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The influence of fibroblast growth factor 4 on hepatogenic capacity of Wharton's jelly mesenchymal stromal cells

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Abstract

Wharton's jelly mesenchymal stromal cells (WJ-MSCs) derived from human umbilical cords could be an appropriate candidate for hepatocyte replacement therapy. Improvement of the efficiency of the cell expression of liver specific genes can be considered in finding new transplantation resources. The present study aimed to differentiate WJ-MSCs toward hepatocyte-like cells on collagen film in the presence of hepatogenic factors, including fibroblast growth factor 4 (FGF4), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1). MSCs derived from Wharton's jelly explants were characterized by flow cytometry. Then, the cells were cultured in the presence of hepatogenic media with or without FGF4 on 2D collagen films for 21 days. The expression of liver-specific genes was evaluated by real-time polymerase chain reaction (RT-PCR) and immunocytochemistry. The functional assays were performed by Periodic Acid–Schiff (PAS) staining and Indocyanin Green (ICG) uptake. The cultures pre-exposed to FGF4 expressed higher levels of endodermal markers, such as albumin, compared to the control cultures. Also, cytokeratin 18 expression was significantly increased in FGF4-treated cells. However, the expression level of other liver-specific markers was not influenced by exposure to hepatogenic media with or without FGF4. In conclusion, it was demonstrated that FGF4 could induce the differentiation of WJ-MSCs toward endoderm. Despite the morphological changes and increase in PAS reaction, WJ-MSCs could not differentiate into hepatocytes by hepatogenic media consisting of IGF-1.

Keywords: Wharton's jelly, mesenchymal stromal cells, 2D, collagen, fibroblast growth factor 4, hepatocyte growth factor.

Introduction

Hepatocytes transplantation therapy may be considered as a promising replacement resource for treatment of end-stage liver diseases. However, the major problems with this procedure are shortage of liver donors and immunological rejection of the transplanted cells [1, 2]. During the last decade, several investigations have been performed to find therapeutic strategies and the best cell source for hepatocyte transplantation [1, 3–5]. Mesenchymal stromal cells (MSCs) from human umbilical cord, Wharton's jelly, are an appropriate candidate for hepatocyte replacement therapy [6, 7]. Umbilical cord can be typically regarded as medical waste post-delivery [8]. This provides an available, non-invasive source without any ethical concern. Wharton's jelly-derived MSCs (WJ-MSCs) have a large expansion and self-renewal potential as well as an extensive differentiation potential toward various fates, including adipogenesis, osteogenesis, and chondrogenesis [9, 10]. Similar to autologous transplantation, allogeneic transplantation of WJ-MSCs can be applicable for cell therapy without immunological cross-reactivity [11, 12]. Wharton's jelly has been shown to express a low level of many liver-specific markers such as albumin, cytokeratins (CKs) 18 and 19, α-fetoprotein (AFP) [6]. These features make human Wharton's jelly a superior resource for isolating stem cells in comparison to other sources, such as bone marrow and amniotic membrane, for therapeutic and clinical applications [13, 14].

Up to now, combinations of growth factors have been used to differentiate MSCs toward hepatocyte-like cells [15]. Hepatocyte growth factor (HGF) can be considered as a critical factor in the early stages of hepatogenesis. It also stimulates hepatoblast proliferation and liver regeneration [10, 16]. The other important factor in hepatocyte differentiation is fibroblast growth factor-4 (FGF4). FGF4 has mitogenic and angiogenic effects and can induce MSCs to proliferate and differentiate toward endoderm and subsequently hepatogenic fate [17, 18]. Insulin-like growth factor-1 (IGF-1) can be considered as an anabolic hormone, which is mainly produced by the liver. IGF-1 plays an important role in *in vitro* hepatocyte differentiation and proliferation [19]. It also stimulates HGF production and expression of liver specific markers, such as albumin [20–22].

In *in vivo* condition, cells are primarily embedded within microenvironment including various components of extracellular matrix (ECM), neighboring cell populations, and different growth factors. The simplicity, convenience, and high viability of the cells in conventional 2D monolayer culture condition have caused that this culture condition to be more popular in many investigations [23]. However, the conventional culture might not mimic the physiological behavior of cells *in vivo*. Therefore, *in vitro* usage of ECM constitutions, such as collagen, has been shown to improve functional efficiency

of the cells [24]. Evidence has demonstrated that *in vitro* culturing of hepatocytes on collagen gel led to higher expression levels of liver specific genes [25]. The role of type I collagen in regulation of hepatocytes cycle and differentiation was also demonstrated [26].

The effects of a combination of HGF, IGF-1, and oncostatin M (OSM) on differentiation of bone marrow-derived MSCs have been shown previously [27]. Pre-exposed MSCs to FGF4 have been detected to differentiate the cells toward endoderm. Besides, dedifferentiation of the hepatocytes derived from stem cells has been reported by various procedures [28]. Therefore, mimicking *in vivo* development of the hepatocytes may lead to differentiation of hepatocyte-like cells that are more suitable for therapeutic purposes. The current study aims to differentiate WJ-MSCs toward hepatocyte-like cells on collagen film in the presence of hepatogenic factors, including FGF4, HGF, and IGF-1.

Materials and Methods

Isolation and primary culture of Wharton's jelly mesenchymal stromal cells

Umbilical cords of the newborn infants, born through cesarean section were obtained after getting written informed consents from their parents. They were then transported to the laboratory in cold phosphate-buffered saline (PBS) containing 100 U/mL Penicillin and 100 µg/mL Streptomycin. After discarding the arteries and amnion completely, the umbilical vein was cut open lengthwise and crushed. Afterwards, the Wharton's jelly of umbilical cord was cut into small pieces for generation of explants with 5 mm dimension. The culture medium, α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS), 1% L-Glutamine, 100 U/mL Penicillin, and 100 µg/mL Streptomycin, was also added to the explants of Wharton's jelly after 15 minutes. The cells derived from Wharton's jelly reached 70–80% confluence after two weeks.

Surface marker characterization

The cell surface markers on the WJ-MSCs were evaluated by flow cytometry at the third passage. An aliquot of the cells at a concentration of 1×10^6 cells/mL was washed in cold PBS containing 10% FBS, as blocking solution, for 20 minutes. After that, the cells were allowed to react with Fluorescein Isothiocyanate (FITC)-conjugated anti-CD44, CD90, and CD144, Phycoerythrin-conjugated anti-CD34, CD73, and CD106, and Peridinin Chlorophyll Protein Complex (PerCP)-conjugated anti-CD105 antibodies (all from Abcam, UK, Cambridge) for 30 minutes. They were then washed with cold PBS, re-suspended in PBS containing 10% FBS, and analyzed by a flow cytometer (BD FACSCalibur™, BD Biosciences). Mouse antibodies IgG1-PE and IgG1-FITC were used as isotype controls.

Adipogenic and osteogenic differentiation potential of WJ-MSCs

For adipocyte and osteocyte differentiation, the WJ-MSCs were cultured in the presence of adipogenic media (StemCell Technologies, Inc., Canada) and osteogenic (MACS, Germany) for three and four weeks, respectively, with the media being changed twice a week. The differentiated adipocytes were stained with Oil Red O (Sigma, USA). Briefly, 0.005% of Oil Red O in isopropanol was prepared and diluted with deionized water at 1:3 ratios. The cells were fixed with 4% formalin at 4^0C for 20 minutes and were then incubated with staining solution for 15 minutes. To detect the calcium deposition, 1% Alizarin Red/S (Sigma, USA) was prepared in 25% ammonium hydroxide. After that, the cells were fixed at 96% methanol for 20 minutes and incubated with staining solution for 15 minutes. The nuclei were counter-stained with Hematoxylin.

Collagen film preparation

In this study, 3 mg/mL type I collagen from rat tail (Gibco, A10483-01) was used to prepare working solution (1 mg/mL). The stock solution was diluted with 10X DMEM at 8:1 ratio on ice by slow and gentle pipetting. Each well of a 4-well culture dish was coated with 125 µL of the working solution and incubated to polymerize at 37^0C for one hour.

Hepatocyte differentiation

WJ-MSCs were seeded on the collagen film at a density of 10^3 cells/mL. The cultures were divided into three groups. The first group was considered as control cultures that were incubated at DMEM (low glucose) supplemented with 5% FBS, 100 U/mL Penicillin, and 100 µg/mL Streptomycin. The second group was exposed to DMEM supplemented with 5% FBS, 20 ng/mL HGF, 100 nM Dexamethasone, 100 U/mL Penicillin, and 100 µg/mL Streptomycin for seven days. Then, 10 ng/mL OSM was added to the hepatocyte differentiation medium and the cells were incubated for an additional two weeks. Finally, the third group was first incubated with DMEM supplemented with 5% FBS and 10 ng/mL FGF4 for two days and thereafter the culture medium was replaced with DMEM containing 5% FBS, 20 ng/mL HGF, 20 ng/mL IGF, 100 nM Dexamethasone, 100 U/mL Penicillin, and 100 µg/mL Streptomycin and was incubated for an additional five days. The differentiation protocol was continued by adding 10 ng/mL OSM and the cells were cultured up to day 21. Half of the culture medium was changed every three days. Figure 1 depicted the experimental design and the steps of the differentiation protocol in briefly.

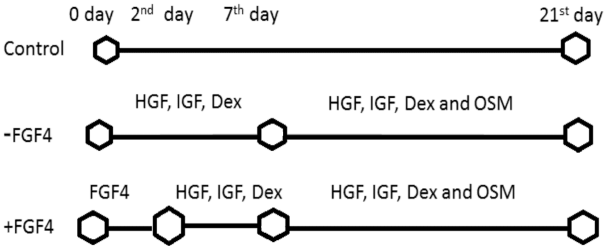

Figure 1 – ***Schematic diagram of the protocol steps for each experimental group.***

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

Total RNA was prepared from WJ-MSCs using Biazol isolation reagent (Bioflux, Japan) according to the manufacturer's instructions. Then, 5 µg of total RNA was

utilized to make cDNA using ABI cDNA Synthesis Kit (USA). The human specific primers were used to amplify cDNA for quantitative real-time PCR (qPCR) reactions. qPCR was performed using the SYBRs green PCR master mix (Applied Biosystems, Rotkreuz, Switzerland) in 50 cycles. After amplification, the melting curve was analyzed to ensure that no primer dimers were produced in the samples and the accuracy of the reactions was confirmed. Subsequently, in order to normalize the qPCR data, each target gene was compared to the housekeeping gene, 18srRNA. Then, the following equation was applied for each data point: $2^{(-\Delta\Delta Ct)}$. The specificity of the primers was determined using NCBI Primer-BLAST software [29] (Table 1).

Table 1 – ***The sequences of primers used for qPCR/real-time PCR***

Gene	Forward primer	Reverse primer	Annealing temperature [^{0}C]
Albumin	5'-ACAGAGACTCAAGTGTGCCAG-3'	5'-GCAAGGTCCGCCCTGTCATC-3'	60
AFP	5'-TTCATATGCCAACAGGAGGC-3'	5'-TGAGAAACTCTTGCTTCATCGT-3'	60
CK18	5'-AAATCCGGGAGCACTTGGAG-3'	5'-CAATCTGCAGAACGATGCGG-3	60
CK19	5'-ACTACACGACCATCCAGGAC-3'	5'-CCGTCTCAAACTTGGTTCGGA-3	60
CYP2B6	5'-TTCTTCCGGGGATATGGTGT-3'	5'-TCCCGAAGTCCCTCATAGTG-3'	55
HNF-4	5'-AAGAAATGCTTCCGGGCTGG-3'	5'-GACGGGGGAGGTGATCTGTC-3'	57
G6P	5'-CGACGAAGCGCAGACAG-3'	5'-GTATCCGACTGATGGAAGGC-3'	60
Claudin	5'-CTTCTTGCAGGTCTGGCTAT-3'	5'-AGGTTGTTTTTCGGGGACAG-3'	60
18S	5'-GTTGATTAAGTCCCTGCCCT-3'	5'-TCCGAGGGCCTCACTAAACC-3'	60

Immunocytochemical and immunofluorescence staining

The cells were fixed with 4% paraformaldehyde (Sigma), washed in PBS, and incubated in 0.3% H_2O_2 in dark for 20 minutes to suppress endogenous peroxidase activity. The cells were incubated in blocking solution containing 5% goat serum at room temperature for 20 minutes. They were then stained with primary antibodies [cytokeratin 18 (CK18), cytokeratin 19 (CK19), and α-fetoprotein (AFP)] for one and a half hours. Afterwards, the super enhancer was added to the cells for 15 minutes and they were incubated in applied Polymer_HRP (EnVision) for 30 minutes. Then, freshly prepared 3,3'-diaminobenzidine (DAB) and H_2O_2 solution were added to the samples for 3 minutes and the cells were washed by distilled water. The cells were counterstained with Hematoxylin.

2D cultured cells were prepared for immunofluorescence staining as follow. The cells were fixed in 4% para-formaldehyde (Sigma) for 15 minutes, and then washed in PBS. The cells were permeabilized with 0.5% Triton X-100 for 10 minutes and blocked with 1% goat serum in PBS for 20 minutes. The anti-albumin FITC-conjugated (Abcam) at 1:100 dilutions were added to the cells and incubated according to the manufacturer's protocol. Then, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and analyzed by the fluorescence microscope (Olympus BX51, Japan).

Periodic Acid–Schiff (PAS) staining for glycogen

The cells were fixed with 4% paraformaldehyde, washed with PBS, oxidized in 1% periodic acid for 5 minutes, and rinsed in several changes of deionized water. Thereafter, the cells were incubated in Schiff reagent (containing basic fuchsine, potassium metabisulfite, and hydrochloric acid) for 15 minutes, washed with tap water for 5 minutes, and their nuclei were counterstained with Mayer's Hematoxylin for one minute.

Indocyanine Green (ICG) uptake

To determine cellular uptake of Indocyanine Green (ICG), 1 mg ICG dry powder (Cardiogreen; Sigma Aldrich, UK) was suspended in 1 mL solvent, 50 µL dimethyl sulfoxide, and 950 µL culture medium. ICG was added to the differentiated cells at 37^{0}C and 5% CO_2 for one hour. Cellular uptake of ICG was checked by inverted microscope. Then, the cells were incubated with fresh William's medium containing 10% FBS. ICG was totally released from the cells six hours later.

Statistical analyses

All the statistical analyses were performed using GraphPad Prism 5 software (GraphPad, USA) and Mann–Whitney test. *P*-value <0.05 was considered as statistically significant.

All the experiments were performed in triplicate.

Results

Characterization of WJ-MSCs

The flow cytometry analysis of WJ-MSCs at the third passage showed that the MSCs phenotype by expressing the surface markers, such as CD90 (thy-1), CD73 (ecto-5'-nucleotidase) [12], CD44 (adhesion molecule for hyaluronic acid) [30], CD106, and CD105 (cell adhesion molecules) [31]. However, these cells were negative for CD34 (hematopoietic stem cell) and CD144 (endothelial cell) markers (Figure 2) [32].

The frequency of the positive cells for each CD marker has been summarized in Table 2.

Table 2 – ***The percentages of the WJ-MSCs showing positive-reaction for CD markers by flow cytometry***

CD markers	*CD44*	*CD105*	*CD106*	*CD34*	*CD144*	*CD90*	*CD73*
%	89.6	74.4	69.2	1.10	2.18	90.5	95.6

The differentiation capacities of WJ-MSCs toward adipogenic and osteogenic lineages were assessed by oil red staining and Alizarin Red/S, respectively (Figure 3). The results revealed that the cells could store lipid droplet in the presence of adipogenic medium. The cells could also differentiate into osteoblast and restore Ca^{2+}. These data confirmed pluripotency of the WJ-MSCs.

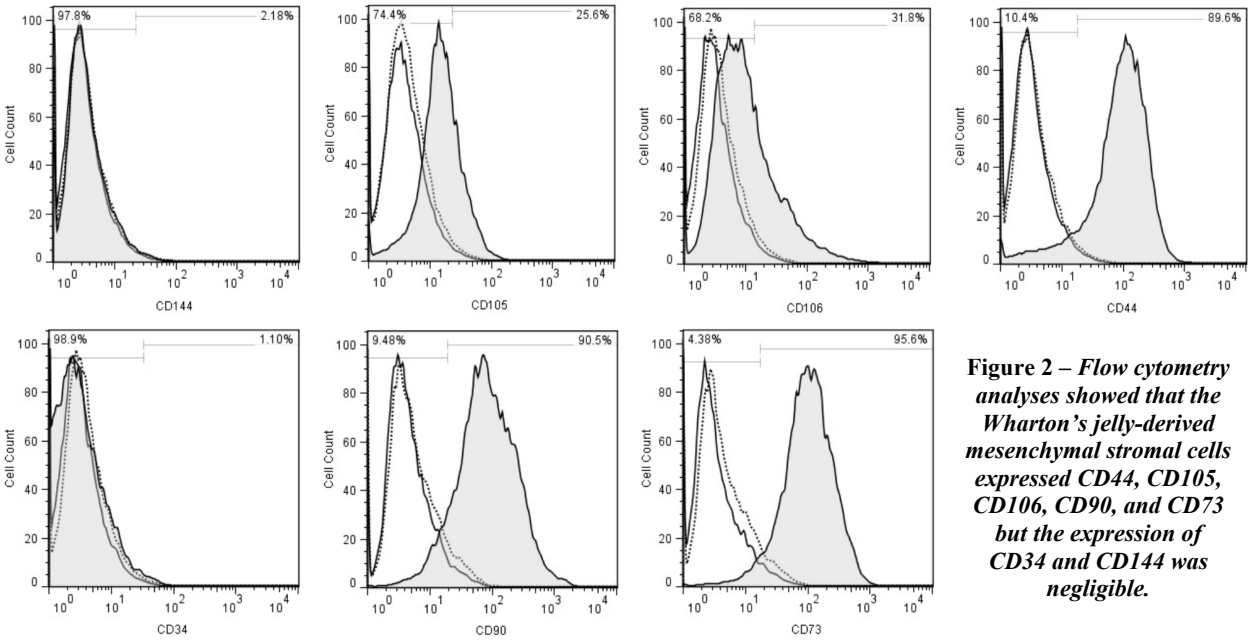

Figure 2 – ***Flow cytometry analyses showed that the Wharton's jelly-derived mesenchymal stromal cells expressed CD44, CD105, CD106, CD90, and CD73 but the expression of CD34 and CD144 was negligible.***

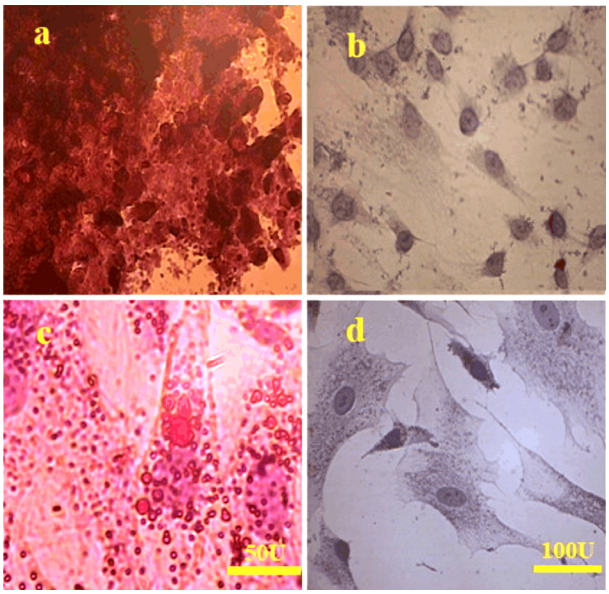

Figure 3 – ***Alizarin Red/S staining revealed Ca^{2+} deposition in the cells cultured in osteogenic medium (a) and its absence in the control cultures (b). Oil Red O revealed the presence of lipid droplets in the cells cultured in adipogenic medium (c) and its absence in the control cultures (d). The nuclei were counterstained with Hematoxylin.***

Cell morphology

The cells in the control group showed the typical characteristic of MSCs; *i.e.*, spindle fibroblast-like morphology. Exposing the cells to hepatogenic media changed the cell morphology from spindle fibroblast-like into a polygonal- and epithelial-like shape (Figure 4). The growth factors presented in the hepatogenic media increased the proliferation rate of the WJ-MSCs.

Quantitative real-time PCR

Albumin, AFP, CK18, CK19, claudin, hepatic nuclear factor 4 (HNF4), glucose-6-phosphatase (G6P) and CYP2B6 expression was assessed in the cells cultured in all conditions for 21 days. Albumin mRNA levels increased after the cells were exposed to the differentiation medium supplemented with FGF4 (26-fold) or without FGF4 (28-fold) compared to the undifferentiated cells. Additionally, in comparison to the control cultures, CK19 expression decreased in the cells pre-exposed to FGF4 and in the cells exposed to hepatogenic medium without FGF4 pre-treatment. A significant decrease in CK18 was also observed in the FGF4-treated cells compared to the control cultures (p=0.01). However, expression of AFP was not changed in the FGF4-pre-treated cells and in the cells treated with hepatogenic medium without FGF4 compared to the control cultures. Naïve human WJ-MSCs expressed a very low level of metabolic enzymes, such as CYP2B6. However, a non-significant increase in CYP2B6 was detected in the pretreated cultures with FGF4 (62.79-fold) compared to naïve and hepatogenic treated-cells. On the other hand, the cells could express claudin in all the cultures (Figure 5). The expression level of HNF4 showed a non-significant increase in the cultures exposed to the differentiation medium without FGF4 compared to the undifferentiated cells (1.6-fold) and also the cells pre-treated with FGF4.

The expression of G6P, a secretary products of the hepatocytes, were increased significantly (p=0.03) in naïve human WJ-MSCs compared to cultures supplemented with FGF4. However, the expression level of G6P was decreased in the cultures exposed to hepatogenic medium without FGF4, it was not significant. The results revealed no significant difference among the three groups regarding genes expression except for G6P and CK18.

Immunocytochemistry and immunofluorescent staining

Expression of AFP, CK18, and CK19 (Figure 6) at protein level was assessed in different culture groups by immunocytochemistry staining. The frequency of the positive cells after immunostaining with CK18, CK19, and AFP has been presented in Table 3.

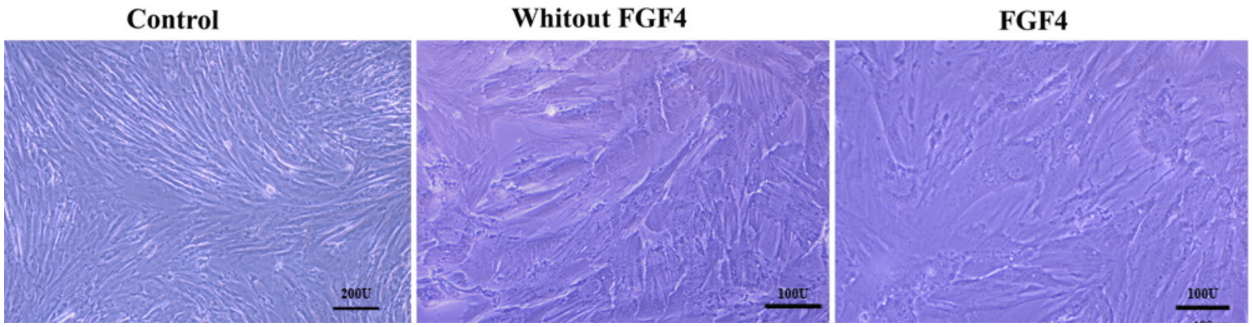

Figure 4 – ***The inverted microscopy showed morphological modification in the cultures treated with hepatogenic media with or without FGF4. The cells in the control cultures were fibroblast-like.***

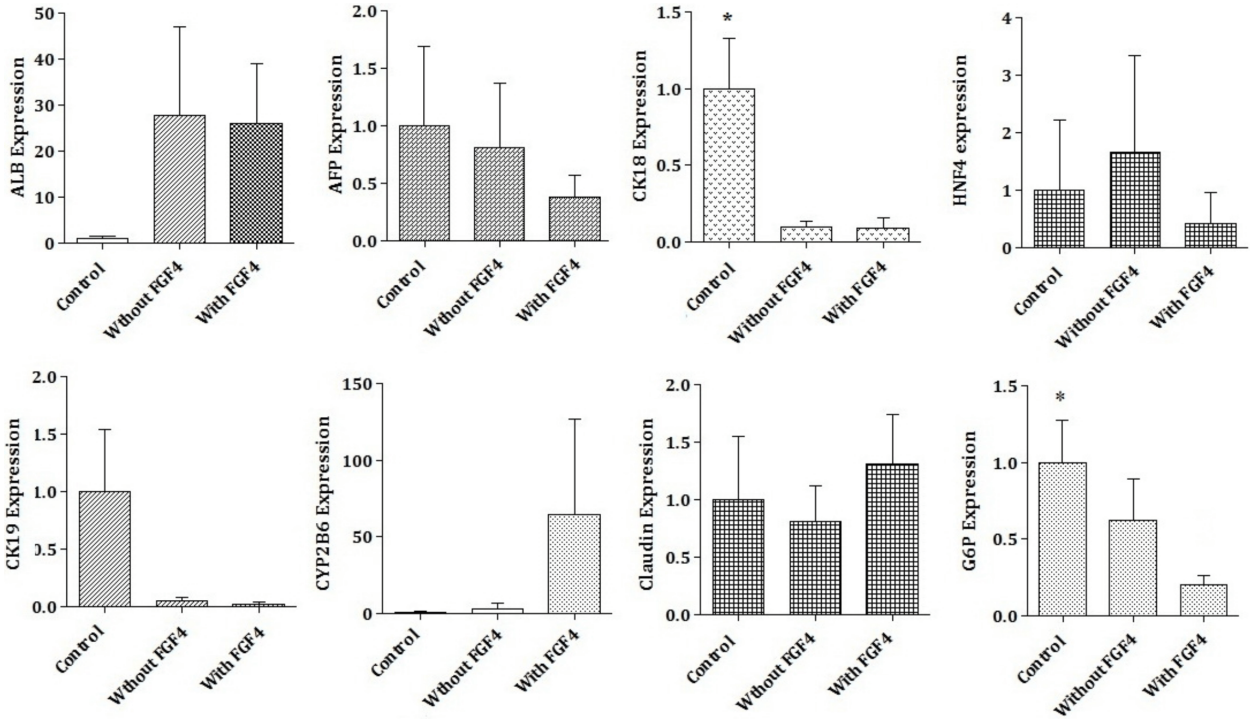

Figure 5 – ***Quantitative real-time PCR showed that the cultures treated with FGF4 expressed higher levels of albumin, CYP2B6 and claudin. The FGF4-treated cells expressed a lower level of CK18 and CK19 and G6P. AFP was expressed by all the cultures.***

Table 3 – ***The mean and standard deviation of the percentage of the reacted-cells to cytokeratins 18, 19, and α-fetoprotein antibodies***

Liver specific markers	With FGF4 (mean ± SD)	Without FGF4 (mean ± SD)	Control (7 days) (mean ± SD)
Cytokeratin 18	63±2.15*	50.5±2.12	27.66±1.15
Cytokeratin 19	41±1.2	41±3.6	30.6±4.5
α-Fetoprotein	53.33±7.37	46.6±6.1	23.3±7.5

*Significant difference with control cultures (p<0.05).

According to the results, the percentage of CK18 protein showed a significant increase by exposing the cells to hepatogenic media with FGF4 compared to control cultures (p=0.046). However, no significant change was observed in expression of CK19 by treating the cells with hepatogenic medium with or without pre-exposure to FGF4. Moreover, AFP was shown to be expressed in the cells treated with hepatogenic media with or without FGF4 pretreatment (53.3% and 46.6%, respectively) and they showed a non-significant increase (p=0.05) in the expression of AFP compared to the control cultures (23.3±7.5).

The accumulation of albumin in the cells was confirmed by immunofluorescent staining (Figure 7). An increase in intensity of albumin staining was shown in cultures exposed to hepatogenic media with FGF4 compared to control.

Functional assay

PAS staining

The differentiation efficiency of WJ-MSCs was evaluated by PAS staining. The WJ-MSCs, which were cultured in hepatogenic media with or without FGF4 (for 21 days), demonstrated the ability to store glycogen (Figure 8).

ICG uptake

ICG uptake assay used to evaluate the functional capacity of the differentiated cells compared to the undifferentiated ones (control cultures) at day 21. The results showed that ICG was released by the cells incubated in the hepatogenic media with or without FGF4 as well as the cells cultured without any hepatogenic stimulation (Figure 8).

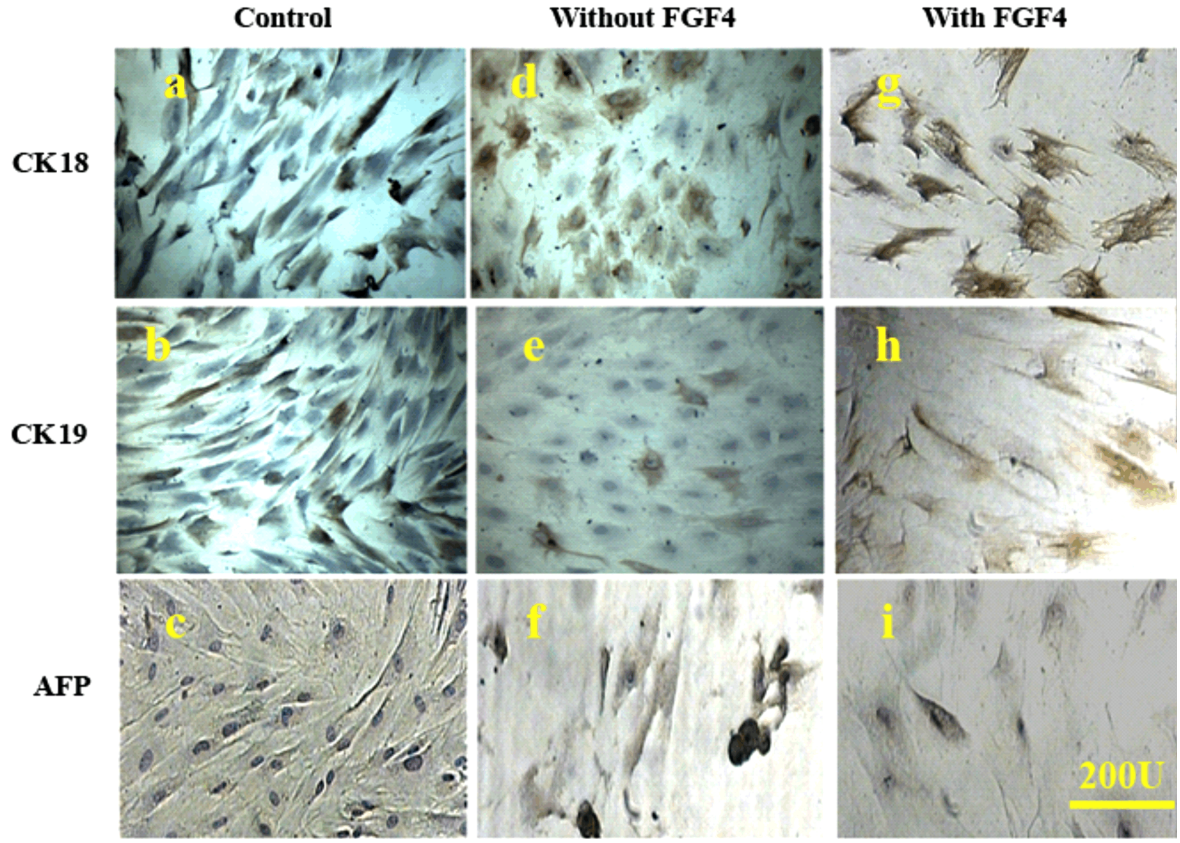

Figure 6 – ***Immunohistochemistry showed that the Wharton's jelly-derived mesenchymal stromal cells expressed cytokeratins 18 and 19 as well as a low level of AFP in the control (a–c), hepatogenic media without FGF4 (d–f), and with FGF4 (g–i). Also, adding FGF4 to the hepatogenic medium increased the percentage of positive cells and the intensity of the reaction to cytokeratin 18 (g–i).***

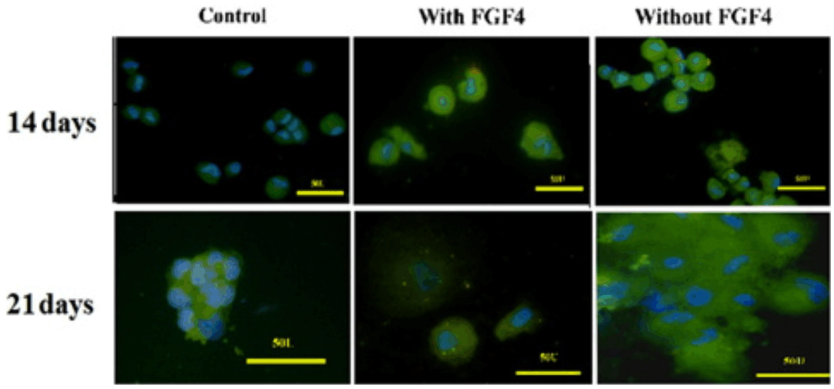

Figure 7 – ***Immunofluorescent staining of WJ-MSCs differentiated toward hepatocyte-like cells using anti-ALB human polyclonal antibody after treatment with hepatogenic media with or without FGF4 and control for 14 and 21 days. The intensity of reaction was increased in cultures treated with FGF4.***

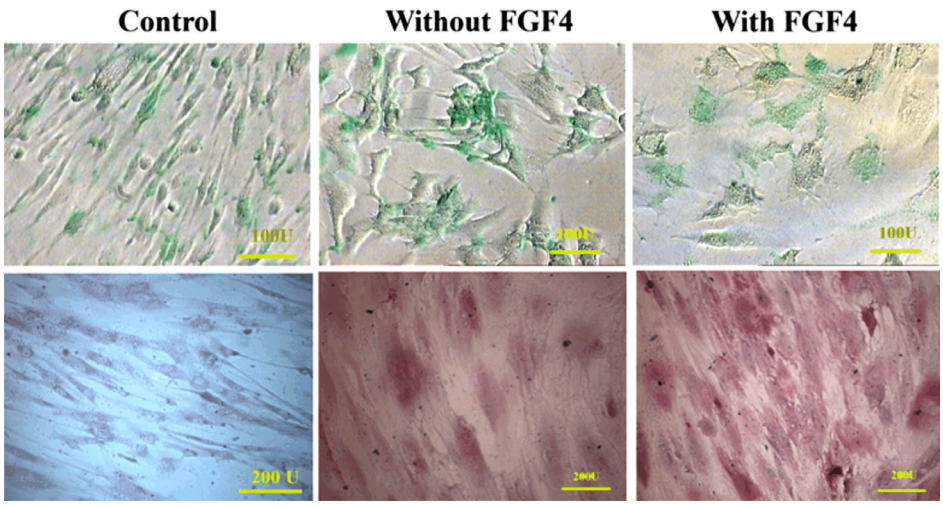

Figure 8 – ***Both cultures treated with hepatogenic media with or without FGF4 showed ICG uptake and elimination (above row) and stored glycogen (below row). The control cultures were weakly stained with PAS and a smaller number of the cells eliminated ICG.***

Discussion

In the last decade, WJ-MSCs were considered as an attractive cell source used for liver regeneration and cell therapy, because of their hepatogenic potential. The findings of the current study showed that these cells expressed a combination of the early- and mid-hepatic genes, including albumin, AFP, CK18, CK19, CYP2B6, HNF4 and claudin, which is in agreement with the results of a previous research [6].

Up to now, many researchers have tried to find the best protocol to make a functional hepatocyte [10, 33, 34]. The WJ-MSCs showed hepatocyte-like cell phenotype (polygonal shape) after being cultured in the hepatogenic medium for 21 days. Similarly, a previous study indicated that hepatogenic media could induce human umbilical cord blood MSCs to change into a small, round, and epithelioid shape [35]. Both hepatogenic protocols used in the present study also changed the cell morphology.

An *in vivo* study showed that polymerization of collagen I into fibrils inhibited the growth of hepatocytes [36, 37]. The mechanical properties of the extracellular matrix could also induce the cells to differentiate by alternation in the signaling pathway and cytoskeletal organization [26]. Furthermore, culturing the mature hepatocytes in the presence of HGF entrapped in 2D collagen/heparin film led to an increase in albumin synthesis [38].

In this study, a combination of growth factors, including, HGF, IGF, and FGF4, were used. HGF and FGF4 have a mitogenic capacity and play a pivotal role in early stage of hepatogenesis [39, 40]. IGF-1 has been reported to play a key role in stimulating the differentiation of embryonic stem cells (ESCs) into hepatogenic lineage. IGF-1 also plays a role in liver development and regeneration [41]. It has been shown to induce the cells to proliferate and express HGF as well as some other liver-specific markers. FGF4 induces the cells to differentiate toward definite endoderm [42]. Administration of FGF4 alone has been shown to exert a slight impact on the expression of hepatic markers, such as albumin. However, when FGF4 was used in combination with the other growth factors involved in hepatogenesis, such as BMP2, it could induce the cells to differentiate from definite endoderm to hepatocyte-like cells [43]. The results of the present study also showed that the combination of FGF4, HGF, and IGF-1 the higher expression of albumin and CYP2B6 as liver-specific genes but not significantly. A previous investigation indicated that the expression of AFP, claudin, and CK19 reduced in human mature hepatocytes compared to hepatoblasts, but they expressed higher levels of albumin and CYP450 [44]. Although CK18 expression reduced in mRNA level, its expression in protein level showed a significant increase compared to the control cultures. This may indicate that CK18 mRNA did not translate to protein in control cells. However, the previous reports showed that a combination of IGF-1 with HGF induced bone marrow-derived MSCs toward hepatogenic lineage [19, 27]. Furthermore, gene and protein expression level changes in umbilical cord from culture to culture. It was indicated that there were heterogeneous populations in the UCMSC (umbilical cord-derived mesenchymal stem cells) [6]. Nonetheless, the present study findings indicated that administration of the hepatogenic media containing IGF-1 and HGF with or without FGF4 had no effects on the expression level of liver-specific markers in WJ-MSCs. We also found that the naïve WJ-MSCs expressed the liver-specific markers, which might be a cause of lack of significant difference between the treated and untreated cells.

Conclusions

The expression of liver-specific markers in WJ-MSCs was not changed significantly after administration of IGF-1 and HGF with or without FGF4. HNF4 can be considered as an important growth factor in differentiation of WJ-MSCs toward hepatocyte in a time- and dose-dependent manner. However, the morphological changes and glycogen storage were observed by treating these cells with hepatogenic media with or without FGF4.

Conflict of interests

The authors declared a potential conflict of interests regarding employment contracts.

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References

[1] Dhawan A, Puppi J, Hughes RD, Mitry RR. Human hepatocyte transplantation: current experience and future challenges. Nat Rev Gastroenterol Hepatol, 2010, 7(5):288–298.
[2] Zhou WL, Medine CN, Zhu L, Hay DC. Stem cell differentiation and human liver disease. World J Gastroenterol, 2012, 18(17):2018–2025.
[3] Hughes RD, Mitry RR, Dhawan A. Current status of hepatocyte transplantation. Transplantation, 2012, 93(4):342–347.
[4] Bilir BM, Guinette D, Karrer F, Kumpe DA, Krysl J, Stephens J, McGavran L, Ostrowska A, Durham J. Hepatocyte transplantation in acute liver failure. Liver Transpl, 2000, 6(1):32–40.
[5] Fitzpatrick E, Mitry R, Dhawan A. Human hepatocyte transplantation: state of the art. J Intern Med, 2009, 266(4):339–357.
[6] Campard D, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. Gastroenterology, 2008, 134(3):833–848.
[7] Anzalone R, Lo Iacono M, Corrao S, Magno F, Loria T, Cappello F, Zummo G, Farina F, La Rocca G. New emerging potentials for human Wharton's jelly mesenchymal stem cells: immunological features and hepatocyte-like differentiative capacity. Stem Cells Dev, 2010, 19(4):423–438.
[8] Semenov OV, Breymann C. Mesenchymal stem cells derived from Wharton's jelly and their potential for cardio-vascular tissue engineering. Open Tissue Eng Regen Med J, 2011, 4:64–71.
[9] Anzalone R, Lo Iacono M, Loria T, Di Stefano A, Giannuzzi P, Farina F, La Rocca G. Wharton's jelly mesenchymal stem cells as candidates for beta cells regeneration: extending the differentiative and immunomodulatory benefits of adult mesenchymal stem cells for the treatment of type 1 diabetes. Stem Cell Rev, 2011, 7(2):342–363.
[10] Prasajak P, Leeanansaksiri W. Developing a new two-step protocol to generate functional hepatocytes from Wharton's jelly-derived mesenchymal stem cells under hypoxic condition. Stem Cells Int, 2013, 2013:762196.

[11] Ryan JM, Barry FP, Murphy JM, Mahon BP. Mesenchymal stem cells avoid allogeneic rejection. J Inflamm (Lond), 2005, 2:8.
[12] Taghizadeh RR, Cetrulo KJ, Cetrulo CL. Wharton's jelly stem cells: future clinical applications. Placenta, 2011, 32(Suppl 4): S311–S315.
[13] Lutjemeier B, Troyer DL, Weiss ML. Wharton's jelly-derived mesenchymal stromal cells. In: Cetrulo CL, Cetrulo KJ, Cetrulo CL Jr (eds). Perinatal stem cells. John Wiley & Sons, Inc., Hoboken, NJ, 2009, 79–97.
[14] Witkowska-Zimny M, Wrobel E. Perinatal sources of mesenchymal stem cells: Wharton's jelly, amnion and chorion. Cell Mol Biol Lett, 2011, 16(3):493–514.
[15] Chivu M, Dima SO, Stancu CI, Dobrea C, Uscatescu V, Necula LG, Bleotu C, Tanase C, Albulescu R, Ardeleanu C, Popescu I. *In vitro* hepatic differentiation of human bone marrow mesenchymal stem cells under differential exposure to liver-specific factors. Transl Res, 2009, 154(3):122–132.
[16] Wu XB, Tao R. Hepatocyte differentiation of mesenchymal stem cells. Hepatobiliary Pancreat Dis Int, 2012, 11(4):360–371.
[17] Kang XQ, Zang WJ, Bao LJ, Li DL, Xu XL, Yu XJ. Differentiating characterization of human umbilical cord blood-derived mesenchymal stem cells *in vitro*. Cell Biol Int, 2006, 30(7):569–575.
[18] Rodrigues M, Griffith LG, Wells A. Growth factor regulation of proliferation and survival of multipotential stromal cells. Stem Cell Res Ther, 2010, 1(4):32.
[19] Ayatollahi M, Soleimani M, Geramizadeh B, Imanieh MH. Insulin-like growth factor 1 (IGF-I) improves hepatic differentiation of human bone marrow-derived mesenchymal stem cells. Cell Biol Int, 2011, 35(11):1169–1176.
[20] Sanz S, Pucilowska JB, Liu S, Rodríguez-Ortigosa CM, Lund PK, Brenner DA, Fuller CR, Simmons JG, Pardo A, Martínez-Chantar ML, Fagin JA, Prieto J. Expression of insulin-like growth factor I by activated hepatic stellate cells reduces fibrogenesis and enhances regeneration after liver injury. Gut, 2005, 54(1):134–141.
[21] Skrtic S, Wallenius K, Gressner A, Jansson JO. Insulin-like growth factor signaling pathways in rat hepatic stellate cells: importance for deoxyribonucleic acid synthesis and hepatocyte growth factor production. Endocrinology, 1999, 140(12): 5729–5735.
[22] Tutau F, Rodríguez-Ortigosa C, Puche JE, Juanarena N, Monreal I, García Fernández M, Clavijo E, Castilla A, Castilla-Cortázar I. Enhanced actions of insulin-like growth factor-I and interferon-α co-administration in experimental cirrhosis. Liver Int, 2009, 29(1):37–46.
[23] Serra M, Brito C, Sousa MF, Jensen J, Tostões R, Clemente J, Strehl R, Hyllner J, Carrondo MJ, Alves PM. Improving expansion of pluripotent human embryonic stem cells in perfused bioreactors through oxygen control. J Biotechnol, 2010, 148(4):208–215.
[24] Koohestani F, Braundmeier AG, Mahdian A, Seo J, Bi J, Nowak RA. Extracellular matrix collagen alters cell proliferation and cell cycle progression of human uterine leiomyoma smooth muscle cells. PLoS One, 2013, 8(9):e75844.
[25] Gómez-Lechón MJ, Jover R, Donato T, Ponsoda X, Rodriguez C, Stenzel KG, Klocke R, Paul D, Guillén I, Bort R, Castell JV. Long-term expression of differentiated functions in hepatocytes cultured in three-dimensional collagen matrix. J Cell Physiol, 1998, 177(4):553–562.
[26] Hansen LK, Wilhelm J, Fassett JT. Regulation of hepatocyte cell cycle progression and differentiation by type I collagen structure. Curr Top Dev Biol, 2005, 72:205–236.
[27] Ayatollahi M, Soleimani M, Tabei SZ, Kabir Salmani M. Hepatogenic differentiation of mesenchymal stem cells induced by insulin like growth factor-I. World J Stem Cells, 2011, 3(12):113–121.
[28] Chen Y, Wong PP, Sjeklocha L, Steer CJ, Sahin MB. Mature hepatocytes exhibit unexpected plasticity by direct dedifferentiation into liver progenitor cells in culture. Hepatology, 2012, 55(2):563–574.
[29] Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics, 2012, 13:134.
[30] Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol, 2003, 4(1):33–45.
[31] Gougos A, St Jacques S, Greaves A, O'Connell PJ, d'Apice AJ, Bühring HJ, Bernabeu C, van Mourik JA, Letarte M. Identification of distinct epitopes of endoglin, an RGD-containing glycoprotein of endothelial cells, leukemic cells, and syncytiotrophoblasts. Int Immunol, 1992, 4(1):83–92.
[32] Lin CS, Xin ZC, Dai J, Lue TF. Commonly used mesenchymal stem cell markers and tracking labels: limitations and challenges. Histol Histopathol, 2013, 28(9):1109–1116.
[33] Khanjani S, Khanmohammadi M, Zarnani AH, Talebi S, Edalatkhah H, Eghtesad S, Nikokar I, Kazemnejad S. Efficient generation of functional hepatocyte-like cells from menstrual blood-derived stem cells. J Tissue Eng Regen Med, 2013, Mar 18.
[34] Lee KD, Kuo TKC, Whang-Peng J, Chung YF, Lin CT, Chou SH, Chen JR, Chen YP, Lee OKS. *In vitro* hepatic differentiation of human mesenchymal stem cells. Hepatology, 2004, 40(6): 1275–1284.
[35] Kang XQ, Zang WJ, Bao LJ, Li DL, Song TS, Xu XL, Yu XJ. Fibroblast growth factor-4 and hepatocyte growth factor induce differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocytes. World J Gastroenterol, 2005, 11(47):7461–7465.
[36] Fassett J, Tobolt D, Hansen LK. Type I collagen structure regulates cell morphology and EGF signaling in primary rat hepatocytes through cAMP-dependent protein kinase A. Mol Biol Cell, 2006, 17(1):345–356.
[37] Wells RG. The role of matrix stiffness in regulating cell behavior. Hepatology, 2008, 47(4):1394–1400.
[38] Hou YT, Ijima H, Matsumoto S, Kubo T, Takei T, Sakai S, Kawakami K. Effect of a hepatocyte growth factor/heparin-immobilized collagen system on albumin synthesis and spheroid formation by hepatocytes. J Biosci Bioeng, 2010, 110(2):208–216.
[39] Jung J, Zheng M, Goldfarb M, Zaret KS. Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science, 1999, 284(5422):1998–2003.
[40] Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeler C. Scatter factor/hepatocyte growth factor is essential for liver development. Nature, 1995, 373(6516):699–702.
[41] Magner NL, Jung Y, Wu J, Nolta JA, Zern MA, Zhou P. Insulin and IGFs enhance hepatocyte differentiation from human embryonic stem cells *via* the PI3K/AKT pathway. Stem Cells, 2013, 31(10):2095–2103.
[42] Morrison GM, Oikonomopoulou I, Migueles RP, Soneji S, Livigni A, Enver T, Brickman JM. Anterior definitive endoderm from ESCs reveals a role for FGF signaling. Cell Stem Cell, 2008, 3(4):402–415.
[43] Cai J, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X, Guo Y, Ding M, Deng H. Directed differentiation of human embryonic stem cells into functional hepatic cells. Hepatology, 2007, 45(5):1229–1239.
[44] Schmelzer E, Wauthier E, Reid LM. The phenotypes of pluripotent human hepatic progenitors. Stem Cells, 2006, 24(8): 1852–1858.

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