

# Efficient Differentiation of Human Induced Pluripotent Stem Cell (hiPSC) Derived Hepatocyte-Like Cells on hMSCs Feeder

Naser Mobarra<sup>1,10</sup>, Masoud Soleimani<sup>2</sup>, Fatemeh Kouhkan<sup>3</sup>, Zahra Hesari<sup>4</sup>, Reyhaneh Lahmy<sup>5</sup>, Majid Mossahebi-Mohammadi<sup>2</sup>, Ehsan Arefian<sup>3,6</sup>, Zahra Jaafarpour<sup>7</sup>, Hajar Nasiri<sup>8</sup>, Reza Pakzad<sup>9</sup>, Rezvan Tavakoli<sup>3</sup>, Parvin Pasalar<sup>11</sup>

<sup>1</sup>Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

<sup>2</sup>Department of Hematology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran

<sup>3</sup>Department of Molecular Biology and Genetic Engineering, Stem Cell Technology Research Center, Tehran, Iran

<sup>4</sup>Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

<sup>5</sup>Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

<sup>6</sup>Microbial Biotechnology Laboratory, Department of Microbiology, School of Biology, College of Science, Tehran University, Tehran, Iran

<sup>7</sup>Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

<sup>8</sup>Hematology-Oncology and Stem cell Transplantation Research Center, Shariati Hospital, Tehran university of Medical Science, Tehran, Iran

<sup>9</sup>Departments of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

<sup>10</sup>Students' Scientific Research Center, Tehran University of Medical Sciences, Tehran, Iran

<sup>11</sup>Metabolic disorder Research center, Endocrinology and Metabolism, Molecular sciences institute, Tehran University of Medical Sciences, Tehran, Iran

**Corresponding author:** Parvin Pasalar, Ph.D. Metabolic disorder Research center, Endocrinology and Metabolism, Molecular sciences institute, Tehran University of Medical Sciences Tehran, Iran

Tel: +98-2188953004

Fax: +98-2164053385

E-mail: ppasalar@yahoo.com

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## ABSTRACT

**Background:** The use of stem cells is considered as an appropriate source in cell therapy and tissue engineering. Differentiation of human induced Pluripotent Stem Cells (hiPSCs) to Hepatocyte-like Cells (HLCs) on mouse embryonic fibroblasts (MEFs) feeders is confronted with several problems that hinder the clinical applications of these differentiated cells for the treatment of liver injuries. Safe appropriate cells for stem cell-based therapies could create new hopes for liver diseases. This work focused on the determination of a capacity/efficiency for the differentiation of the hiPSCs into Hepatocyte-like Cells on a novel human adult bone marrow mesenchymal stem cells (hMSCs) feeder.

**Materials and Methods:** Undifferentiated human iPSCs were cultured on mitotically inactivated human adult bone marrow mesenchymal stem cells. A three-step differentiation process has been performed in presence of activin A which added for 3 days to induce a definitive endoderm formation. In the second step, medium was exchanged for six days. Subsequently, cells were treated with oncostatin M plus dexamethasone for 9 days to generate hepatic cells. Endodermic and liver-specific genes were assessed via quantitative reverse transcription-polymerase chain reaction and RT-PCR, moreover, immunocytochemical staining for liver proteins including albumin and alpha-fetoprotein. In addition, functional tests for glycogen storage, oil red examination, urea production and alpha-fetoprotein synthesis, as well as, cells differentiated with a hepatocyte-like morphology was also performed.

**Results:** Our results show that inactivated human adult bone marrow mesenchymal stem cell feeders could support the efficient differentiation of hiPSCs into HLCs. This process induced differentiation of iPSCs into definitive endocrine cells that expressed sox17, foxa2 and expression of the specific genes profiles in hepatic-like cells. In addition, immunocytochemical analysis confirmed albumin and alpha-fetoprotein protein expression, as well as, the hiPSCs-derived Hepatocyte-like Cells on human feeder exhibited a typical morphology.

**Conclusions:** we suggested a successful and efficient culture for differentiation and maturation of hepatocytes on an alternative human feeders; this is an important step to generate safe and functional hepatocytes that is vital for regenerative medicine and transplantation on the cell-based therapies.

**KEYWORDS:** Induced pluripotent stem cells; Hepatocyte-like Cells; Bone marrow mesenchymal stem cells

## INTRODUCTION

Hepatocyte cell transplantation is a potential way can be a replacement target until patients can receive whole organ transplantation.<sup>1, 2</sup> Stem cell-derived cells have a potential for multi-directional differentiation and self-renewal for replacement therapy which are considered an alternative and proper cell source for generating hepatocytes.<sup>3, 4</sup>

Generation of human induced pluripotent stem cells from dermal fibroblasts by epigenetic reprogramming that is ethically acceptable, holds great promise for advancements in regenerative medicine and disease modeling.<sup>5-8</sup> Thus, they are an infinite source for hepatocyte production in vitro and may serve as a basic component for cell therapy. Cultivation methods for human pluripotent stem cells (hPSCs) have been developed on the basis of mouse embryonic stem cells (mES). Human PSCs are typically derived and propagated on mitotically treated or by  $\gamma$ -irradiation inactivated mouse embryonic fibroblasts (MEFs) as feeder layer cells, which can secrete various factors to prevent PSCs cells from spontaneous differentiation without losing their stemness.<sup>9-11</sup> Despite these advantages, MEFs has a limited potential for clinical use because they are not proper to support human pluripotent stem cells using for therapeutic purpose because they may transfer the danger of exogenous antigens, zoonosis and viruses to hiPSCs which leads to decrease their clinical use.<sup>9, 12</sup> Therefore, to circumvent these problems the use of primary human derived living cells seems to be a hopeful approach. Human tissue-based feeder layers need to be developed for human pluripotent cells as clinical purposes. At the first, Mesenchymal stem cells (MSCs) were identified in Bone Marrow(BM),<sup>13</sup> also they are multipotent cells that can be isolated from bone marrow, adipose tissue, umbilical cord blood and etc, which can replicate as undifferentiated cells in vitro<sup>14-16</sup>.

Thus, a successful differentiation of hiPSCs - derived Hepatocyte-like cells (HLCs) on bone

marrow (BM) feeder can be readily accepted as a great advantage for their potential in vivo differentiation and regenerative medicine. Especially, the differentiation of human iPSCs into hepatocyte-like cells on hMSCs feeder cells has not yet been reported. In this study, we determine whether hMSCs could be used as feeder layers to support the differentiation of hiPSCs to Hepatocyte-like Cells. Here, we show that hMSCs can perform as an appropriate feeder cells instead of MEFs to support the propagation and efficient differentiation of hiPSCs and may be a promising strategy for cell therapy in liver diseases.

## MATERIALS AND METHODS

### Culture, Expansion and passage of cells

Human adult bone marrow mesenchymal stem cells (hMSCs) (from Stem Cells Technology Research Center, Tehran, Iran. Passage 5) which applied as a feeder was plated onto gelatin-coated dishes in DMEM (Gibco, 12491-015) supplemented with 15% FBS (Gibco, 10270106). When cells reach to 60–70% confluency, they inactivated via mitomycin-C treatment for 3h (10 $\mu$ g/ml). After washing with PBS, undifferentiated human iPSCs (a gift from Stem Cells Technology Research Center, Tehran, Iran. The passage number of cells was used from 8 to 14)<sup>17</sup> were cultured on the hMSCs, in DMEM/F12 (Gibco, 31330038) supplemented with 20% knockout serum replacement (Gibco, 10828), 1mM L-glutamin (Gibco, 25030), 1% non-essential amino acids (PAA, M11-003), 0.1mM  $\beta$ -mercaptoethanol, 100U penicillin/streptomycin (Gibco, 15140-122) and 10 ng/ml basic fibroblast growth factor (Peprotech, 100-18B). Then, cells were incubated under standard condition at 95% humidity and 5% CO<sub>2</sub> at 37<sup>o</sup>C. HiPSCs colonies for embryoid body (EB) formation or transferred onto new hMSCs were detached with collagenase IV (Gibco, 15140-122) every 5-7 days.

### EB formation

In order to evaluate the in vitro differentiation ability of grown hiPSCs cells on bone marrow-derived MSCs were detached by collagenase IV, Aggregated EB forms were transferred into 4-well culture plates in iPSC medium without bFGF for dispose to differentiation.

### Hepatic differentiation procedure

To induce hepatic differentiation, a three-step protocol was performed. Endoderm induction was performed according to Song et al.<sup>4</sup> Briefly, EBs were transferred onto gelatin-coated plates in six well plates for 24h in serum-free RPMI 1640 medium (Gibco, 11875119) supplemented with B27 (Gibco, 0080085SA) and 100 ng/ml activin A (Stemgent 03-001). On the following 2 days, 0.5 and 1% insulin-transferrin-selenium (ITS, Gibco, 41400045) was added to this medium, respectively.

Following activin A treatment, hepatogenic differentiation was applied based on Farzaneh et al.,<sup>18</sup> In the second step, the medium was replaced with Hepatocyte Culture Medium (HCM, Lonza, CC-3198) supplemented with 2% FBS, 20 ng/ml hepatocyte growth factor (HGF, R&D, 294-HGN-005) and 20 ng/ml fibroblast growth factor-4 (FGF-4, R&D, 235-F4-025) for a period of six days. The medium was changed every 2 days.

Afterwards, to achieve hepatic cell maturation, differentiated cells were allowed to mature more for an additional nine days in hepatocyte culture media (HCM) that contained 5% FBS, 20 ng/ml oncostatin M (OSM, R&D, 295-OM-010) plus 0.1  $\mu$ M dexamethasone (Dex, R&D, 2749/10), 1% non-essential amino acids and L-glutamine. The medium was changed every two days.

### Total RNA isolation, reverse transcription and quantitative Real-Time PCR and RT-PCR

Total RNA was extracted from human iPSCs, HepG2 cells and each of steps to examine expression of specific genes. Reverse transcription PCR and Real-Time PCR were performed as described previously.<sup>19-21</sup> Total RNA was isolated from cells using TRIzol Reagent (Gibco, 15596018). In the reverse transcription reaction, 3  $\mu$ g of total

RNA was used with the revert aid first strand cDNA synthesis Kit (Fermentas, k1621) and oligo dt primers according to the manufacturer's instructions. RT-PCR amplification was carried out by a standard procedure with denaturation at 95 $^{\circ}$ C for 3 min, annealing temperature from 57 $^{\circ}$ C to 65 $^{\circ}$ C for 35s according to the primers and extension at 72 $^{\circ}$ C for 30s and followed by a final extension at for 72 $^{\circ}$ C. The number of cycles varied between 30 and 40 depending on the abundance of particular mRNA. PCR-products were electrophoreses on 2% agarose gel and visualized by a UV transilluminator (oviduct, UK). Real-time RT-PCR was performed by using Takara SYBR Premix ExTaq Master (RR820A). Gene expression levels were quantified by Rotor Gene 6000 (Corbett, Concorde, NSW, Australia) and analyzed by rest 2009. Target gene expression values were normalized to those for the housekeeping gene Beta-Actin (ActB) and calibrated to the iPSCs. Primers and product lengths are listed in Table 1.

### Immunofluorescence Staining

After completing the differentiating process, the hHLCs were rinsed twice with PBS and fixed with 4% paraformaldehyde (Sigma, 30525-89-4) for 20 min at 4 $^{\circ}$ C, then 5 min at room temperature (RT). After washing, the cells were permeabilized with 3% Triton X-100 in PBS.

The fixed cells were blocked for 30 min at 37 $^{\circ}$ C with 10% goat serum (Gibco, 16210064)/PBS and then remove it. They were next incubated overnight at 4 $^{\circ}$ C in a humidity chamber with respective primary antibodies: mouse monoclonal anti-fetoprotein (AFP, 1:200, R&D, MAB1368), mouse monoclonal anti-albumin (ALB, 1: 200, R&D, MAB1455). At the end of the incubation time, the cells were rinsed 3 times with PBS-Tween 20 (0.1%) and incubated with the PE conjugated as a secondary antibody, goat anti-mouse (1:100; R&D, F0102B) as appropriate for 45 min at 37  $^{\circ}$ C in the dark. Subsequently, these subjected cells were washed with PBS-Tween %0.1, 3 $\times$ 5 min. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole [(DAPI) (1  $\mu$ g/ml)] (Sigma, 28718-90-3). Nuclear staining and the cells were analyzed with a fluorescent microscope (Nikon; Japan).

**Table1:** Primer Sequences and conditions used for qRT-PCR and RT-PCR

Genes	Sequences	Annealing Temperature °C	Gene bank code
	5' → 3' F R		
<b>OCT-4</b>	TTC GCA AGC CCT CAT TTC AC CCA TCA CCT CCA CCA CCT G	60	NM_002701.5
<b>SOX17</b>	CAA GAT GCT GGG CAA GTC TGG TCC TGC ATG TGC TG	60	NM_022454.3
<b>FOXA2</b>	AGC GAG TTA AAG TAT GCT GG GTA GCT GCT CCA GTC GGA	58	NM_153675.2
<b>ALB</b>	CTT TGG CAC AAT GAA GTG GGT AAC GCA GTC AGC CAT TTC ACC ATA GG	62.8	NM_000477.5
<b>AFP</b>	GCC AAG ATA AAG GAG AAG AAG TGT GTA AGC AAC GAG AAA CG	59	NM_001134.2
<b>TDO</b>	TGG GAA CTG CCT GCA TTT GG ACA GAA TCC AAC TCC CAG AGG	65	NM_005651.3
<b>AAT</b>	GGA AAA TGA AGA CAG AAG GTC CCT TAG TGA TGC CCA GTT G	60	NM_001127707
<b>PEPCK</b>	GCG GCT GAA GAA GTA TGA C GGA ACC TGG CAT TGA ACC	57	XM_006723802
<b>CY18</b>	TGG CGA GGA CTT TAA TCT TGG CTC AGA ACT TTG GTG TCA TTG G	55	NM_199187.1
<b>Act B</b>	GTC CTC TCC CAA GTC CAC AC GGG AGA CCA AAA GCC TTC AT	60	XM_006715764

### Cellular Low-Density Lipoprotein (LDL) uptake assay

We assayed Low-Density Lipoprotein (LDL) uptake in matured cells by using the Dil-Ac-LDL staining kit according to the manufacturer's instructions (Biomedical Technologies, BT-904)

### Functional Assays

#### Periodic Acid-Schiff (PAS) stain for glycogen synthesis

Glycogen storage was measured by using PAS staining at day 18. Briefly, hiPSCs-HLCs-derived cells toward the end of the 3rd week were fixed with 4% paraformaldehyde for 20 min at room temperature and then washed with PBS. Fixed cells were oxidized in 1% periodic acid (Sigma, 10450-60-9) for 5 min and rinsed 3 times in deionized water. Samples were then treated with Schiff's reagent (Sigma, 3952016) for 10-15 minutes. At the end, washed in running water for 5 minutes and assess under light microscope (Olympus, Japan).

#### Oil red

Differentiated cells were examined for one of the hepatocytes characteristics by oil red staining. HLCs-

derived cells were fixed with 4% paraformaldehyde for 20 min at room temperature and then washed with PBS and incubated for 45 min with oil red reagent. Afterward, cells were washed with running water and analyzed by a light microscope (Olympus, Japan).

### Secretion analysis

Conditioned media was collected on the day zero, 3rd, 13th and 18th days from differentiated cells and then were stored at -20<sup>oC</sup> until assay day. The conditioned media was assayed for Alpha-fetoprotein (AFP) production via a Chemiluminescence Immunoassay kit (Diasorin.UK). The cell urea concentration was measured by a Colorimetric Assay Kit (Pars Azmun, 1400029) according to manufacturer's recommendations. The conditioned media of undifferentiated iPSCs (on the day zero) and HepG2 cells were used as negative and positive controls. The data was normalized based on defined cell number.

### Statistical Methods

Triplicate samples from the subjected cells were conducted. Data was expressed as the mean ±

standard deviation (SD). The comparisons of parameters were performed using Student's t-test.  $p$  value  $\leq 0.05$  was considered as significant results. Data was analyzed using SPSS 16 (SPSS Inc., Chicago, IL, USA) software.

## RESULTS

hMSCs illustrated fibroblastic morphology (Figure 1A) and hiPSCs cells respectively cultured on MSCs, formed tightly packed and flat colonies with sharp edges that exhibited large nuclei and limited cytoplasm (Figure 1/Day 0).

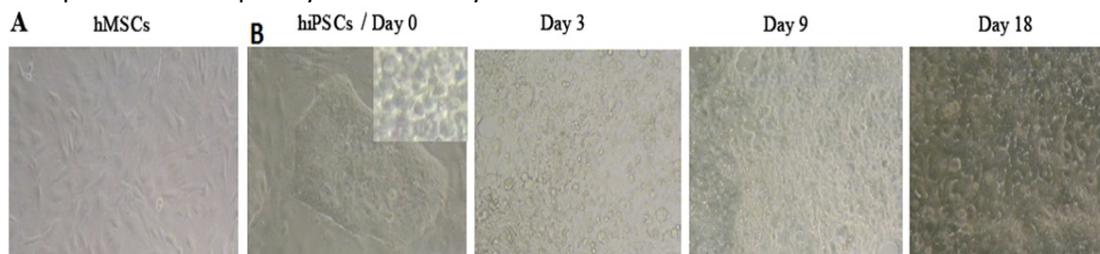
After passaging and remain for 4-5 days in suspension culture, the hiPSCs clones formed ball-shaped and round structures known as embryoid bodies (EBs) (Figure 1C).

Three stage differentiation processes are illustrated in Figure 2. Three days after starting, the attached cells derived from the EBs (Figure 1/Day 3) were detected through Quantitative RT-PCR. Forkhead box A2 (FOXA2) and SRY-box containing gene 17 (SOX17) which are endodermis markers were increased the expression level ( $P < 0.05$ ), whereas OCT-4 was remarkably reduced ( $P < 0.05$ ) (Figure 3A). These results demonstrated that derived hiPSCs efficiently could be differentiated into endoderm in vitro.

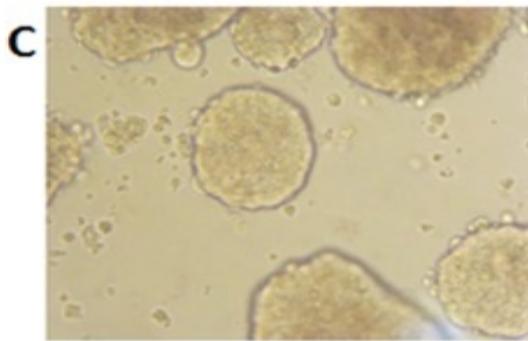
As shown in Figure 1B, human iPSCs began to show morphological changes at the 3rd day and pre-matured progressively (Figure 1/Day 9). In the second stage, Albumin (ALB), Alpha-fetoprotein (AFP), tryptophan 2, 3-dioxygenase (TDO), cytokeratin 18 (CY 18), Alpha-1 antitrypsin (AAT) and Phosphoenolpyrovate Carboxykinase (PEPCK) genes expression were used to evaluate differentiation efficiency. However, combination of two growth factors in this stage, led to increase AFP expression specially. Maximum expression of AFP was shown in pre-mature hepatocyte on the day 9

before starting final stage (Figure 3B). These results demonstrated that the derived hiPSCs could be differentiated into pre-mature hepatocyte.

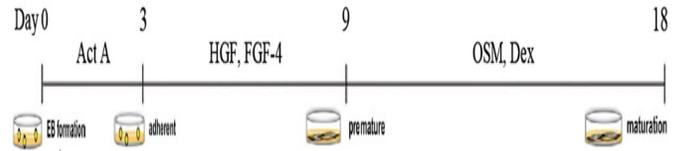
Finally, in the beginning of the day 18th, morphology of cells was analyzed. They resembled flattened shapes typical of hepatocytes (Figure 1/Day 18). Furthermore, we next investigated the expression of molecular and biochemical hepatic markers in the end stage. RT-PCR analysis demonstrated that ALB, TDO, AAT, CY18 and PEPCK were also quite low initially but were dramatically increased further subsequently which resembled the HepG2. Importantly, AFP expression was presented in pre-mature hepatocyte of monolayer culture, decreased in final period (Figure 3B). After confirming the expression of HLC-derived markers culture pellets at the morphological and mRNA level, we next examined the capability to perform hepatic biochemical function assays for use in clinic. Alpha-fetoprotein was not secreted considerably at the day 3, whereas significantly increased at day 9 ( $p < 0.05$ ) in compared to the end of stage 3 ( $p < 0.05$ ) (Figure 4A) that also verified with RT-PCR analysis of AFP expression. Undifferentiated cells did not secrete Urea whereas after induction of hepatic differentiation, urea production continuously increased ( $p < 0.05$ ) and it reached nearly to the positive control amount at the day 18 (Figure 4B). Moreover, in the induced culture at stage 3 to prove more, we examined the ability of differentiated cells to uptake LDL. The most of matured cells uptake LDL and more differentiated cells expressed early fetal liver cell markers such as AFP and ALB (Figure 5); moreover, glycogen and lipid storage exhibited strong positive staining (Figure 6). These results could prove hepatocyte maturation.



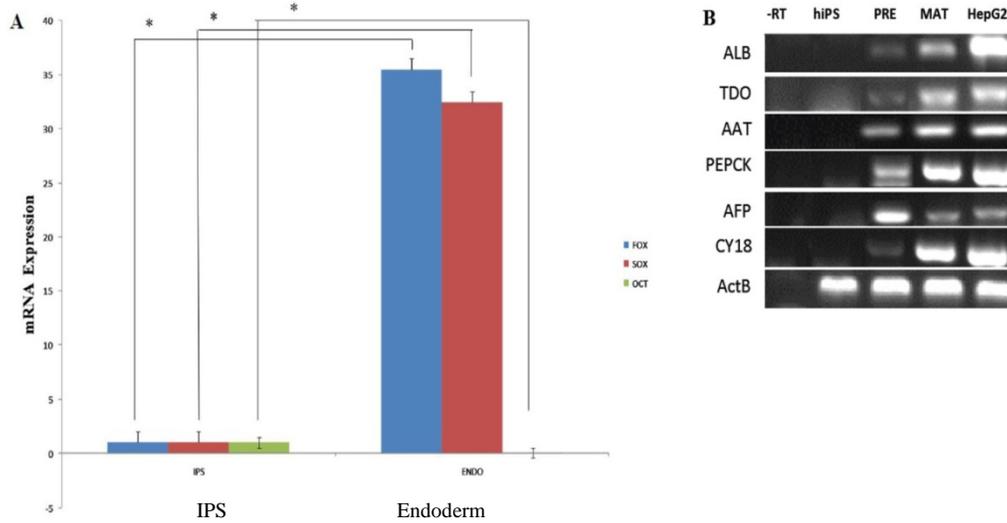
**Fig1:** Phase contrast microscopy images of **A)** hMSCs, **B)** hiPSC colonies on hMSCs feeders and morphological changes of iPSC cells at different stages of differentiation ( $\times 100$  magnifications)



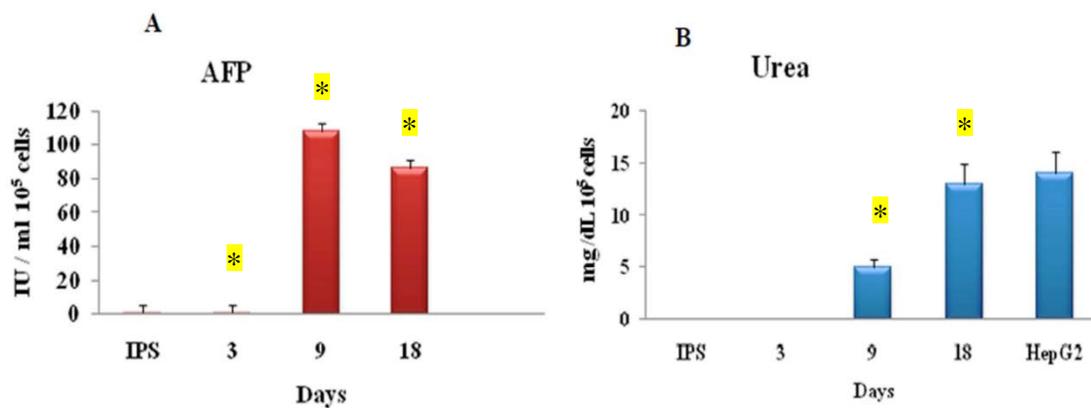
**Fig1c.** Phase contrast microscopy images of EBs ( $\times 100$  magnifications)



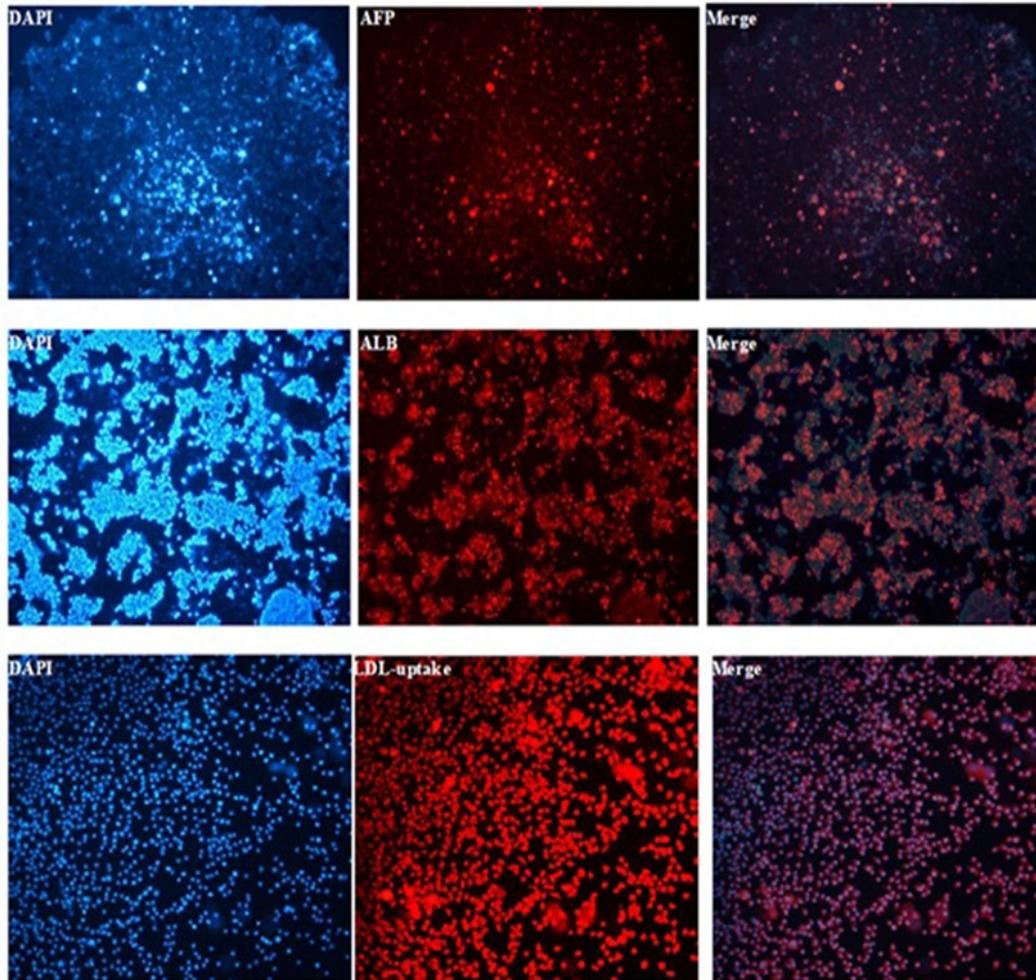
**Fig2.** Schematic flow chart of the 3-stage differentiation procedure



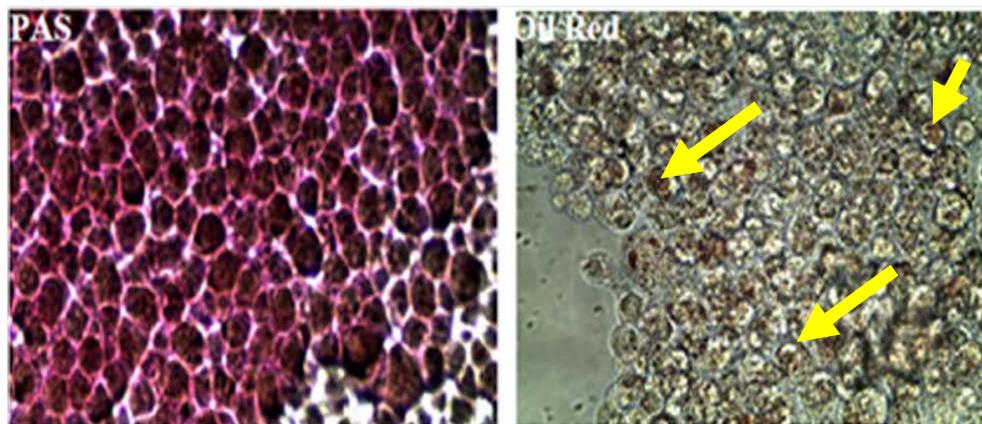
**Figure 3.** A) Quantitative transcriptional analysis of pluripotent and endodermal markers. B) RT-PCR characterization of hepatocyte markers. \* Significance was defined as  $P < 0.05$  and values represent means  $\pm$  SD. PRE: Pre-mature. MAT: Mature



**Fig 4.** A) Alpha-fetoprotein and B) Urea production by hepatocytes at developmental stages \* Significance was defined as  $P < 0.05$  and values represent means  $\pm$  SD.



**Fig5.** Immunocytochemistry staining showed Albumin expression, Alpha-fetoprotein and Lipoprotein uptake, the main markers of hepatocytes in different cells. Nuclei were stained with DAPI. ( $\times 100$  magnifications)



**Fig6.** Staining analysis of matured HLCs include glycogen (PAS) and lipid storage (Oil red). ( $\times 100$  magnifications)

**DISCUSSION**

Liver transplantation (LT) since 1992 until now, has been the best and only therapeutic possibility

for patients with end-stage of liver diseases.<sup>22, 23</sup> Nevertheless, Hepatocyte-based therapies derived from stem cells are emerging as an alternative strategy to whole-organ transplantation for

decreasing mortality in acute liver failure and treatment of metabolic liver diseases.<sup>24</sup> Cultivated hepatocyte-like cells transplantation is an effective way to restore an intact hepatocyte which are already engineered from iPSCs in clinical use.<sup>23</sup> The safe and efficient derivation of hiPSCs into specialized cell types will be extremely important in transplantation therapy using hiPSCs.

Human induced pluripotent stem cells are well known for its pluripotency and self renewal capability which are beneficial them in regenerative medicine; Moreover, it is useful for functional studies and transplantation.<sup>25, 26</sup> Mostly, expansion and differentiation of pluripotent stem cells require complex culture systems which used SNL or murine-derived cells as feeders<sup>27, 28</sup> which is not proper for regenerative medicine because of the ethical reasons, as well as, they may transfer the chance of zoonosis, exogenous antigens, bear a risk of immune response, infection and viruses to hiPSCs.<sup>29</sup> Considering the capabilities and applications of iPSCs, researchers have focused on improvement iPSCs in vitro culture system. In this study, unlike previous reports which typically used MEF or SNL to derivation of pluripotent stem cells,<sup>28</sup> we were looking at the fact that whether hMSCs can maintain the differentiation potency of iPSCs to HLCs. This study is a first report of hiPSCs- HLCs administration on human based-tissue feeder.

In our study, the expansion efficiency of hiPSCs on hMSC feeder cells was in consistent with a previous report by Cheng et al<sup>12</sup> which indicated the hiPSCs were growing improved on hMSC feeder cells; furthermore, we report that hiPSCs can be efficiently differentiated on hMSC feeder to the hepatic cells. The cells were differentiated on novel feeders in three steps; In order to evaluate the in vitro differentiation ability of iPSCs to endoderm, we checked endodermic specific genes. QRT-PCR detected that EBs expressed marker pluripotency such as Oct-4. Although, Foxa2 and Sox17 expression were noticeably low down. After 3 days, the results were reversed. Similar to our study, expression of Foxa2 and Sox17 in endoderm differentiation observed in these cells derived from human pluripotent stem cells is consistent with previous reports during hepatogenic differentiation of human ips.<sup>23, 30</sup> In final steps of differentiation in

consist with Kazemnejad,<sup>31</sup> multiple examinations were employed to identify the hepatic identities. First, HPLCs exhibit a polygonal epithelial morphology and large cytoplasm that contained various granules. Interestingly, the differentiated cells have shown characteristic of hepatocyte markers including being positive for examined the biochemical markers ALB and AFP production, LDL-uptake, Glycogen and lipid storages which are other characteristics of hepatocytes. Differentiated cells were able to secrete Urea into the medium which was similar to a hepatoma cell line HepG2 as well. However, AFP secretion in the final steps of differentiation is much lower than primary hepatocytes. Additionally, molecular characterization for gene markers AAT, PEPCK, TDO and CY18 (Figure 3B) were used as an evidence for functionality of efficient differentiation. However, these results were similar to other researchers<sup>32</sup> suggested that the differentiated cells in our culture exhibit characteristics of HLCs-like cells.

At first , MEFs were used<sup>10, 33</sup> followed<sup>12</sup> Autologous fibroblasts and immortalized human skin fibroblasts have been reported and could be used as feeder layers to establish and maintain hiPSCs.<sup>25, 29</sup> In our culture system and differentiation process, using hMSCs is clearly an advanced feeder for iPSCs culture techniques that can be applied in regenerative medicine because in contrast to MEFs or SNL which are pathogens, xeno and allo-free. However, with the mentioned benefits, the hMSCs could be readily derived from healthy adult donors and expanded manifold before use as feeder cells. Moreover, these feeders have been widely used in clinics for many years.

Our results proved our hypothesis that hiPSCs can be induced with high efficiency the differentiation into functional hepatocyte-like cells. This basic research might minimize ethical problems and overcomes the current limitations of human iPSCs cells for future therapeutic usage. We believe that using high-quality, hiPSCs-HLCs-derived cells on human-derived MSCs feeder cells will improve the safety of hepatocyte transplantation in the treatment of acute liver diseases.

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## CONFLICT OF INTEREST

All authors declare no conflict of interest.

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