** | Sense | 5'-GAGACCAGAGGTTGATGTGATG -3' |
| | Antisense | 5'-AGGCAGGCAGCTTTATCAGCA-3' |
| **Alpha-fetoprotein** | Sense | 5'-CATGAGCACTGTTGCAGAGGAGA -3' |
| | Antisense | 5'-CGTGGTCAGTTTGCAGCATTCTG -3' |
| **Cytokeratin 18** | Sense | 5'-TTGATGACACCAATATCACACGA- 3' |
| | Antisense | 5'-TATTGGGCCCGGATGTCTG-3' |
| **Cytokeratin 19** | Sense | 5'-GCGGCCAACGGCGAGCTA -3' |
| | Antisense | 5'-GCAGGACAATCCTGGAGTTCTC -3' |
| **Hepatocyte nuclear factor-4α** | Sense | 5'-CTTCTTTGACCCAGATGCCAAG-3' |
| | Antisense | 5'-GAGTCATACTGGCGGTCGTTG-3' |
| **β-actin** | Sense | 5'-CTGGAACGGTGAAGGTGACA-3' |
| | Antisense | 5'-AAGGGACTTCCTGTAACAATGCA -3' |

The next day, cells were treated with Alexa Flour 594 donkey anti-mouse IgG (1:500, Abcam, USA) for 1 h and counterstained with 4,6-diamidino-2-phenylindole to visualize nuclei. Finally, the cells were examined with an inverted fluorescence microscope (Labomed TCM 400). At least 3 images form each cover slips, 3 cover slips per each sample and 3 samples (biological replicates, n=3) per each treatment were considered for quantification of data [24]. In addition, all images were taken with exactly the same acquisition parameters including exposure time, gamma and saturation. Finally, image J software (version 1.42 V, NIH, USA) was used for quantification.

**Hepatic functional tests**

**Glycogen staining**

At day 21, the cells were evaluated for detection of glycogen deposit using periodic acid-schiff (PAS) staining. Firstly, the cells were fixed with 4% paraformaldehyde solution for 10 min, followed by oxidation using 1% periodic acid for 5 min. After washing with PBS, the cells were treated with Schiff's reagent (Sigma–Aldrich, USA) for 15 min. and counterstained using hematoxylin. Finally, the cells were visualized under an inverted microscope [23].

**Urea production**

Urea concentrations were measured in the culture media of induced ASCs at different time points (on days 1, 7, 14, and 21). First, the cells were incubated in a culture medium containing 3mM $NH_4CL$ for 24h at 37ºC. After collecting the supernatants, urea production was evaluated with a colorimetric assay kit (Zistchem, Iran) following the manufacturer's instruction. As a negative control, untreated hASCs were used and the amount of urea was normalized to the number of cells in each well [23]. This protocol was approved by Hormozgan University of Medical Sciences Ethics Committee.

**Statistical analysis**

The data are expressed as mean±SD. One way-analysis of variance followed by Dunnett post

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hoc test was conducted for analysis of statistical comparison and the p-value <0.05 was considered to be significant.

## Results

### Morphological changes and expression of hepatocyte-specific markers during hepatic differentiation of hASCs

To induce differentiation of hASCs into hepatocyte-like cells, medium containing FGF4, HGF, dexamethasone, and oncostatin M was used over the course of 21 days (Fig.1A). At various stages of differentiation protocol, cells were evaluated morphologically. During the first week of induction, the cells exhibited a fibroblast-like shape without any significant morphological changes. After 2 weeks, however, the induced hASCs slowly lost fibroblastic morphology and started becoming broad and flattened. Notably, morphological changes were remarkable during the last week of differentiation and some of the cells exhibited hepatocyte-like morphology such as a polygonal and round shape and cytoplasmic granulation (Fig. 1B).

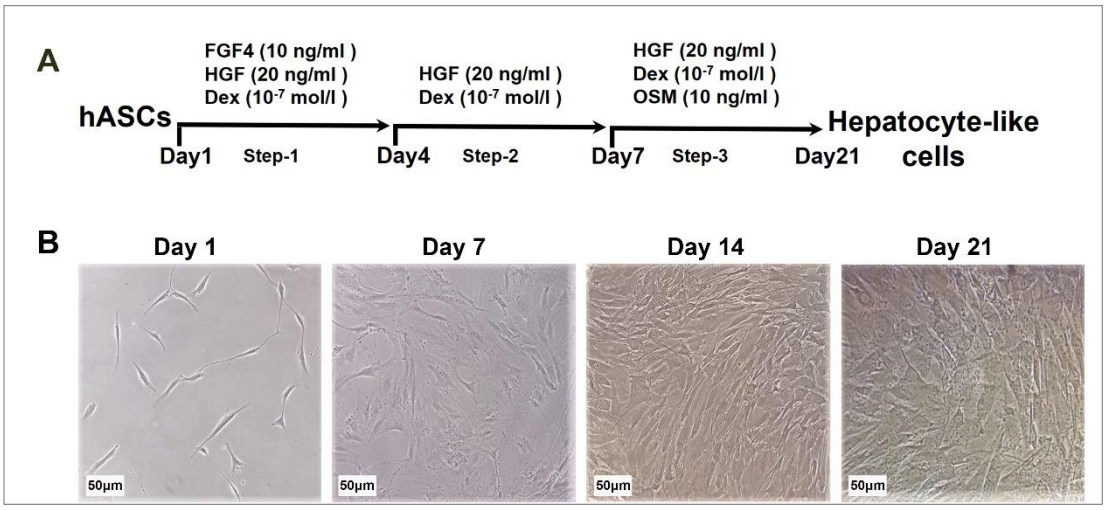


**Fig. 1.** Hepatic differentiation of hASCs. A: Schematic representation of the hepatic differentiation protocol. B: Morphology of hASCs treated with HGF= hepatocyte growth factor, Dex= dexamethasone, OSM= oncostatin M, and FGF4= fibroblast growth factor at days 1, 7, 14, and 21. The cells exhibited remarkable changes toward round and polygonal shapes at days 14 and 21 upon hepatic induction.

The differentiation status was also confirmed by evaluating the expression of several hepatocyte specific genes, including albumin, alpha-fetoprotein, cytokeratin 18, cytokeratin 19, and hepatocyte nuclear factor-4α to examine whether the morphological changes were associated with the hepatic genes expression. Therefore, total RNA of treated cells were extracted at various time points (on days 1, 7, 14, 21) and the expression of the mentioned genes was measured. As a negative and positive controls, untreated hASCs and Hep G2 cells were used, respectively.

As illustrated in Fig. 2A, an early increase was observed for alpha-fetoprotein mRNA, an early hepatic progenitor marker at day 7 to 3-fold ($p<0.05$) with further increase at days 14 and 21 to 8 ($p<0.001$) and 9.5 fold ($p<0.001$) respectively. Moreover, statistical analysis validated a significant upregulation

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for albumin expression level at day 14 to 5.5 fold (p<0.01) that reached maximum level of 13-fold (p<0.001) at day 21 compared to day 1 of hepatic differentiation induction (Fig. 2B). However, upon hepatic differentiation of hASCs, no significant difference in cytokeratin 19 expression level was detected at all-time points (Fig. 2D). By contrast, the mRNA level of cytokeratin 18, as an adult hepatic marker, was found to significantly upregulate at day 21 to approximately 5-fold (p<0.01) in comparison to day 1 (Fig. 2C). Significant expression level of hepatocyte nuclear factor α was noted starting at day 14 (3.5-fold, p<0.05) with a maximum increase at day 21 to nearly 8-fold upon hepatic induction of hASCs (Fig. 2E).

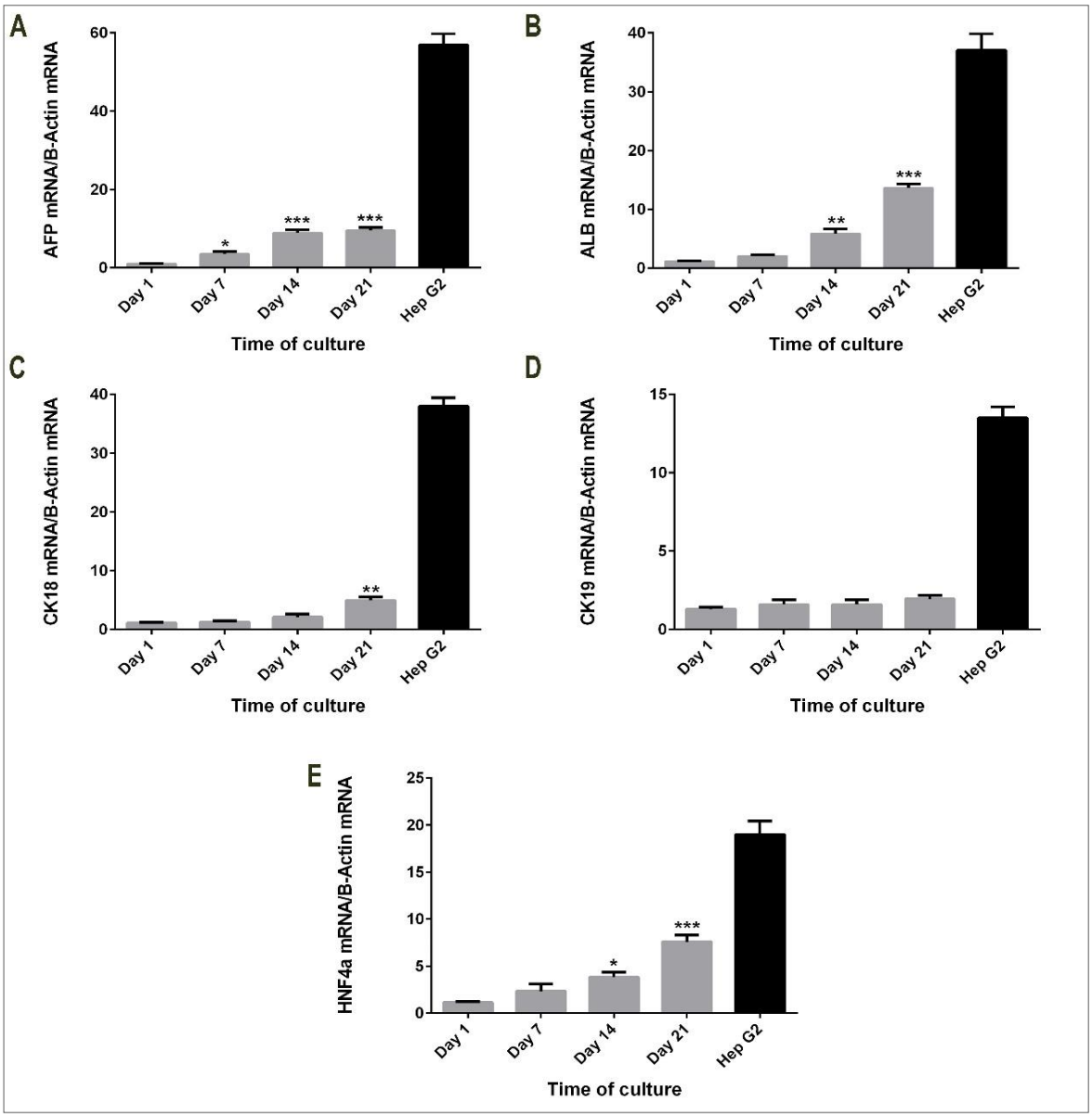


**Fig. 2.** qRT-PCR analysis of several hepatocyte-specific genes upon the treatment of hASCs with a combination of hepatocyte growth factor (HGF), dexamethasone (DEX), oncostatin M (OSM), and fibroblast growth factor (FGF4). The expression levels of target genes were normalized with β-actin. The data are shown as mean±SD in three independent experiments (n=3). ALB= Albumin; AFP= Alpha-fetoprotein; CK= Cytokeratin. *p<0.05, **p<0.01, ***p<0.001 relative to day 1.

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In addition, the expression of albumin and alpha-fetoprotein genes were further confirmed at protein level. Consistent with the expression of mentioned genes, the treated cells stained positive for both alpha-fetoprotein (Fig. 3A) and albumin (Fig. 4A) at day 21. Also, quantification of data revealed the high percentage of alpha-fetoprotein (Fig. 3B) and albumin (Fig. 4B) positive cells in hepatocyte-like cells. By contrast, untreated hADSCs were negative whereas the staining was intensely positive for HepG2 cells (Figs. 3, 4).

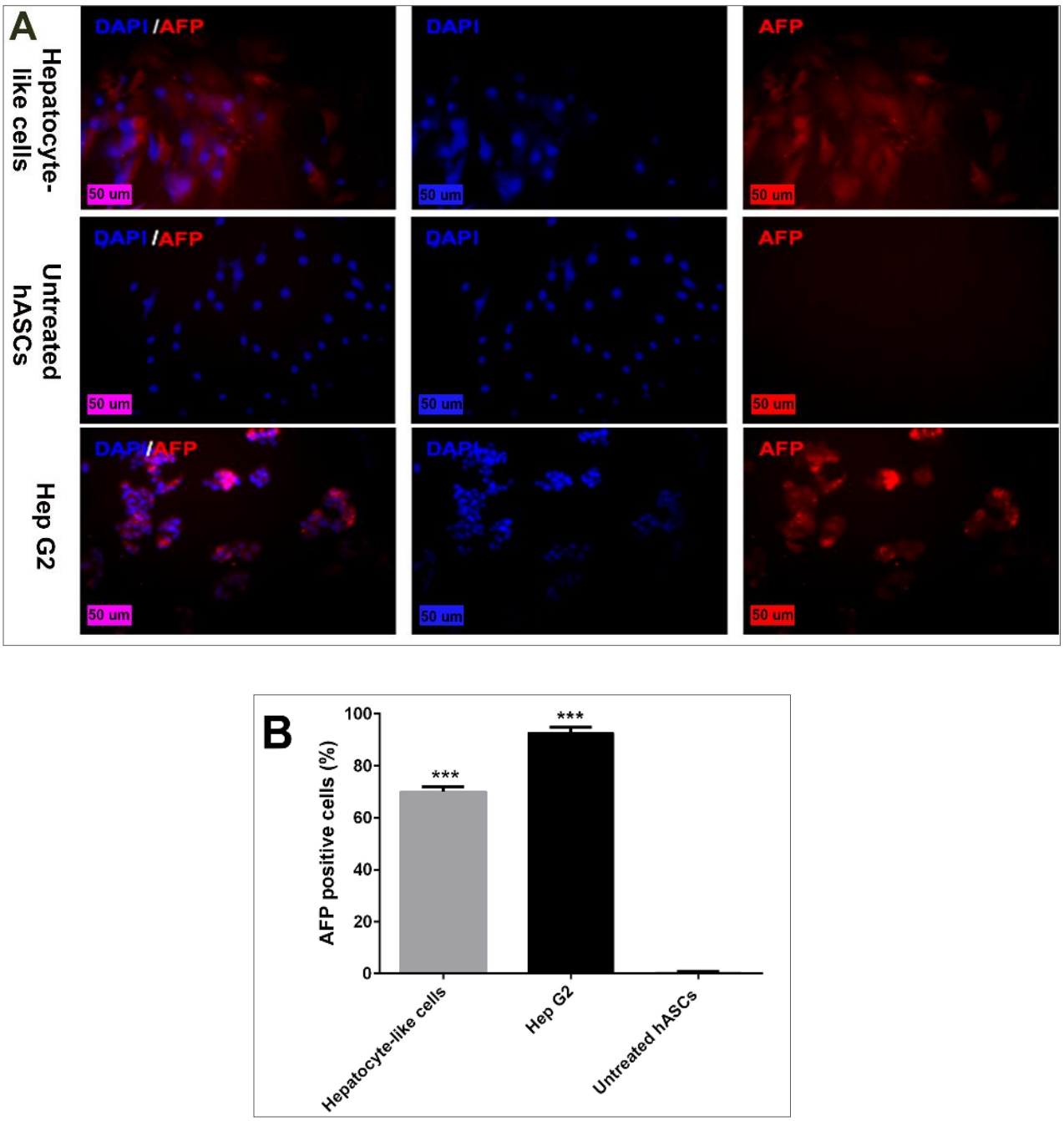


**Fig. 3.** Immunofluorescence staining of alpha-fetoprotein (AFP). A: More intensive staining was detected for AFP in hepatocyte-like cells compared to the untreated cells. B: Quantification of immunostaining results provided in A. ***p<0.001 relative to untreated hASCs, (n=3).

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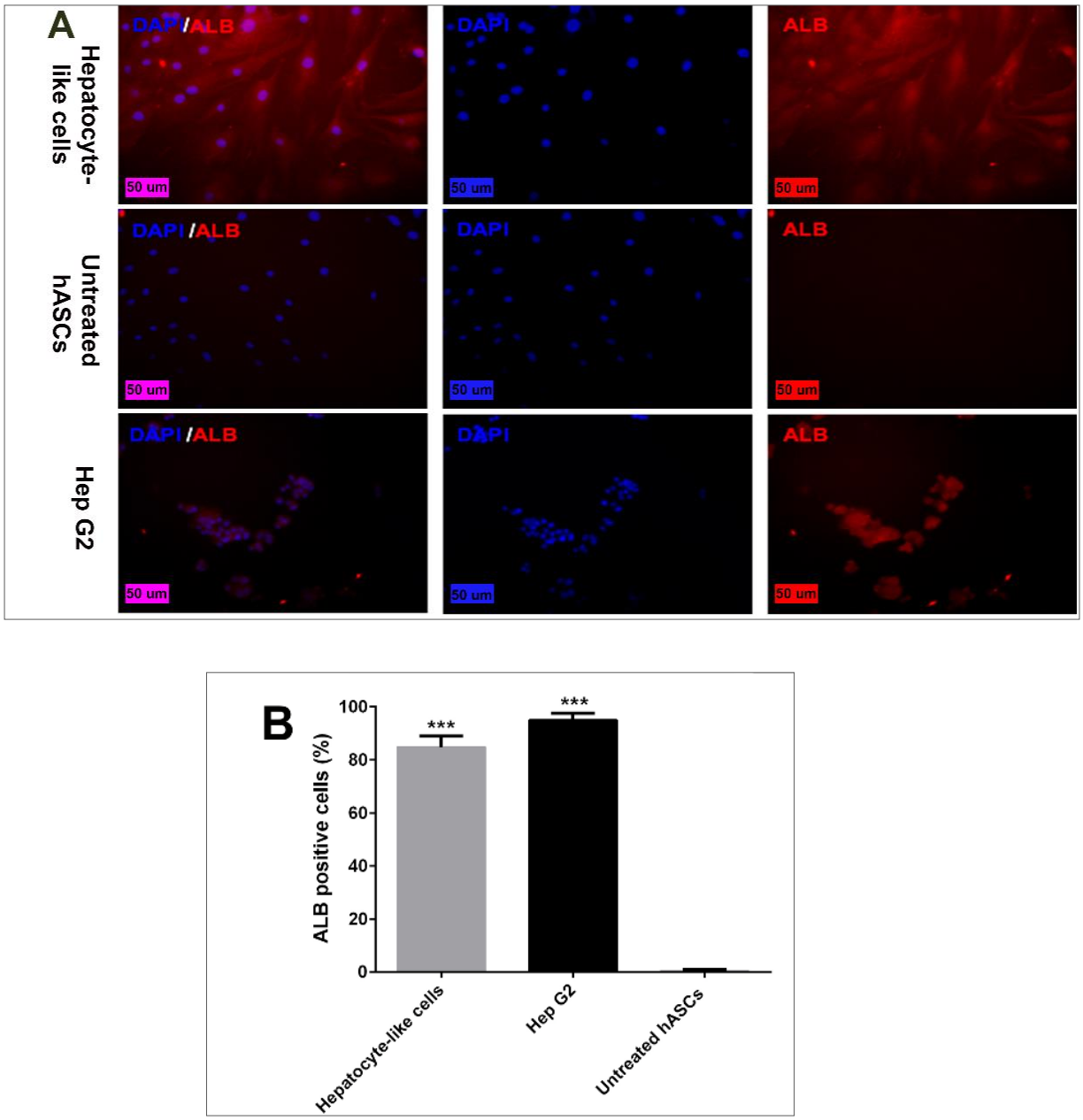


**Fig. 4.** Immunofluorescence staining of albumin. A: More intensive staining was detected for albumin in hepatocyte-like cells compared to the untreated cells. B: Quantification of immunostaining results provided in A. ***p<0.001 relative to untreated hASCs, n=3.

**Functional characterization of hepatocyte-like cells derived from hASCs**

To verify the biological functions of treated cells, urea production and glycogen storage ability of the cells were examined. For this reason, PAS was applied to assess the glycogen deposit in the cells. As expected, hepatic differentiation of hASCs led to strongly positive staining in the treated cells at day 21. However, no deposit of glycogen was found in non-induced cells, while Hep G2 cells were intensely positive for PAS staining (Fig. 5A).

In addition to these results, metabolic function of hepatocyte-like cells was also evaluated using urea assay at various stages of

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hepatic differentiation. Following 2 weeks of hepatic induction, urea secretion elevated to approximately 15 mg/dl/50000 cells ($p<0.05$) and continued to increase gradually with a maximum level to 17 mg/dl/50000 cells ($p<0.01$) compared to day 1 (Fig. 5B).

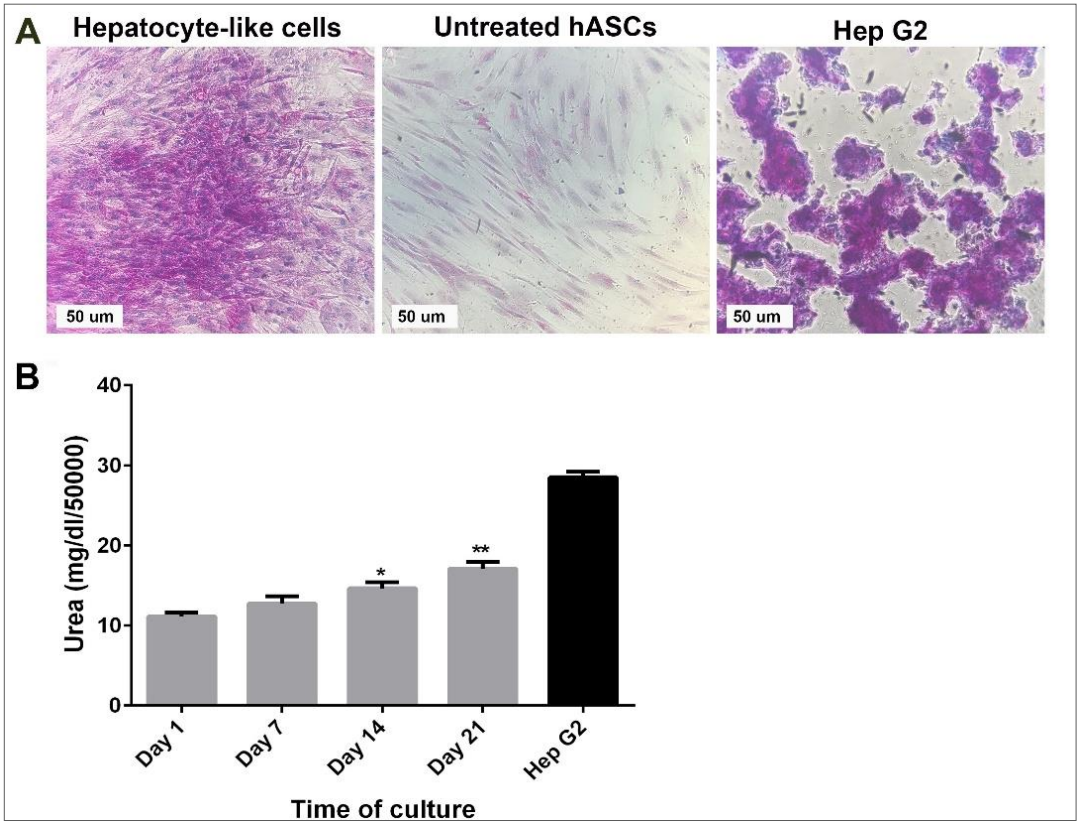


**Fig. 5.** Functional assays of hepatocyte-like cells. A: Glycogen storage ability was detected using periodic acid-schiff staining which showed more positive cells in hepatocyte-like cells compared to untreated hASCs. B: A significant increase in urea production was detected in hepatocyte-like cells at day 14 and day 21 relative to day 1. The data are shown as mean±SD in three independent experiments (n=3). *p <0.05, **p <0.01, ***p <0.001 relative to day 1.

## Discussion

As it has been extensively reported, ASCs possess the potential to differentiate into hepatocyte-like cells using a cocktail of growth factors and inductive agents [11-13, 15]. Interestingly, the expression of several hepatocyte-specific markers in naïve hASCs shows them to become one of the most attractive sources for cell-based therapy of liver diseases [10]. Whereas these findings are exciting, the function of the generated hepatocyte-like cells is often at a far lower level than a real hepatocyte mainly due to using inefficient induction conditions. We have recently documented the hepatic differentiation of hASCS using a combination of HGF, dexamethasone, and oncostatin M over the course of 21 days [23]. In the current study, we report the differentiation efficacy of new culture medium by adding FGF4 to previous inductive medium to promote differentiation of hASCs toward functional hepatocyte-like cells.

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FGFs, secreted from the cardiac mesoderm, are known to be involved in early liver development [18]. In this regard, it has been well documented that FGFs are required for albumin expression and eventually hepatic induction at early somatic stages of development [25]. Of particular interest is FGF4, a member of FGF superfamily, acting as a mitogenic, angiogenic, and survival factor and involving in cell proliferation and differentiation [26]. Notably, the striking role of FGF4 in enrichment and propagation of hepatic progenitors has been reported [27]. Added to this, improvement in liver regeneration has been demonstrated upon transplantation of FGF4 transduced BM-MSCs in cirrhotic rats [22]. In support of this, FGF4 together with HGF were found to promote hepatic differentiation of MSCs and these pretreated cells have been shown as having the capacity to improve liver function in $CCl_4$-induced liver fibrosis [21].

Based on the mentioned results clearly confirming the important role of FGF4 in liver development and regeneration, in this study we examined a new protocol to induce hASCs toward functional hepatocyte-like cells. For this reason, a three-step protocol was designed in which hASCs were treated with a combination of HGF, dexamethasone, FGF4, and oncostatin M, over a course of 21 days and thereafter differentiation status was evaluated at various time points. Upon using this new condition, treated cells exhibited remarkable morphological changes. While we did not observe any change in the cytokeratin 19 expression level (a cholangiocytes gene), a significant increase in alpha-fetoprotein, albumin, cytokeratin 18, and hepatocyte nuclear factor-4α expression levels was detcted in a time-dependent manner. Our study also revealed that new differentiation condition induces hASCs toward functional hepatocyte-like cells as shown by the presence of glycogen storage and increase in urea production as being two prominent features of hepatocyte. These findings indicate that using a cocktail of the mentioned inductive agents results in differentiation of hASCs into functional hepatocyte-like cells.

In this context, it is important to mention that growth factors and regulators used in this study are known to be involved in development and differentiation of hepatocyte. In addition to acting as a hepatocyte mitogen, HGF is also required for hepatoblast migration and proliferation [28, 29]. As mentioned before, FGFs exert a major influence on hepatic specification and also promote liver bud growth [25, 27]. Moreover, it is well established that oncostatin M, secreted by haematopoietic cells in liver, promotes hepatocyte differentiation, acting in collaboration of HGF and glucocorticoid hormones [30].

## Conclusion

A number of *in vitro* studies have reported the generation of hepatocyte-like cells using a combination of growth factors and regulators, including HGF, EGF, FGF, oncostatin M, dexamethasone, etc. However, given the complexity of liver development, the produced hepatocyte-like cells exhibit only a certain

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level of differentiation mainly due to inefficient culture conditions. In the current study, we report the differentiation efficacy of a new, simple and cost-effective culture medium containing FGF4, HGF, dexamethasone, and oncostatin M to induce hepatic differentiation of hASCs toward functional hepatocyte-like cells. However, the involvement of additional growth factors in hepatocyte differentiation remains to be studied. Certainly, given the advances in establishing efficient protocols, it is possible to generate mature functional hepatocyte from stem cells.

## Conflict of Interest

The authors declare no conflict of interest to disclose.

## Acknowledgements

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