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Original Article/Liver

Generation of functional hepatocyte-like cells from human bone marrow mesenchymal stem cells by overexpression of transcription factor HNF4α and FOXA2 ☆

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ABSTRACT

Background: Our previous study showed that overexpression of hepatocyte nuclear factor 4α (HNF4α) could directly promote mesenchymal stem cells (MSCs) to differentiate into hepatocyte-like cells. However, the efficiency of hepatic differentiation remains low. The purpose of our study was to establish an MSC cell line that overexpressed HNF4α and FOXA2 genes to obtain an increased hepatic differentiation efficiency and hepatocyte-like cells with more mature hepatocyte functions.

Methods: Successful establishment of high-level HNF4α and FOXA2 co-overexpression in human induced hepatocyte-like cells (hiHep cells) was verified by flow cytometry, immunofluorescence and RT-PCR. Measurements of albumin (ALB), urea, glucose, indocyanine green (ICG) uptake and release, cytochrome P450 (CYP) activity and gene expression were used to analyze mature hepatic functions of hiHep cells.

Results: hiHep cells efficiently express HNF4α and FOXA2 genes and proteins, exhibit typical epithelial morphology and acquire mature hepatocyte-like cell functions, including ALB secretion, urea production, ICG uptake and release, and glycogen storage. hiHep cells can be activated by CYP inducers. The percentage of both ALB and α-1-antitrypsin (AAT)-positive cells was approximately 72.6%. The expression levels of hepatocyte-specific genes (*ALB, AAT*, and *CYP1A1*) and liver drug transport-related genes (*ABCB1, ABCG2*, and *SLC22A18*) in hiHep cells were significantly higher than those in MSCs-Vector cells. The hiHep cells did not form tumors after subcutaneous xenograft in BALB/c nude mice after 2 months.

Conclusion: This study provides an accessible, feasible and efficient strategy to generate hiHep cells from MSCs.

Introduction

Liver transplantation is currently the only curative treatment for the end stages of liver diseases. However, liver transplantation is not available for a large fraction of liver failure cases due to a limited supply of organs and a long wait time [1,2]. In particular, fulminant liver failure (FLF) is a clinically life-threatening disease, and liver transplantation is the only definitive treatment. Due to the scarcity of donor livers and the timing of transplantation, liver transplantation cannot be performed within a suitable time period. To save the lives of patients and prolong survival during the period of waiting for liver transplantation, stem cell-based therapy by transplantation of functional hepatocyte-like cells has attracted increasing attention and has been evaluated in the clinic as an alternative to organ transplantation [3]. Moreover, functional hepatocyte-like cells are widely used in the field of regenerative medicine, personalized disease modeling and drug development [4,5]. Unfortunately, all these applications are limited

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by the fact that the supply of available donor cells is inadequate. Therefore, generation of surrogate functional hepatocyte-like cells is urgently needed.

Mesenchymal stem cells (MSCs) are a rich source of self-renewing stem cells and can be isolated from a variety of tissues, including bone marrow, adipose tissue, umbilical cord blood, skin, tendon, muscle, and dental pulp [6,7]. MSCs have been extensively studied due to their easy accessibility and feasibility for autologous transplantation. In 1999, Petersen et al. first proposed bone marrow as a potential source of hepatic oval cells [8]. Increasingly more studies have demonstrated that human hepatocyte-like cells can be derived from MSCs by directed differentiation *in vitro* [9–11]. The transplantation of MSC-derived hepatocytes (MDHs) by a hepatic differentiation system improved short-term liver function in animal experiments [12]. However, the low efficiency of hepatic differentiation is noted *in vitro* in mouse and human MSCs [5,11]. Moreover, the extent of functional liver repopulation has been modest; however, MSC-derived hepatocyte-like cells exhibit numerous characteristics of mature hepatocytes and can be engrafted *in vivo*. The transdifferentiation into myofibroblasts [13] and the malignant transformation [14] of transplanted MSCs at the site of injury are major concerns. Therefore, the differentiation of MSCs into functional hepatocyte-like cells adequate for clinical therapy without formation of myofibroblasts and malignant transformation remains a challenge.

Hepatocyte nuclear factor (HNF) is a transcription factor that regulates liver-specific gene expression and is essential for differentiation and maturation processes in the liver. Previous studies demonstrated that hepatocyte-like cells are directly converted from mouse and human fibroblasts by overexpression of liver-enriched transcription factors [15–18]. Moreover, transduction of those factors could efficiently generate metabolically functional hepatocyte-like cells from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) [19]. Overexpression of HNF4α appears to be crucial for improving hepatic transdifferentiation from MSC [20]. Huang et al. reported that human induced hepatocytes (hiHeps) can be generated from adult adipose tissue-derived mesenchymal stem cells (AD-MSCs) by Foxa3, HNF1α, and HNF4α [17]. However, the efficiency of hepatic conversion from MSCs is still not sufficient for clinical application. To the best of our knowledge, few reports have examined the generation of functional human hepatocyte-like cells from MSCs by overexpression of liver-enriched transcription factors [20,21]. In our previous study [22], we transduced HNF4α into immortalized human bone marrow MSC (BM-MSCs) UE7T-13 with a lentiviral vector and confirmed that overexpression of HNF4α could directly promote human BM-MSCs to differentiate into hepatocyte-like cells. The differentiated cells have the functionality of partial mature hepatocyte-like cells. However, the efficiency of hepatic differentiation remains low. Approximately 28% of differentiated cells expressed albumin (ALB) and α-1-antitrypsin (AAT) protein.

According to previous analyses [21,23–25], HNF4α and FOXA2 play important roles in liver differentiation. In this study, to obtain increased hepatic differentiation efficiency and hepatocyte-like cells with more mature hepatocyte functions, we constructed human BM-MSCs by co-overexpressing *HNF4α* and *FOXA2* genes, thereby generating hiHep cells.

Methods

Cell culture

The immortalized human BM-MSCs UE7T-13 were kind gifts from the RIKEN Bioresource Center, Japan [26] and were cultured at 37 °C and 5% CO_2. The culture medium contained Dulbecco's modified Eagle medium (LG-DMEM, HyClone, Pennsylvania, USA) with 100 IU/mL penicillin (HyClone), 10% fetal bovine serum (FBS) (HyClone), and 100 mg/mL streptomycin (HyClone). Once adherent cells reached approximately 80%–90% confluence, they were harvested with 0.25% trypsin-EDTA and replanted for continuous passage. Human hepatocellular carcinoma BEL-7402 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. Primary hepatocytes (Invitrogen, Massachusetts, USA) were maintained under standard conditions as described previously [22].

Table 1
Primers used in RT-PCR analysis.

Gene	Forward (5′-3′)	Reverse (5′-3′)
HNF4α	TTAGCCGGCAGTGCGTGGTG	CTGGGAACGCAGCCGCTTGA
FOXA2	GTGAAGATGGAAGGGCACGA	AGTTCATGTTGGCGTAGGGG
GAPDH	CGGAGTCAACGGATTTGGTCGTAT	AGCCTTCTCCATGGTGGT

RT-PCR: real-time polymerase chain reaction; HNF4α: hepatocyte nuclear factor 4α; FOXA2: forkhead box protein A2; GAPDH: Glyceraldehyde3-phosphate dehydrogenase.

Table 2
Primers used in q-PCR analysis.

Gene	Forward (5′-3′)	Reverse (5′-3′)
ALB	CCTATGGTGAAATGGCTGACTG	ATCACATCAACCTCTGGTCTCAC
AAT	CTCTTCCAGTCCATTACCGC	GTCTGGTAGAACAAGTTCAGCATC
CYP1A1	GGTCAAGGAGCACTACAAAAC	CAAAGAGGTCCAAGACGATG
ABCB1	CACCACTGGAGCATTGACTAC	TACAGCAAGCCTGGAACCT
ABCG2	CCTGTGGAGGAACTGGGTA	TAAGGATGTAAATGTTGGGATG
SLC22A18	CTCCATCGTGCCATACCT	TGCGAACCTGCCAAATAC
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

q-PCR: real-time quantitative PCR detetive system; ALB: albumin; AAT: alpha-1 antitrypsin; CYP1A1: cytochrome P450 family 1 subfamily A member 1; ABCB1: ATP binding cassette subfamily B member 1; ABCG2: ATP-binding cassette subfamily G member 2; SLC22A18: solute carrier family 22 member 18; GAPDH: glyceraldehyde3-phosphate dehydrogenase.

Vector constructs and transduction of UE7T-13 cells

According to the previous report [27], complementary DNA of *Homo sapiens HNF4α* and *FOXA2* (also known as *HNF3β*) were separately cloned into pDONRTMP4–P1R (Invitrogen) by utilizing the Gateway BP recombination reaction. Constructed plasmids were then introduced into 293FT cells together with ViraPowerTM Lentiviral packaging mix (Invitrogen) and Lipofectamine 2000 (Invitrogen). After 72-h incubation, lentiviruses were harvested from the medium, passed through a 0.45 μm filter and concentrated by ultracentrifugation (50 000× *g* for 120 min at 4 °C). Lentivirus particles of *HNF4α* and *FOXA2* were used to infect UE7T-13 cells. The Genebank accession numbers of *HNF4α* and *FOXA2* were NM_178849.2 and NM_021784.4, respectively.

UE7T-13 cells were infected by pLV/Final-puro-hFOXA2-mCherry and pLV/Final-puro-hHNF4α-hrGFP lentivirus particles cultured in a three-plasmid system (Invitrogen). After transduction and fluorescence-activated cell sorting (FACS) analysis, these cells were developed in hepatocyte culture medium until the cells doubled to generate hiHep cells. At the same time, an empty vector control was established by UE7T-13 cells infected with pLV/Final-puro-mCherry and pLV/Final-puro-hrGFP lentivirus particles. After being subjected to the same treatment as hiHep cells, these cells were named MSCs-Vector cells.

RT-PCR assay and q-PCR assay

According to the manufacturer's instructions and our previous study [22], a total RNA extraction kit (Sangon Biotech Co., Ltd., Shanghai, China) was used to extract total RNA from cells after

Table 3
Primary antibodies used.

Antigen	Primary antibody	Company	Dilution
HNF4α	Hnf4α Rabbit mAb	Cell Signaling	1:1000
FOXA2	Foxa2 XP®Rabbit mAb	Santa	1:100
ALB(ICC)	Goat x-Human Albumin	Bethyl(A80-229A)	1:250
AAT	Polyclonal Rabbit Anti-Human AAT	Dako(Code IR505)	Ready-to-Use
CK18	Anti-Cytokeratin 18 Antibody	Abcam(ab82254)	1:100
CK8	Rabbit Monoclonal Antibody	Epitomics	1:50

Table 4
Secondary antibodies used.

Antigen	Secondary antibody	Company	Dilution
HNF4α	Alexa Fluor®350 Donkey Anti-RabbitIgG($H+L$)	Invitrogen	1:1000
FOXA2	Alexa Fluor®633 Donkey Anti-GoatIgG($H+L$)	Invitrogen	1:1000
ALB(ICC)	Alexa Fluor®633 Donkey Anti-GoatIgG($H+L$)	Invitrogen	1:1000
AAT	Alexa Fluor®633 Goat Anti-RabbitIgG($H+L$)	Invitrogen	1:1000
CK18	Alexa Fluor®633 Goat Anti-MouseIgG($H+L$)	Invitrogen	1:1000
CK8	Alexa Fluor®633 Goat Anti-RabbitIgG($H+L$)	Invitrogen	1:1000

HNF4α: hepatocyte nuclear factor 4α; FOXA2: forkhead box protein A2; ALB: albumin; AAT: alpha-1 antitrypsin; CK: cytokeratin.

isolation using Trizol reagent (Invitrogen). Reverse transcription-polymerase chain reaction (RT-PCR) was run on a SuperScript® III One-Step RT-PCR System (Invitrogen) under the following conditions at 94 °C for 3 min: 30 cycles at 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 35 s. The primer sequences for RT-PCR used in this study are described in Table 1.

Quantitative real time PCR(q-PCR) was run on the 7500 real time system (Applied Biosystem, Massachusetts, USA) using the QuantiTect SYBR Green PCR Master Mix (Qiagen, Venlo, Netherlands). Quantified values were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for q-PCR are presented in Table 2.

Immunofluorescence

The cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. After incubation with PBS containing 0.2% Triton X-100 (Sigma, Missouri, USA) and blocking by 3% BSA in PBS for 60 min, cells were incubated with primary antibodies at 4 °C overnight. Cells were then incubated with appropriate fluorescence-conjugated secondary antibody for 1 h at room temperature in the dark. Nuclei were stained with DAPI (Sigma). All antibodies are listed in Tables 3 and 4.

PAS stain, urea secretion, albumin, ALT, AST, LDH measurement and ICG uptake and release assays

Cells were stained by periodic acid-Schiff (PAS, Sigma) according to the manufacturer's instructions. The amounts of urea in the culture media were measured after the addition of 5 mmol/L ammonium chloride (Sigma) using a commercially available kit (MAKER Science Technology Co., Ltd., Chengdu, China). The amounts of albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactic dehydrogenase (LDH) in the supernatant were measured by human ELISA kits (Bethy1 Laboratory, Georgia, USA). For the indocyanine green (ICG) uptake and release assay, cells were incubated in culture medium with 1 mg/mL ICG (Sigma) for 1 h at 37 °C. Microscopy was used to examine ICG uptake by cells. Then, cells were incubated at 37 °C for 6 h. Microscopy was used to analyze the ICG excretion.

Flow cytometry analysis

Both green fluorescent protein (GFP)- and red fluorescent protein (mCherry)-positive cells were determined and further purified by fluorescence-activated cell sorting analysis (BD Biosciences, New Jersey, USA) according to the manufacturer's instructions. Cells were collected and washed thrice with PBS. Then, 5×10^5 cells were incubated with 500 μL flow cytometry fixation buffer at room temperature for 10 min and permeabilized with 100 μL flow cytometry permeabilization/wash buffer I (Sigma) for 15 min. Then, samples were incubated with ALB and AAT antibodies at room temperature for 2 h. Flow cytometry analysis using the BD Influx System was detected after two washes with permeabilization/wash buffer I.

CYP induction assay

Cells in 6-well plates were treated with fresh culture medium containing omeprazole (Sigma, 100 mmol/L) or phenobarbital (Sigma, 500 mmol/L) in a 37 °C and 5% CO_2 incubator for 48 h. The control group was treated in the same manner with the fresh culture medium with dimethyl sulfoxide (DMSO, Sigma, 500 μmol/L). q-PCR was used to analyze the activation of CYP2C9 and CYP3A4 in response to phenobarbital and CYP1A1, CYP1A2, and CYP1B1 in response to omeprazole.

Animals

All conditions and experimental procedures involving animals in this study were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Sun Yat-sen University. The female BALB/c nude mice (15–20 g) were obtained from the animal center of Sun Yat-sen University (Guangzhou, China).

Tumor generation assay

BALB/c nude mice were subcutaneously transplanted with 6×10^7 hiHep cells and 6×10^7 Bel-7402 cells (6 mice per group) in the right thigh, separately. Tumors were counted 2 months after transplantation.

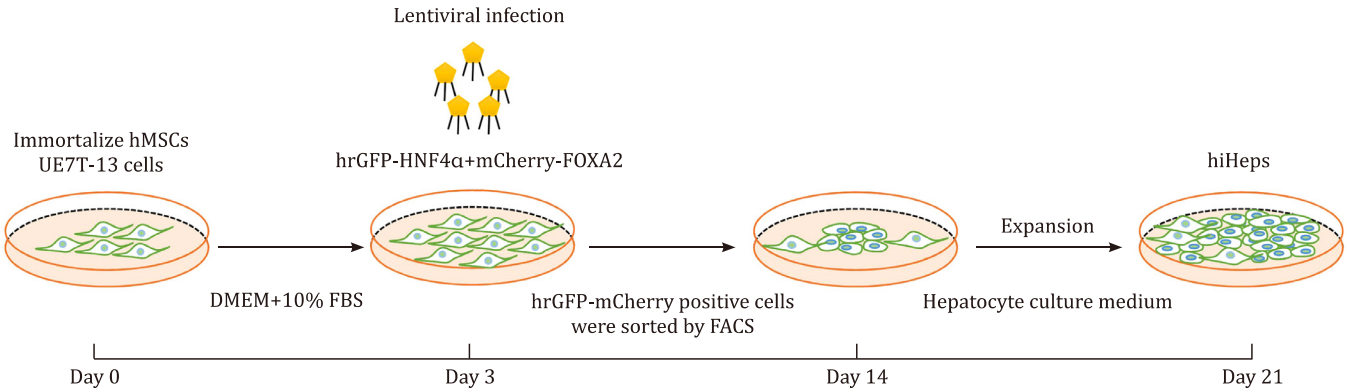


Fig. 1. Flow chart of hiHep cell generation. General process of lentiviral packaging, transduction of target cells, sorting for both hrGFP- and mCherry-positive cells by flow cytometry analysis, and amplification in hepatocyte culture medium. GFP: green fluorescent protein; HNF4α: hepatocyte nuclear factor 4α; FOXA2: forkhead box protein A2; FACS: flow cytometry.

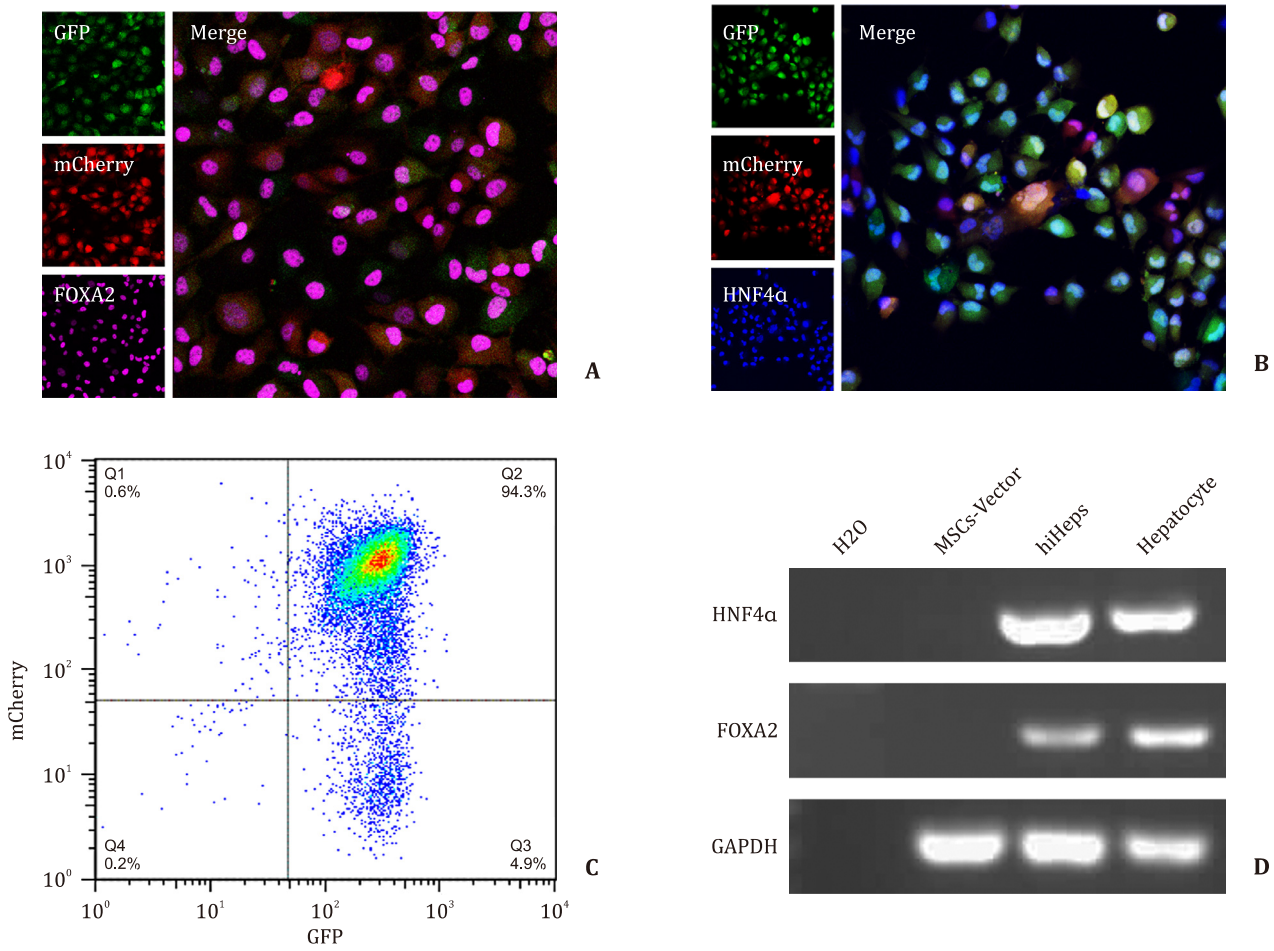


Fig. 2. Successful establishment of stable, high-level expression of HNF4α and FOXA2 in hiHep cells. **A**: Immunofluorescence analyses revealed strong protein expression of GFP, mCherry and FOXA2 in hiHep cells (original magnification ×200); **B**: Immunofluorescence analyses revealed strong expression of GFP, mCherry and HNF4α protein in hiHep cells (original magnification ×200); **C**: Approximately 94.3% of hiHep cells express both GFP and mCherry proteins, as assessed by flow cytometry; **D**: RT-PCR was performed to verify that HNF4α and FOXA2 mRNA were robustly expressed in hiHep cells but not in MSCs-Vector cells. HNF4α: hepatocyte nuclear factor 4α; FOXA2: forkhead box protein A2; GFP: green fluorescent protein; hiHeps: human induced hepatocyte-like cells; MSC: mesenchymal stem cells; RT-PCR: real-time polymerase chain reaction.

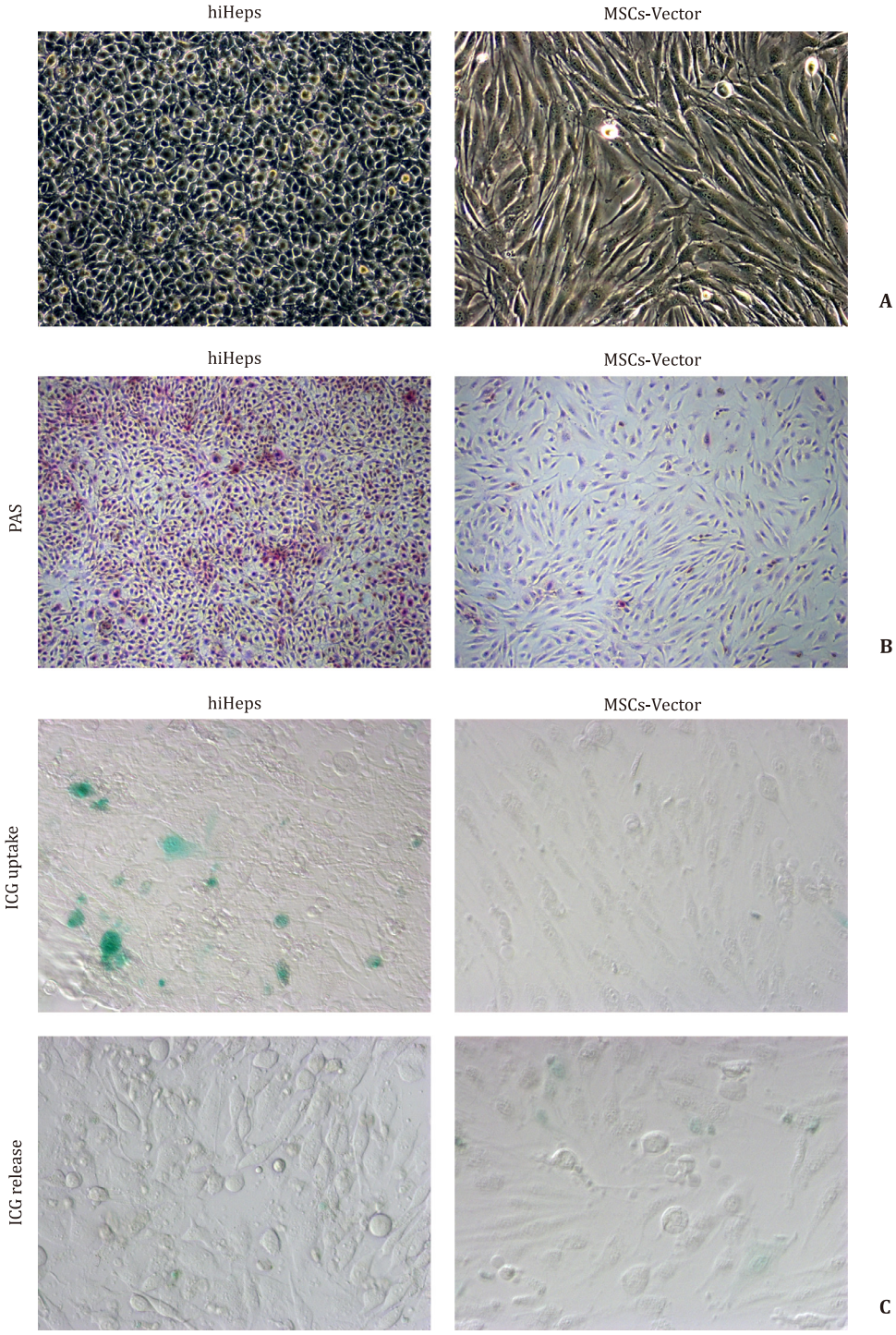


Fig. 3. Hepatic functions of hiHep cells *in vitro*. **A**: hiHep cells displayed morphological phenotypes of epithelial cells, while MSCs-Vector cells still exhibited their spindle shape (original magnification ×100); **B**: hiHep cells exhibited a strong ability for glycogen storage, whereas MSCs-Vector cells were negative for PAS staining (original magnification ×40); **C**: hiHep cells showed a significant increase in ICG uptake and were released when incubated in fresh culture medium lacking ICG after 6 h. However, MSCs-Vector cells exhibited negative results for ICG uptake and release tests (original magnification ×200). hiHeps: human induced hepatocyte-like cells; MSC: mesenchymal stem cells; PAS: periodic Acid-Schiff; ICG: indocyanine green.

Statistical analysis

A statistical software package (SPSS version 13.0, SPSS Inc., Chicago, IL, USA) and Student's *t*-test were used to perform statistical analysis. Data were expressed as the mean± standard deviation (SD). A $P < 0.05$ (two-tailed) was considered significantly different.

Results

Successful establishment of hiHep cells with stable, high-level co-overexpression of hHNF4α-hrGFP/hFOXA2-mCherry genes

MSCs containing hrGFP and mCherry blank vectors are called MSCs-Vector cells and serve as an empty vector control. Cells with

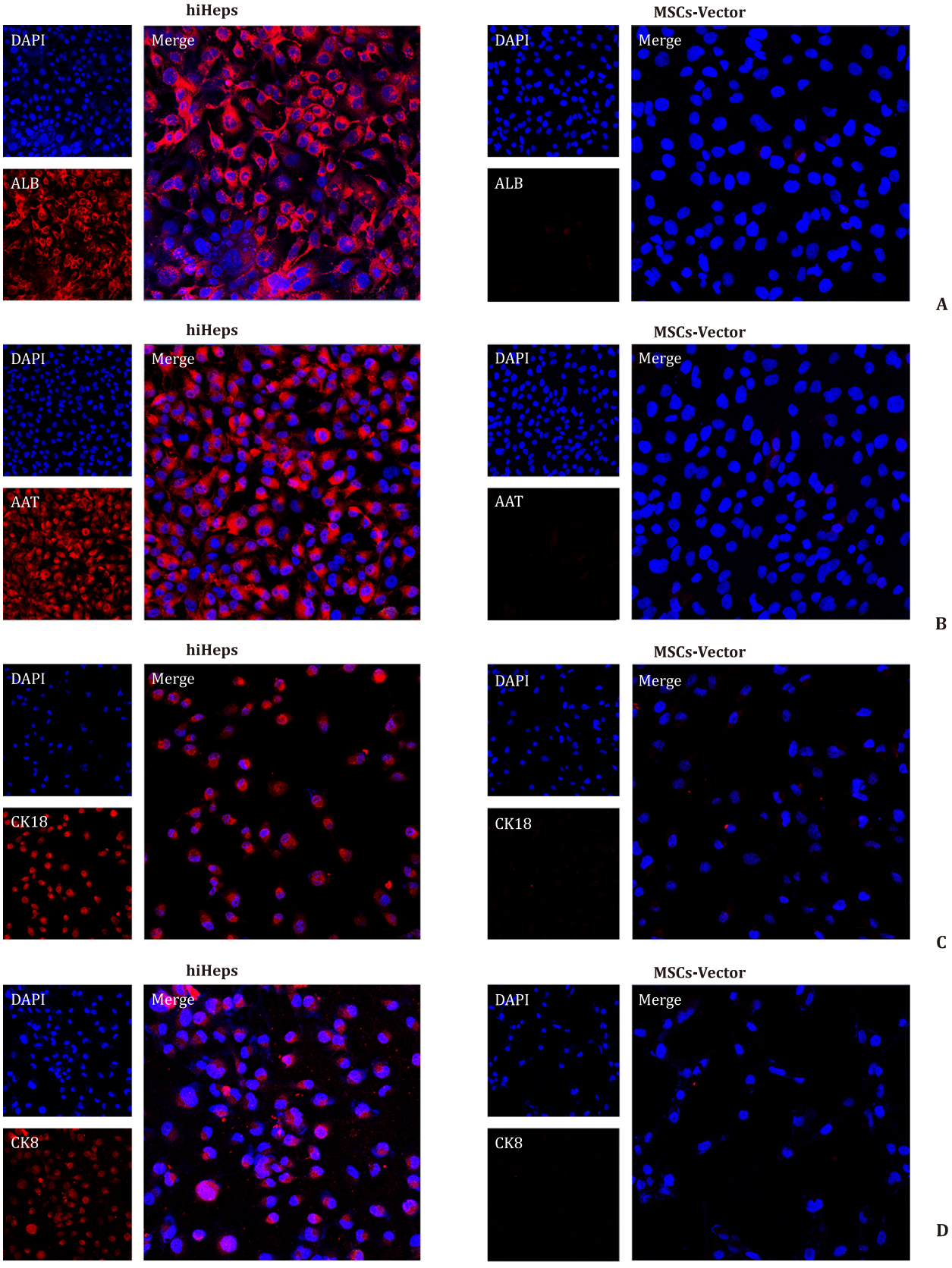


Fig. 4. Positive protein expression of hiHep cells *in vitro* based on immunofluorescence analysis. Immunofluorescence analysis revealed positive protein expression of ALB (**A**), AAT (**B**), CK18 (**C**), and CK8 (**D**) in hiHep cells and negative protein expression in MSCs-Vector cells (original magnification ×200). hiHeps: human induced hepatocyte-like cells; MSC: mesenchymal stem cells; ALB: albumin; AAT: alpha-1 antitrypsin; CK: cytokeratin.

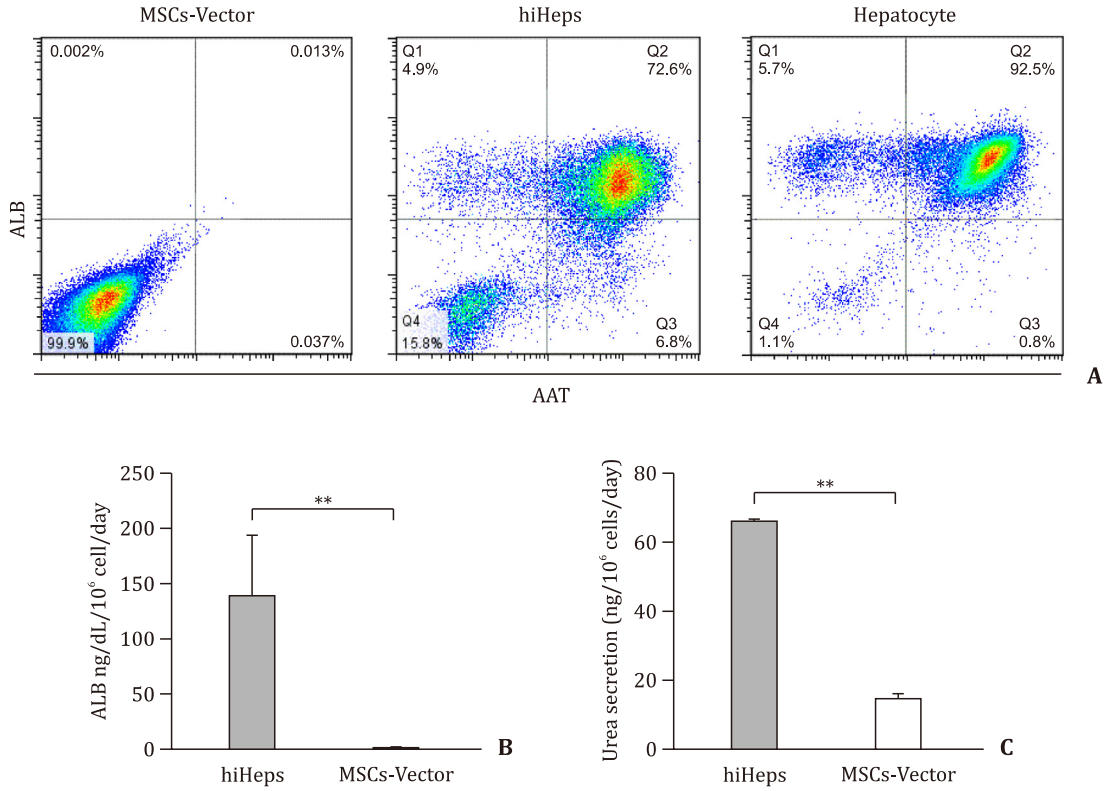


Fig. 5. Hepatic function of hiHep cells *in vitro*. **A**: The percentage of both ALB- and AAT-positive cells was approximately 72.6% in hiHep cells as assessed by flow cytometry. MSCs-Vector cells and hepatocytes were used negative and positive controls, respectively; **B**: The ALB levels produced by hiHep cells were significantly higher than those in MSCs-Vector cells ($P < 0.01$); **C**: The urea secretion rate produced by hiHep cells was significantly higher than that in MSCs-Vector cells ($P < 0.01$). hiHep: human induced hepatocyte-like cells; MSC: mesenchymal stem cells; ALB: albumin; AAT: alpha-1 antitrypsin.

hHNF4α-hrGFP and hFOXA2-mCherry vectors are termed hiHep cells. A flow chart for the generation of the hiHep cells includes the following steps: lentivirus transduction, GFP-mCherry positive cell selection and hiHep cell expansion in hepatocyte culture medium (Fig. 1). The empty vector controls (MSCs-Vector cells) were treated exactly as described for hiHep cells. Immunofluorescence analysis revealed strong protein expression of GFP, FOXA2, HNF4α and mCherry in hiHep cells (Fig. 2A, B). Approximately 94.3% of hiHep cells expressed both GFP and mCherry proteins, as assessed by FACS (Fig. 2C). RT-PCR verified that HNF4α and FOXA2 mRNA were robustly expressed in hiHep cells but not in MSCs-Vector cells. Primary hepatocytes served as positive control cells (Fig. 2D). Therefore, we established hiHep cells with stable, high-level co-overexpression of HNF4α and FOXA2 proteins via lentiviral transduction.

Hepatic functions in hiHep cells in vitro

The hiHep cells displayed morphological phenotypes of epithelial cells, whereas MSCs-Vector cells still exhibited their spindle shape (Fig. 3A). Glycogen storage is an important function of hepatocyte-like cells. hiHep cells exhibited a strong ability for glycogen storage. ICG uptake and release tests were used to evaluate hepatic function in clinical examinations. Uptake and excretion of ICG is an important feature of mature hepatocyte-like cells [28]. ICG is significantly absorbed by hiHep cells and excreted after 6 h in fresh culture without ICG. However, PAS-positive cells generally do not exist, and ICG metabolism is deficient among MSCs-Vector cells in PAS staining and ICG uptake and release tests (Fig. 3B, C). We confirmed the expression of ALB, AAT, CK18, and CK8 in hiHep cells using immunofluorescence and observed negative expression in MSCs-Vector cells (Fig. 4).

To analyze the transformation efficiency, ALB and AAT double-positive cells were detected by flow cytometry. The percentage of both ALB- and AAT-positive cells was approximately 72.6% in hiHep cells. MSCs-Vector cells and hepatocytes were used as negative and positive controls, respectively (Fig. 5A). According to the results of our previous study [22], the proportion of coexpressing ALB and AAT cells was approximately 28% in HNF4α-overexpressing MSCs. Thus, co-overexpression of HNF4α and FOXA2 significantly increases the hepatic differentiation efficiency of MSCs. In addition, hiHep cells displayed several hallmark functions of mature hepatocytes, which secreted ALB (Fig. 5B) and produced urea (Fig. 5C). The ALB levels and urea secretion rate produced in hiHep cells were significantly higher than those in MSCs-Vector cells ($P < 0.01$).

We next analyzed whether hiHep cells were responsive to a CYP inducer. hiHep cells could be activated by omeprazole. CYP1A2 and CYP1B1 mRNA expression was significantly higher in hiHep cells than that in MSCs-Vector cells ($P < 0.01$; Fig. 6A, B). CYP1A1 mRNA expression levels were not significantly different between hiHep cells and MSCs-Vector cells (Fig. 6C). hiHep cells were activated by phenobarbital, and CYP3A4 mRNA expression was significantly higher in hiHep cells than that in MSCs-Vector cells ($P < 0.01$; Fig. 6D). CYP2C9 mRNA expression levels were not significantly different between hiHep cells and MSCs-Vector cells (Fig. 6E).

Gene expressions of hiHep cells in vitro

We used q-PCR to quantitatively detect the mRNA expression levels of hepatocyte-specific genes, namely, *ALB, AAT*, and *CYP1A1*,

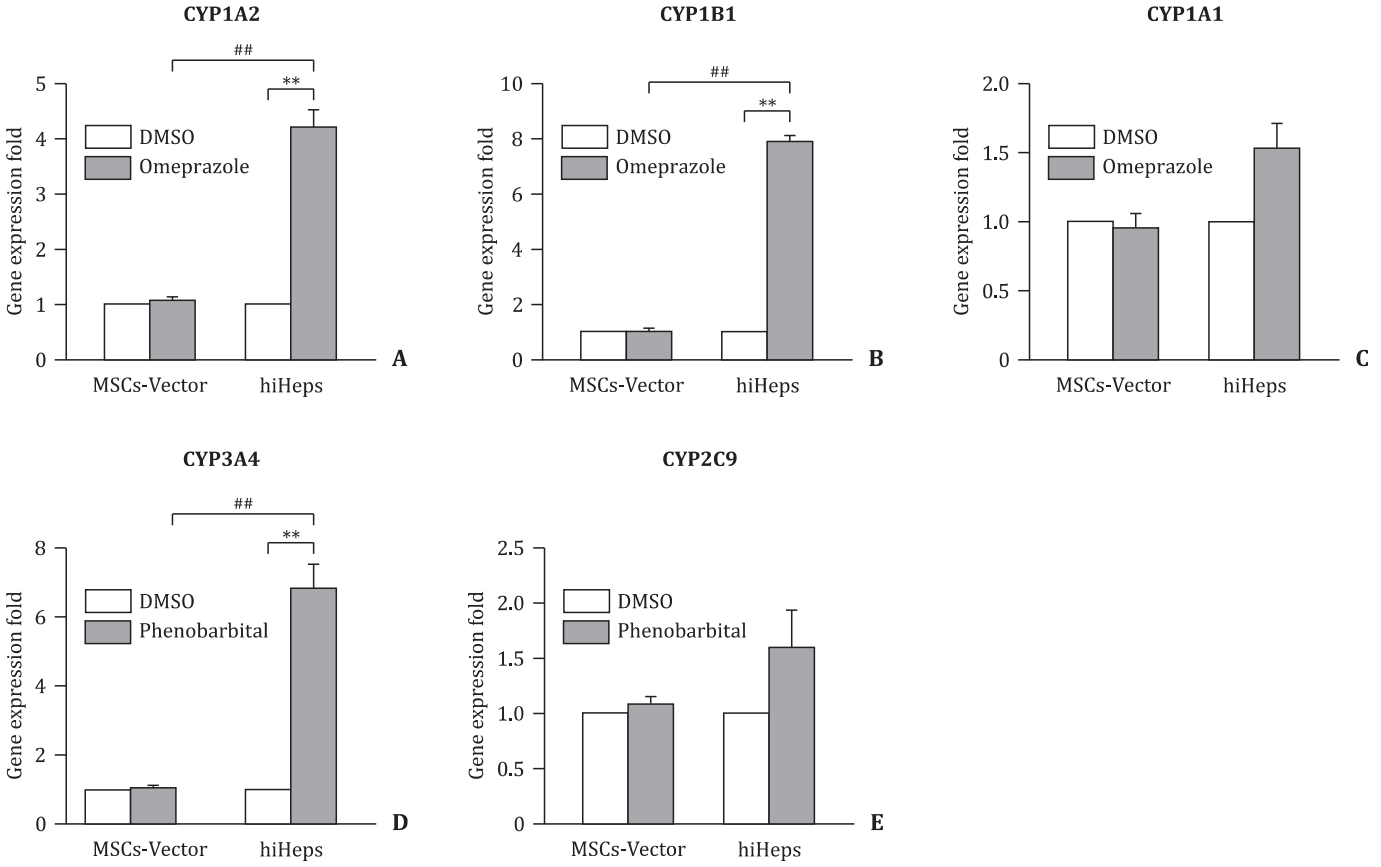


Fig. 6. Hepatic functions of hiHep cells *in vitro*. hiHep cells can be activated by Omeprazole. CYP1A2 (**A**) and CYP1B1 (**B**) mRNA expression was significantly higher than that in MSCs-Vector cells ($P < 0.01$), and CYP1A1 mRNA expression levels were not significantly different between hiHep cells and MSCs-Vector cells (**C**). hiHep cells could be activated by phenobarbital, CYP3A4 mRNA expression was significantly higher in hiHep cells than that in MSCs-Vector cells ($P < 0.01$) (**D**), and the CYP2C9 mRNA expression level was not significantly different between hiHep cells and MSCs-Vector cells (**E**). **: $P < 0.01$, compared to DMSO-treated hiHeps cells; ##: $P < 0.01$, compared to omeprazole/Phenobarbital MSC-Vector cells. CYP: cytochrome P450; MSC: mesenchymal stem cells; hiHeps: human induced hepatocyte-like cells; DMSO: dimethylsulfoxide.

and liver drug transport related genes, namely, *ABCB1, ABCG2*, and *SLC22A18*, in hiHep cells. As shown in Fig. 7, the expression levels of *ALB, AAT, CYP1A1, ABCB1, ABCG2* and *SLC22A18* genes in hiHep cells were significantly higher than those in MSCs-Vector cells ($P < 0.01$) and were slightly lower than those in hepatocytes ($P > 0.05$).

hiHep cells did not form tumors in 2 months after subcutaneous xenograft in mice

Twelve nude mice were randomly divided into two groups, the hiHep group and Bel-7402 cell group, with 6 mice in each group. hiHep cells (6×10^7) or Bel-7402 cells (6×10^7) were injected subcutaneously into the right thigh of each nude mouse. As shown in Fig. 8, after 2 months of cell tumorigenesis, 6 nude mice in the Bel-7402 cell group exhibited tumors, whereas none of the mice in the hiHep group exhibited tumors. This result indicates that hiHep cells do not form tumors after subcutaneous xenograft in BALB/c nude mice and are biologically safe *in vivo*.

Discussion

The generation of functional hepatocyte-like cells and establishment of an efficient hepatic differentiation technology from human MSCs are important for the supply of sustainable and readily available donor cells and cell-based therapies for liver diseases. Previous studies have demonstrated that the efficiency of hepatic differentiation from MSCs is improved by modifying culture protocols [29,30]. However, the low efficiency of hepatic differentiation from MSCs is insufficient for clinical application. Further investigation is required to increase the efficiency and repeatability of hepatic differentiation of MSCs. Multiple studies [16,17,31] confirmed that HNF4α and FOXA2 bind to other transcription factors and play important roles in transdifferentiation into hepatocyte-like cells in mouse fibroblasts and human fibroblasts. Co-overexpression of HNF4α and FOXA2 promotes differentiation of mouse embryonic stem cells into hepatocyte-like cells [32]. In our previous study [22], we verified that HNF4α-overexpressing MSCs (E7-hHNF4a cells) expressed ALB, CYP2B6, AAT and FOXA2 mRNA and exhibited some liver cell functions, such as ICG uptake and release, glycogen uptake, urea production and ALB secretion. However, the efficiency of hepatic differentiation remained low. In this study, to obtain increased hepatic differentiation efficiency and obtain hepatocyte-like cells with more mature hepatocyte functions, we established HNF4α-FOXA2 co-overexpression MSCs (hiHep cells) by lentiviral transduction. The combination of HNF4α and FOXA2 transduction can promote efficient hepatic differentiation and maturation of immortalized human BM-MSCs (UE7T-13 cells).

In recent years, given the success of reprogramming somatic cells to pluripotent cells, numerous studies have demonstrated cell fate conversion between terminally differentiated lineages by overexpression of specific transcription factors. For example, cardiomyocytes and blood cells are induced from mouse fibroblasts by overexpression of defined transcription factors [33,34].

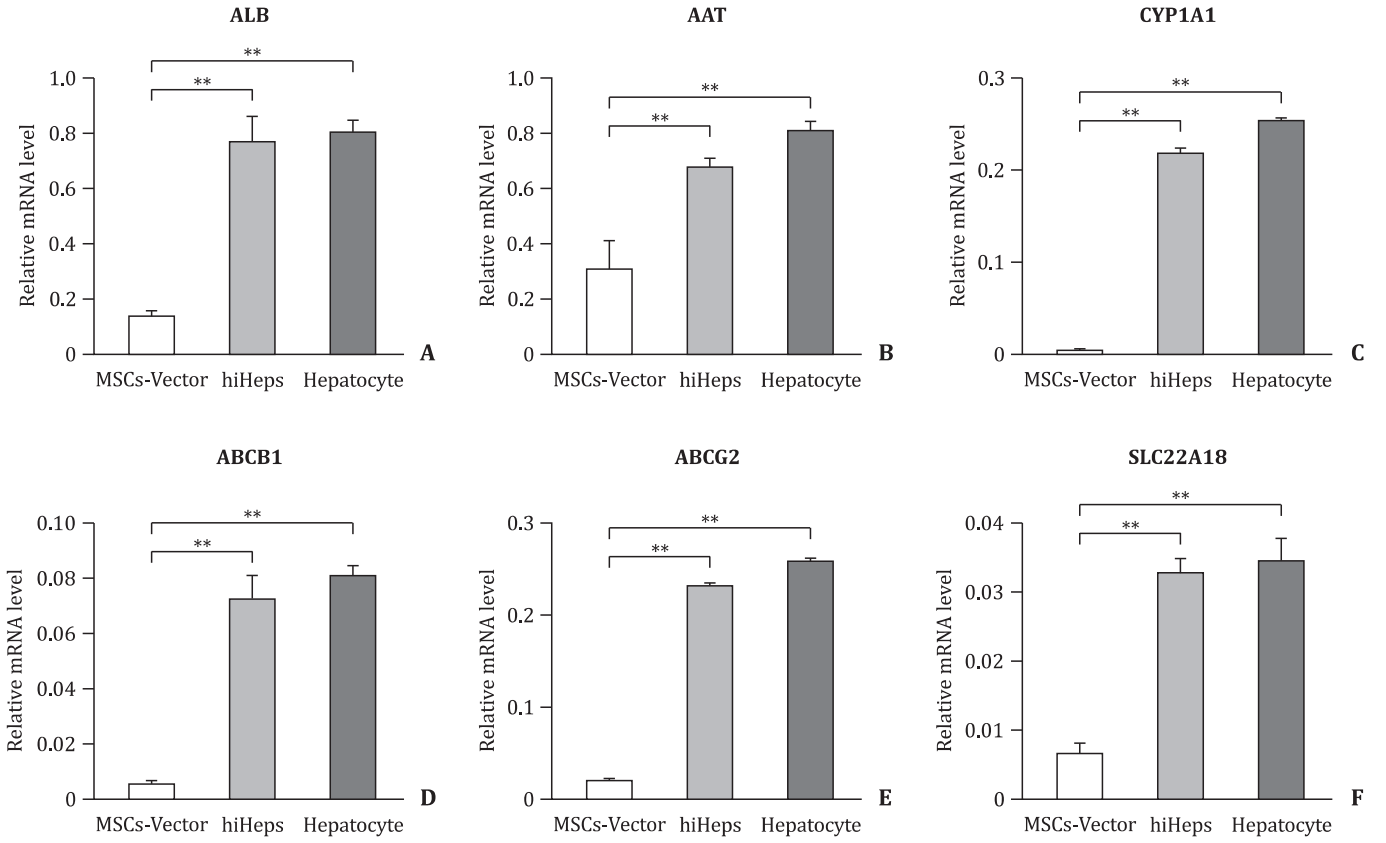


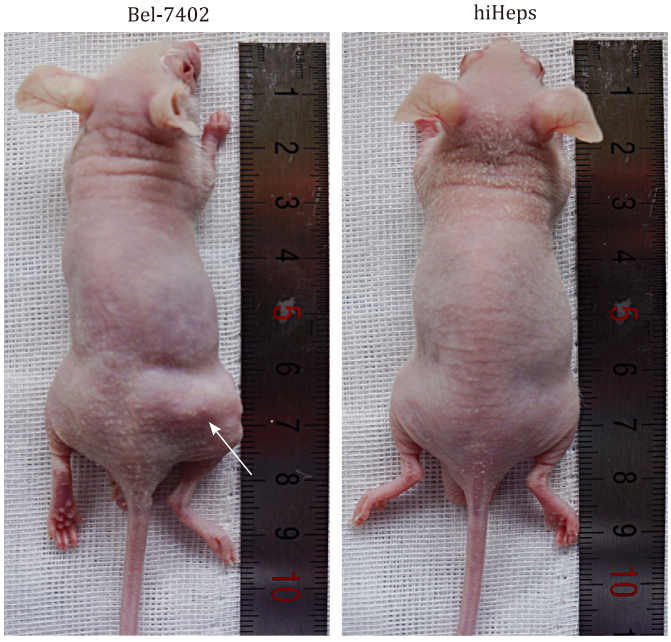
Fig. 7. The expression of hepatocyte-specific genes and liver drug transport-related genes in MSCs-Vector hiHep cells and hepatocytes. q-PCR analysis was used to quantitatively detect mRNA expression levels of hepatocyte related genes, namely, *ALB, AAT*, and *CYP1A1*, and liver drug transport-related genes, namely, *ABCB1, ABCG2*, and *SLC22A18*. ALB (**A**), AAT (**B**), CYP1A1 (**C**), ABCB1 (**D**), ABCG2 (**E**) and SLC22A18 (**F**) mRNA expression levels were significantly higher in hiHep cells than those in MSCs-Vector cells and were lower or slightly lower than those in hepatocytes. **: $P < 0.01$, compared to MSCs-Vector cells. ALB: albumin; AAT: alpha-1 antitrypsin; CYP: cytochrome P450; ABCB1: ATP binding cassette subfamily B member 1; ABCG2: ATP-binding cassette sub-family G member 2; SLC22A18: solute carrier family 22 member 18; MSC: mesenchymal stem cells; hiHeps: human induced hepatocyte-like cells.

In 2011, two groups generated hepatocyte-like cells from mouse fibroblasts by co-overexpression of Foxa3/Gata4/Hnf4α [15] or Hnf4α/Foxa1/Foxa2/Foxa3, separately [16]. Moreover, two studies published in *Cell Stem Cell* demonstrated an additional approach by directly reprogramming fibroblasts into human induced hepatocytes [17,18]. Compared with the results of our previous study [22], the proportion of cells co-overexpression ALB and AAT is approximately 28% in HNF4α-overexpressing MSCs. In this study, the combination of HNF4α and FOXA2 transduction could achieve increased hepatic differentiation of MSCs with percentage of both ALB- and AAT-positive cells being approximately 72.6% in hiHep cells.

HNF4α and FOXA2 transcription factors play important roles in liver development, differentiation and homeostatic function [24,25]. HNF4α is essential for morphological and functional differentiation of hepatocytes [23]. FOXA2 directly regulates the expression of a number of hepatocyte-specific genes that are involved primarily in hepatic metabolism and early developmental events [24]. In our study, combination of HNF4α and FOXA2 transduction directly upregulated hepatocyte-specific genes, namely, *ALB, AAT*, and *CYP1A1*, and liver drug transport-related genes, namely, *ABCB1, ABCG2*, and *SLC22A18*. hiHep cells exhibited many characteristics of mature hepatocyte-like cells, including epithelial morphology, glycogen storage, urea production, ALB secretion, ICG uptake and release, and CYP450 activity. In particular, CYP3A4 is critical for drug metabolism [35]. Phenobarbital treatment markedly induced CYP3A4 mRNA expression in hiHep cells.

MSCs are the most important stem cells because autologous stem cells can be easily isolated. Autologous MSCs can be extensively expanded and transplanted back into patients. Previous studies have demonstrated that MSCs have the ability to transdifferentiate into hepatocytes [29]. Thus, these cells are expected to be a useful source of cells for transplantation and drug discovery. The efficiency of hepatic differentiation of MSCs has been improved by chemically defined conditions or by overexpression of liver-specific regulatory factors [20,21,29,30,36,37]. However, the differentiation of MSCs into mature hepatocyte-like cells for clinical therapy remains a challenge. Moreover, it is important to develop a simple strategy for the efficient induction of hepatic differentiation. In this study, we generated hiHep cells from immortalized BM-MSCs UE7T-13 by lentiviral transduction of HNF4α and FOXA2. This novel method offers a great advantage for liver tissue engineering, as it can bypass all the complicated steps involved in hepatic differentiation *in vitro*. Approximately 72.6% of hiHep cells were both ALB and AAT positive. hiHep cells express hepatocyte-specific genes and acquire mature hepatocyte function.

In summary, our method, which involves co-overexpression of HNF4α and FOXA2 by lentivirus transduction, is a valuable tool for the efficient generation of functional hepatocyte-like cells derived from human MSCs. hiHep cells exhibit typical epithelial morphology, express hepatocyte-specific genes and acquire functions characteristic of mature hepatocytes, including glycogen storage, urea production, ALB secretion, ICG uptake and release, and CYP450 enzyme activity. In addition, the hiHep cells are not tumorigenic after subcutaneous xenograft in mice.



Groups	Tumor-bearing mice per injected mice
Bel-7402	6/6
hiHeps	0/6

Fig. 8. Tumorigenesis assay. hiHep cells did not form tumors 2 months after subcutaneous xenograft in BALB/c nude mice.

Contributors

SH proposed the study. XPY and HXJ performed the research and wrote the first draft. LD collected and analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. XPY and HXJ contributed equally to this work. SH is the guarantor.

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Ethical approval

All conditions and experimental procedures involving animals in this study were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Sun Yat-sen University.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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