Expression of Endoderm and Hepatic Specific Genes after *in vitro* Differentiation of Human Embryonic Stem Cells

Hossein Baharvand[*], Seyed Mahmoud Hashemi and Saeid Kazemi Ashtiani

Dept. of Stem Cells, Royan Institute, P.O. Box: 19395-4644, Tehran, Iran

ABSTRACT

Background: Human embryonic stem cells (hESC), which are derived from the inner cell mass of the blastocysts, have been considered to be pluripotent cells. In this study we examine the differentiating potential of hESC into hepatocytes by characterization of the expression of endoderm and liver-specific genes. **Methods:** hESC were cultivated in suspension to form aggregates, the embryoid bodies. They were allowed to outgrowth on the plated culture with the stepwise addition of growth factors such as acidic fibroblast growth factor (aFGF), hepatocyte growth factor and oncostatin M into the culture medium. The expressions of endodermal and liver specific genes such as hepatocyte nuclear factor 3β, alpha-fetoprotein (AFP), albumin (ALB), cytokeratin 8 (CK-8), CK-18, transthyretin, glucose 6-phosphatase and tyrosine aminotransferase were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The expressions of ALB and CK-18 in the cytoplasm were analyzed by Immunohistochemistry. **Results:** The immunoblotting and chemiluminescence of the conditioned media indicated the secretion of ALB and AFP. RT-PCR analysis revealed that hepatic gene expression related to early and late-stage liver development were enhanced through *in vitro* differentiation of hESC. **Conclusion:** Our results showed the expression of endoderm and hepatic specific genes after *in vitro* differentiation of hESC into hepatocyte-like cells through addition of various growth factors in three dimensional culture systems (collagen type I). hESC could be a new potential source of hepatocyte for transplantation in patients with liver failure. *Iran. Biomed. J. 10 (3): 117-124, 2006*

Keywords: Embryonic stem cell, Hepatocyte, Endoderm, Gene expression, Differentiation

INTRODUCTION

Human embryonic stem cells (hESC) are a type of pluripotent stem cell lines isolated from the inner cell mass of blastocysts. They are the original source of all other cells and have capacity to be indefinitely maintained in undifferentiated state. They also have the potential to differentiate into a variety of cell lineage [1-3]. The pluripotency of hESC has been proved both *in vivo* and *in vitro*. Directed differentiation of embryonic stem (ES) cells is a powerful tool to analyze the mechanisms controlling the development of mammalian organs. *In vitro* aggregation of hESC brings about the formation of the embryoid bodies (EB) which have shown to express molecular markers specific to three embryonic germ layers [4, 5]. The hESC, injected *in vivo* into immune deficient mice, generate teratomas which contains ectoderm, mesoderm and endoderm [2]. It has recently been shown that some endodermal differentiations toward the hepatic cells occur upon the spontaneous differentiation of hESC [4-6]. The differentiation of ES cells into hepatocyte-like cells has been reported in several papers. Mouse ES cells have shown a potential to develop into hepatocyte-like cells *in vitro* on the basis of hepatic gene expression after adding several growth factors. [7-14]. The research of hepatic differentiation from hESC has not yet been performed except for the resent reports of Lavon *et al.* [15] and Rambhatla *et al.* [6]. The hESC could provide a source of hepatocytes and enable us to study hepatic differentiation *in vitro*. The aim of this study was to investigate the expression of endoderm and hepatic specific genes after *in vitro* differentiation of hESC into hepatocyte-like cells through addition of various growth factors, such as

*Corresponding Author; Fax: (+98-21) 224 09313; E-mail: Baharvand50@yahoo.com

acidic fibroblast growth factor (aFGF), hepatocyte growth factor (HGF) and oncostatin M (OSM) in three dimensional culture system (collagen type I). Also, to explore whether hESC could be a new potential source of hepatocyte for transplantation in patients with liver failure. In this study, we analyze the expressions of albumin (ALB) and cytokeratin 18 (CK-18) in the cytoplasm by immuno-histochemistry and ALB and alpha-fetoprotein (AFP) production in the conditioned media of hESC by immunoblotting, ELISA and chemiluminescence.

MATERIALS AND METHODS

Culture of hESC. Royan H1, hESC line, as previously described [16], was grown on a mitomycin-C treated mouse embryonic fibroblast (MEF) feeder layer in gelatin-coated tissue culture dish. Royan H1 cells were maintained in hES medium consisting of 80% Knockout DMEM supplemented with 20% ES-FCS, 2 mM glutamine, 0.1 mM β–mercaptoethanol, 1% nonessential amino acid stock (Gibco/BRL, USA), 5 mg/ml insulin, 5 mg/ml transferrin, 5 μg/ml selenium, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown in 5% CO_2 and 95% humidity, and then further propagated as clumps of 200-500 stem cells on MEF about every seven days. All chemicals were purchased from (Gibco, BRL, USA) and Sigma (USA).

Induction of hepatic differentiation in vitro. For differentiation, the clumps of stem cells were dissociated with a combined approach of mechanical slicing by a pipette. Then, the clumps were cultured in the absence of MEF feeder layers in "hanging drops" (1 clump/20 μl drop) to produce EB for 2 days. The EB in the "hanging drops" were transferred into the suspension culture in 100 mm Petri dishes and cultured for an additional 3 days. The resulting EB were cultured in three-dimensional culture system in 24-well tissue culture dishes containing Vitrogen (type I collagen) (Cohesion, Palo Alto, CA, USA). By the method of Hamazaki *et al.* [7], several growth factors were added into the culture medium for hepatocyte differentiations at the following times: aFGF (R and D Systems, Minneapolis, MN) (100 ng/ml) were added between the days nine and 12 as an early stage factor for hepatic maturation. HGF (R and D Systems, Minneapolis, MN) (20 ng/ml) was added

between days 12 and 20 as a mid-stage factor. OSM (R and D Systems, Minneapolis, MN) (10 ng/ml), dexamethasone (Sigma, USA) (10^{-7} M), insulin (5 mg/ml), transferrin (5 mg/ml) and selenium (Gibco, BRL, USA) (5 μg/ml) mixture were added as late stage factors between the days 15 and 20. The differentiated cells were allowed to grow up to the day 28. For *in vitro* differentiation without addition of growth factors, the EB in "hanging drops" were transferred into the suspension culture dishes and cultured for an additional 3 days and grown in tissue culture plates for 28 days without addition of growth factors.

RNA extraction and RT-PCR analysis. Total RNA was extracted from cultured cells using NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Then, the extracted RNA was treated by DNase I (Roche Diagnostic, Germany) to avoid cross contamination of RNA by genomic DNA and 2 μg of RNA was reverse transcribed by random hexamer priming using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Germany) based on the protocol of the manufacture. The cDNA samples were subjected to PCR amplification with DNA primers specific to the human genes using a pair of oligomers, each from a different exon. Amplification conditions were as follows: initial denaturation at 94ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 59-70ºC for 45 s (depending on the primers), extension for 45 s at 72°C and a final polymerization at 72ºC for 10 min. Each PCR was performed as triplicates. PCR products were analyzed by gel electrophoresis on 2% agarose and stained with ethidium bromide, visualized and photographed on a UV transluminator (Uvidoc, UK). A description of primers and size of final products are described in Table 1. As positive control, human hepatoma cell line, HepG2, obtained from the Pasture Institute of Iran (Tehran) was used.

Immunohistochemical staining. Formalin-fixed paraffin-embedded collagen matrix was cut into 4-μm-thick sections and mounted on the glass slides coated with poly-L-lysine. The sections were deparafinized in xylene and rehydrated in 100, 96 and 70% ethanol for 5 min and finally brought to PBS. The sections were incubated in blocking buffer (0.1 M PBS containing 0.5% normal goat serums, 0.2% Triton X-100) for 1 h, and thereafter

Table 1. Human primers used in RT-PCR studies.

Genes	Primer sequence (5'→3')	Annealing Temperature (°C)	Length (bp)	Gene bank code
GAPDH	CCA GGT GGT CTC CTC TGA CTT CAA CAG AGG GTC TCT CTC TTC CTC TTG TGC TCT	62	224	NM002046
HNF3β	CCA CCA CCA ACC CCA CAA AAT G TGC AAC ACC GTC TCC CCA AAG T	60	294	NM021784
AFP	GCA GCC AAA GTG AAG AGG GAA GA GTC ATA GCG AGC AGC CCA AAG AAG	69	216	NM001134
AAT	CCA TGT TTG TCA AAG AGC AAC T GGA AGT AAG GTA TAG TCA GGT GAT	61	345	NM001085
TTR	GGT GAA TCC AAG TGT CCT CTG AT GTG ACG ACA GCC GTG GTG GAA	61	352	NM000371
ALB	CTGCTTGAATGTGCTGATGACAG GGCATAGCATTCATGAGGATCTG	60	365	NM000477
CK-8	CAG ATC AAG TAT GAG GAG CTG CA AGC TGG TGC GGC TGA AGG AT	63	503	NM002273
CK-18	CCA TGC GCC AGT CTG TGG AG GTG GTG CTC TCC TCA ATC TGC T	65	322	X12881
TAT	GCT AAG GAC GTC ATT CTG ACA AG GTC TCC ATA GAT CTC ATC AGC TAA G	67	354	NM000353
G6P	GCT GAA TGT CTG TCT GTC ACG AA GCA GAA GGA CAA GAC GTA GAA GA	60	494	NM000151
OCT4	CTT GCT GCA GAA GTG GGT GGA GGA A CTG CAG TGT GGG TTT CGG GCA	65	187	NM002701

GAPDH, Glyceraldehyde-3-phosphate-dehydrogenase; HNF3β, Hepatocyte nuclear factor 3β; AFP, Alpha fetoprotein; AAT, Alpha-1-anti-trypsin; TTR, Transthyretin; ALB, Albumin; CK, cytokeratin; TAT, Tyrosine aminotransferase; G6P, Glucose 6 phosphate.

in primary antibodies diluted in blocking buffer at 4°C for 12 h. The antibodies used in this study were mouse monoclonal anti-ALB (1:200) from (R and D Systems, Minneapolis, MN) and mouse monoclonal anti-CK-18 (1:200) from (Chemikon, Temecola, CA). Adjacent sections served as negative controls and were processed using identical procedures, except for incubation without the primary antibody. The IgG/FITC goat anti-mouse (1:100) from (Sigma, USA) was added as secondary antibody. Incubation with secondary antibody was done at 25°C for 30 minutes. The sections were counter stained with propodium iodide and the images were captured using a fluorescent microscope (Nikon, Japan).

AFP and human ALB secretion by differentiated hESC. The media in triplicate wells, conditioned for 24 h, were assayed for AFP, marker of endoderm, using chemiluminescence kit (Liaison, Italy). Human ALB production in medium was analyzed by immunoblot. The samples were fractionated on 10% SDS-PAGE gels and transferred into polyvinylidene difluoride membrane. After washing, the membrane was blocked with 10% skim milk at room temperature for 1 h and incubated with mouse monoclonal antibody specific for human ALB (1:1000 Sigma, USA), at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse antibody (1:1000, Sigma, USA) at room temperature for 1 h. After washing, the immunoreactive bands were detected by 0.5 mg/ml 3, 3' diaminobenzidine (Sigma, USA) and 0.1% H_2O_2 in PBS. Conditioned medium from hESC cultured on a mitomycin-C (Sigma, USA)

treated mouse MEF feeder layer was used as control group (undifferentiated cells). We also detected human ALB production using quantitative enzyme-linked immunoassay kit (Albumin ELISA, Orgentech Diagnostica, Germany) according to manufacturer's recommendations.

RESULTS

Endoderm and hepatic specific gene expression. We detected the mRNA expression of endodermal- and liver-specific genes including: hepatocyte nuclear factor 3β (HNF3β), transthyretin (TTR), alpha-1-anti-trypsin (AAT), AFP, ALB, CK-8, CK-18, glucose-6-phosphatase (G6P) and tyrosine aminotransferase (TAT) by RT-PCR. Figure 1 illustrates the pattern of endodermal and hepatic-specific gene expressions at the day 28 in differentiating EB with and without the additional growth factors. In this study, the undifferentiated hESC did not express mRNA of the endodermal or hepatocyte lineage genes while, the hESC expressed OCT4 mRNA which is a marker of undifferentiated hESC and HNF3β, which is a liver-enriched transcription factor. In the spontaneously differentiated EB (without addition of growth factors) after the day 28, TAT and G6P, late differential markers of hepatocyte, were not detectable. Endoderm-specific genes expression was detected in EB differentiating without additional growth factors. These data indicate that hESC differentiated toward hepatic lineage cells, without addition of growth factors, but did not differentiate into mature and functional hepatocytes. As shown in Figure 1, the expression of TAT is low and G6P gene expression is not detected at the day 21 with additional growth factors. However, in differentiated EB at day 28, both TAT and G6P, markers of mature hepatocyte, were detectable. We used glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), a key enzyme in glycolysis as internal control. The mRNA encoding GAPDH is moderately abundant message in cells. This house-keeping gene is constitutively expressed in many tissues and has been reported to be a useful internal control.

Immunohistochemistry. To confirm the hepatic differentiation from EB, we performed immuno-staining using primary antibody to human ALB and CK-18. ALB is the most abundant protein synthesized by mature and functional hepatocytes
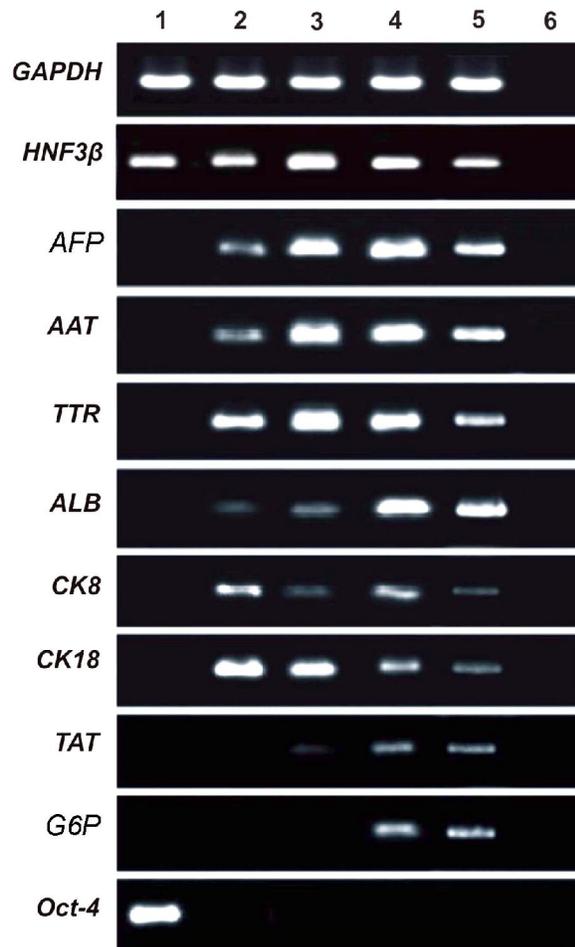

Fig. 1. Expression of endodermal and hepatic specific genes in hESC cells before and after differentiation by RT-PCR. Cells were harvested at the indicated time (days). Lane 1, undifferentiated hESC; Lane 2, spontaneously differentiated (without addition of growth factors) hESC; Lane 3, hESC differentiated (with addition of growth factors) for 21 days; Lane 4, hESC differentiated (with addition of growth factors) for 28 days; Lane 5, hepG2 cell line as positive control; Lane 6, RT-negative. GAPDH, glyceraldehyde-3-phosphate-dehydro-genase; HNF3β, Hepatocyte Nuclear Factor 3β; AFP, alpha fetoprotein; AAT, alpha-1-anti-trypsin; TTR, transthyretin; ALB, albumin; CK, cytokeratin; TAT, tyrosine aminotransferase; G6P, glucose 6 phosphate.

and CK-18 is special skeleton protein of hepatocyte. The obtained results showed that the EB differentiated with growth factors were positively stained for ALB (Fig. 2A) and for CK-18 (Fig. 2B). The EB differentiated without growth factors were weekly positive for ALB (Fig. 2C) and for CK-18 (Fig. 2D).
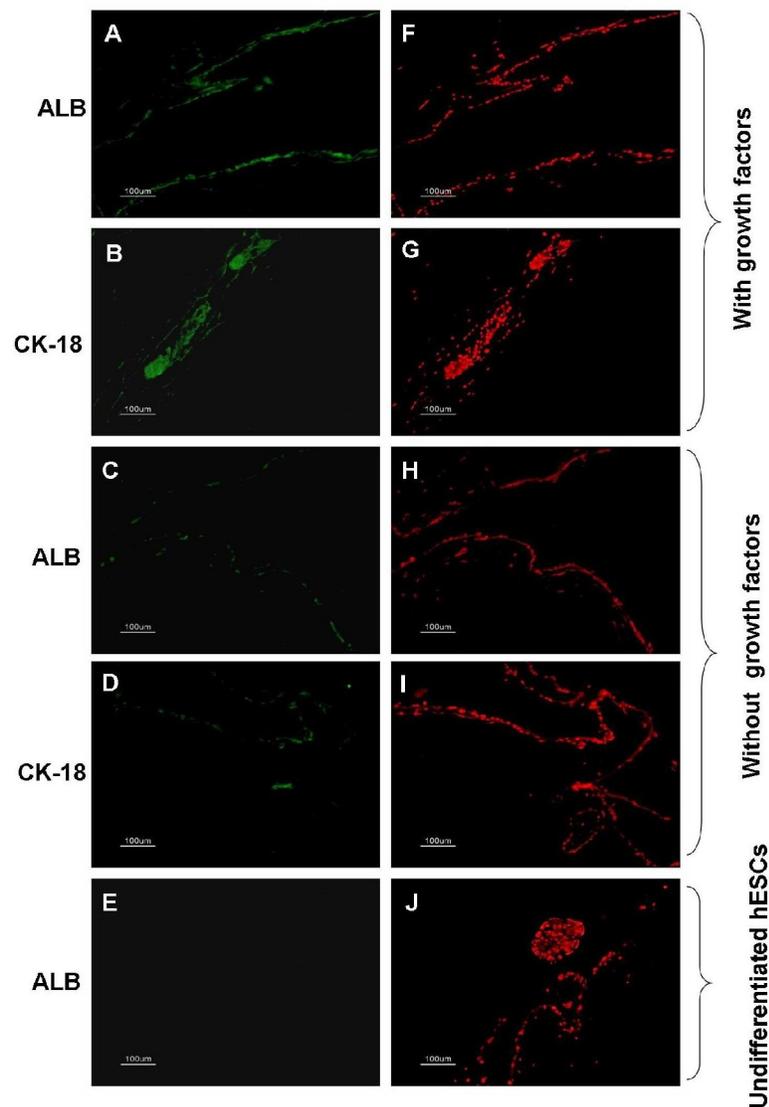
Fig. 2. Immunohistochemical staining of formalin-fixed paraffin-embedded cells in collagen matrix with anti-ALB and anti-CK-18 antibody. The EB differentiated with growth factors were positively stained for ALB **(A)** and for CK-18 **(B)**. The EB differentiated without growth factors were weakly positive for ALB **(C)** and for CK-18 **(D)**. Undifferentiated hESC were negative for ALB **(E)** and for CK-18 (data not shown). The Nuclei were stained with propidium iodide **(F-J)**. ALB, albumin; CK, cytokeratin.

AFP and ALB secretion by differentiated hESC. AFP was detected (>1000 IU/ml) in the conditioned medium of the differentiated EB at the days 21 and 28, indicating endoderm differentiation. No AFP was detected in conditioned medium from hESC cultured on mouse MEF feeder layer. We also detected the presence of human ALB in the conditioned medium of differentiated hESC at the days 21 and 28 using immunoblot analysis. Human ALB in the conditioned medium of the differentiated hESC at the day 21 is lower than that of day 28. No human ALB was detected in the conditioned medium from hESC cultured on mouse MEF feeder layer (Fig. 3). We measured ALB production by ELISA at days 0, 21 and 28 following differentiation. Human ALB produced by differentiated hESC with growth factors at days 21 and 28 was 1.2 ± 0.25 μg/ml and 3.33 ± 0.46 μg/ml, respectively. Differentiated and undifferentiated hESC without growth factors did not secrete ALB. Since ALB is a functional protein produced by mature hepatocytes, its production at the day 28 led to an implication that EB-derived hepatocytes-like cell began to be functionally mature at this time.

1   2   3   4   5

Fig. 3. Western-blot analysis of albumin production. Western-blot analysis was carried out on the conditioned medium of cells. Lane 1, normal human serum (1/100 diluted) as positive control; Lane 2, undifferentiated hESC cultured on mouse embryonic fibroblast (MEF) feeder layer; Lane 3, differentiated hESC without growth factors; Lane 4, differentiated hESC at day 21; Lane 5, differentiated hESC at day 28.

DISCUSSION

In this study, we investigated the endodermal and hepatic differentiation of hESC *in vitro* with and without additional growth factors. Mature hepatocyte markers, TAT and G6P, were clearly detectable in the presence of the growth factors at the day 28. In this condition, production of ALB indicated the presence of mature and functional hepatocytes-like cells. Recently, it has been reported that mouse ES cells can generate cells with hepatocyte characteristics indicating that derivatives of definitive endoderm can develop in this model system [7-14]. The embryonic endoderm gives rise to many organ systems such as liver, pancreas and thyroid [17]. The hESC can spontaneously differentiate into endoderm *in vitro*. Therefore, this *in vitro* system should be useful for the identification and characterization of the endodermal and hepatic differentiations [8]. More recently, Lavon *et al.* [15] have analyzed the differentiation of hESC cells into hepatocytes by suspension culture of EB for 20 days and then dissociated and plated for an additional 10 days with aFGF. In another study, Rambhatla *et al.* [6] induced hepatic differentiation by treatment with sodium butyrate while in the present study, we used 5-day-old EB and differentiation induced by the method of Hamazaki *et al.* [7]. The hepatic endoderm develops next to the cardiac mesoderm and is affected by secreted factors from the mesodermal cells during normal development, [18, 19]. The growth factors, aFGF and bFGF, are secreted from the cardiac mesoderm at the time of hepatic induction. In mouse embryos, it has been shown that FGF signaling from the cardiac mesoderm is necessary and sufficient to induce a hepatic fate within the endoderm. The response to this factor is restricted to the endoderm tissue, which uniquely expresses the receptors for these factors [20]. When visceral endoderm and hepatic

differentiation were examined by gene expression during *in vitro* mouse ES cells differentiation, an early marker such as TTR was detected within 3 days after induction of differentiation. AFP and AAT were first detected around days 6 to 9. AFP is a marker of the endodermal differentiation as well as an early fetal hepatic marker, and its expression decreases as the liver develops into adult phenotype [7, 21]. TTR and AAT represent endodermal differentiation and those expressed throughout liver maturation. It was reported, ALB mRNA expression first appeared within the day 12 [22]. ALB, the most abundant protein synthesized by mature hepatocytes, is the marker of the functional hepatocytes. CK-8 and CK-18 are special skeleton proteins of hepatocytes that play very important roles in maintaining the skeleton of the hepatocytes which expressed in the cytoplasm along with the differentiation of fetal liver *in vivo* [21, 22]. G6P is predominantly expressed in the liver at a late gestational stage and considered as a marker of hepatic mature differentiation [23,24]. TAT represents an excellent enzymatic marker for mature and functional hepatocyte and represents an important function of liver. In the present study, the time of expression of G6P was different from the other studies on mouse ES cells [7, 8]. This difference could be due to the genuine species differences in pluripotent cell phenotype, and variation of culture condition. It may also reflect that the ES cells correspond to a different stage of embryonic development in the two species. Differentiating hESC without additional growth factors expressed endodermal specific genes such as AFP, TTR, AAT, CK-8, CK-18, and ALB, but not G6P and TAT at the day 28, demonstrating that hESC can spontaneously be differentiated into visceral endodermal cells or early hepatic lineage cells; however, they can hardly be differentiated into mature and functional hepatocyte. Currently, there is much interest in the generation of mature hepatocyte

from stem cells. Hepatocyte transplantation has been shown to be a plausible treatment in human metabolic liver diseases [25] and also been shown to be effective in the animal models of hepatic failure and metabolic liver diseases [26, 27]. However, primary cultures of hepatocytes either do not replicate sufficiently *in vitro* to produce the number of cells necessary for transplantation or do not maintain their differentiated properties *in vitro*. [8] Our findings suggested that hESC might be an alternative source of hepatocytes for transplantation therapy, but additional research is needed to improve the efficiency of differentiation.

ACKNOWLEDGMENTS

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