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Induction of Hepatocyte-Like Cells From Mouse Embryonic Stem Cells by Lentivirus-Mediated Constitutive Expression of Foxa2/Hnf4a

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ABSTRACT

Hepatocytes can be generated from embryonic stem cells (ESCs) using inducers such as chemical compounds and cytokines, but issues related to low differentiation efficiencies remain to be resolved. Recent work has shown that overexpression of lineage-specific transcription factors can directly cause cells phenotypic changes, including differentiation, trans-differentiation, and de-differentiation. We hypothesized that lentivirus-mediated constitutive expression of forkhead box A2 (Foxa2) and hepatocyte nuclear factor 4 alpha (Hnf4a) could promote inducing mouse ESCs to hepatocyte-likes cells. First, ESC lines that stably expressed Foxa2, Hnf4a, or Foxa2/Hnf4a were constructed via lentiviral expression vectors. Second, observations of cell morphology changes were made during the cell culture process, followed by experiments examining teratoma formation. Then, the effects of constitutive expression of Foxa2 and Hnf4a on hepatic differentiation and maturation were determined by measuring the marker gene expression levels of Albumin, α-fetoprotein, Cytokeratin18, and α1-antitrypsin. The results indicate that constitutive expression of Foxa2 and Hnf4a does not affect ESCs culture, teratoma formation, or the expression levels of the specific hepatocyte genes under autonomous differentiation. However, with some assistance from inducing factors, Foxa2 significantly increased the hepatic differentiation of ESCs, whereas the expression of Hnf4a alone or Foxa2/Hnf4a could not. Differentiated CCE-Foxa2 cells were more superior in expressing several liver-specific markers and protein, storing glycogen than differentiated CCE cells. Therefore, our method employing the transduction of Foxa2 would be a valuable tool for the efficient generation of functional hepatocytes derived from ESCs. J. Cell. Biochem. 114: 2531–2541, 2013.

KEY WORDS: TISSUE-ENGINEERED LIVER; MICRAGRAVITY BIOREACTOR; TRANSPLANTATION

Orthotopic liver transplantation is the only proven effective treatment for end-stage liver disease. However, the lack of donors, surgical complications, tissue rejection and the high cost of transplantation are serious problems associated with this procedure. Temporary extracorporeal liver support, hepatocyte transplantation and engineered liver tissue are employed to bridge the time until liver transplantation or, ideally, to recover the patient's own liver. One of the most difficult problems associated with the clinical application of these therapies is the availability of a sufficient number of high-quality hepatocytes [Liu et al., 2010].

Embryonic stem cells (ESCs), with their unlimited proliferative capacities and multi-directional differentiation abilities, are expected to become a new source of hepatocytes [Thomson et al., 1998]. Many studies have made substantial contributions to the process of ESCs differentiation into hepatocytes by continuously improving inducers of differentiation and optimizing their combinations and sequences [Li et al., 2010; Liu et al., 2010; Wang et al., 2012; Zhang et al., 2013]. Most of the induction plans try to imitate the routine embryonic development process of liver within a few days of in vitro culture. Because there are many differences between in vivo embryonic

Tao Liu and Shichang Zhang contributed equally to this work.

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development and in vitro culture, most of the induction processes are ineffective, complicated, time-consuming and expensive. These conventional hepatic differentiation methods are also limited by the difficulties involved in scaling up the procedures.

Thanks to the recent research accomplishments in cellular reprogramming, previous studies have shown that cells are highly plastic [Hanna et al., 2011]. This plasticity is subject to the influence of critical transcription factors whose enforced expression can directly cause phenotypic changes in cells, including differentiation [Lavon et al., 2006; Liew et al., 2008], trans-differentiation [Ho et al., 2011; Huang et al., 2011; Murry and Pu, 2011; Sekiya and Suzuki, 2011], and de-differentiation [Hanna et al., 2008]. Therefore, it can be speculated that there are certain specific transcription factors, or a combination of transcription factors, that can facilitate the differentiation of ESCs, or even direct reprogram ESCs, into hepatocyte-like cells. Furthermore, because the two main hepatocyte nuclear transcription factors, forkhead box A2 (Foxa2) and hepatocyte nuclear factor 4 alpha (Hnf4a), begin to be expressed in the early- and mid-phases of liver development, respectively, they play critical roles to support hepatic development and maintain hepatocyte functions [Zhao and Duncan, 2005; Sekiya and Suzuki, 2011]. We also speculate that the constitutive expression of Foxa2 and Hnf4a could have positive effects on the directed differentiation of ESCs into hepatocyte-like cells and that such facilitating influence may transcend regular induction modes to directly speed up the differentiation of ESCs into hepatocyte-like cells.

To prove our hypotheses, we first constructed ESC lines capable of stably expressing Foxa2, Hnf4a, and Foxa2/Hnf4a via lentiviral expression vectors. Next, we observed cell morphology changes during the cell culture process, followed by teratoma formation assays. Lastly, the effects of constitutive expression of Foxa2 and Hnf4a on the differentiation and maturation of the cells were determined by the levels of expression of the marker genes Albumin (ALB), α-fetoprotein (AFP), Cytokeratin 18 (CK18), and α1-antitrypsin (AAT) under different differentiation modes.

MATERIALS AND METHODS

ETHICS STATEMENT

All tissue cultures with ES cells and experiments involving animals and their tissues were approved by the institutional animal care and research ethics committees of Third Military Medical University.

PLASMID VECTORS AND CELLS

pALB/EGFP was kindly provided by Takahiro Ochiya [Yamamoto et al., 2003] (Section for Studies on Metastasis, National Cancer Center Research Institute, Tokyo, Japan); $2K7_{bsd}$, $2K7_{neo}$, EF1-α/eGFP were kindly provided by Suter et al. [2006] (Biology of Aging Laboratory, Department of Rehabilitation and Geriatrics, University of Geneva Medical School, Geneva, Switzerland); pLP1, pLP2, and pLP/VSVG were isolated from ViraPower Packaging Mix (Invitrogen, Carlsbad, CA); pCMV-Hnf4a (containing a cDNA encoding the full-length open reading frame of Hnf4a) was from BPRC (Beijing Proteome Research Center, Beijing, China); and pD-Foxa2 (containing a cDNA encoding the full-length open reading frame of Foxa2) was from PTG (Proteintech Group, Inc, Wuhan, China). 293FT cell line was obtained from Invitrogen (Carlsbad). CCE mouse ESC line was obtained from StemCell Technologies (Vancouver, BC, Canada), and 129 mice ESC line was purchased from Cyagen Biosciences (Guangzhou, China). MEFs (mouse embryonic fibroblasts) were isolated from 13.5 days post-coitus pregnant mice (Kunming White strain).

REAGENTS

Mouse ESC growth medium was from Cyagen Biosciences (Guangzhou, China). LIF (leukemia inhibitory factor), ESGRO Complete™ PLUS Clonal Grade Medium, ESGRO Complete™ Basal Medium and ESGRO Complete™ Accutase were from Millipore (Billerica, MA). Knockout™ D-MEM, Knockout™ Serum Replacement, B27 (50×), RPMI 1640, TRIZOL, sodium butyrate, and BSD (blasticidin) were from Invitrogen (Carlsbad). G418 was from Merck (Whitehouse Station, NJ). Mitomycin C was from Roche (Indianapolis, IN). DMSO, sodium butyrate, polybrene and gelatin were from Sigma–Aldrich (St. Louis, MO). Activin A was from Peprotech (Rocky Hill, NJ). Mouse anti-beta-actin was from Novus (Littleton, CO); and anti-mouse IgG-HRP and anti-rabbit-HRP were from Sigma–Aldrich (St. Louis). All other antibodies and DAPI were from Santa Cruz (Santa Cruz, CA). Reverse transcriptase kits and SYBR Green Real-Time Quantitative PCR kits were from Toyobo (Tokyo, Japan).

PLASMID CONSTRUCTION

A PCR product containing the coding sequences of mouse ALBep (albumin enhancer-promoter) and EGFP (Enhanced Green Fluorescent Protein) from pALB/EGFP [Yamamoto et al., 2003] was ligated into SpeI-SacII-cleaved $2K7_{bsd}$ lentivector. Human elongation factor 1 alpha promoter (EFp) from EF1-α/eGFP was ligated into *Spe*I–*Bam*HI-cleaved lentiviral construct. cDNA encoding the full-length open reading frame of Hnf4a replaced the EGFP sequence in the lentiviral construct. A PCR-amplified woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) cassette modified with *Mlu*I ends was inserted into the *Mlu*I-cleaved lentiviral construct in the forward direction [Zufferey et al., 1999]. The final vector was named $2K7_{bsd}$-EFp-Hnf4a. The plasmid $2K7_{neo}$-EFp-Foxa2 was constructed by the same method.

LENTIVECTOR PRODUCTION AND TRANSDUCTIONS

Lentivector particles were produced by transient transfection in 293FT cells, as previously described [Salmon and Trono, 2007]. The lentivector-containing supernatant was collected after 72 h, filtered through a 0.45-μm pore size polyethersulfone membrane, and concentrated 1,000-fold by ultracentrifugation (50,000*g*, 90 min at 16°C) in the Avanti J-30I High-Performance Centrifuge System (Beckman Coulter http://www.beckmancoulter.com). The pellet was resuspended in complete ESC cell culture medium and subsequently added to the target cells. Titers of the concentrated lentivector were estimated by HeLa cell transduction and ranged from 1×10^7 to 5×10^7 transducing units per milliliter. The multiplicity of infection ranged from 100 to 200 for transduction of CCE cells or 129 cells. Mouse ESCs were transferred onto an irradiated MEF feeder layer in gelatin-coated six-well plates and 1 day later were split onto an irradiated MEF feeder layer. Four days after transduction, blasticidin or neomycin was added to the culture medium of the ESCs. Blasticidin

or neomycin selection was maintained for 4 days. Antibiotics were used at concentrations of 6 and 800 μg/ml for blasticidin and G418, respectively. Irradiated MEFs were added every other day.

CELL CULTURE

CCE cells were maintained on irradiated MEFs in DMEM supplemented with 10% fetal calf serum, L-glutamine, nonessential amino acids, β-mercaptoethanol, penicillin and streptomycin, and leukemia inhibitory factor. Irradiated MEFs were reseeded at a density of 2×10^5 cells/cm^2 1 day before plating ESCs. Mouse ESCs were cultured in ESGRO Complete™ PLUS Clonal Grade Medium in the absence of serum and feeder cells, according to the manufacturer's protocol, before differentiation. Elementary induction medium was composed of Knockout™ DMEM medium supplemented with Knockout™ serum replacement, L-glutamine, nonessential amino acids, β-mercaptoethanol, penicillin and streptomycin.

TERATOMA FORMATION

Cells were harvested by ESGRO Complete™ Accutase treatment, collected into tubes, and centrifuged for 5 min at 1,200 rpm. Two confluent wells from one six-well cell culture plate were injected into the rear leg muscle of 4-week-old male BALB/C-nu/nu mice a using 21 g needle. Four weeks after injection, resulting teratomas were dissected and fixed with PBS (phosphate-buffered saline) containing 4% paraformaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin (HE).

ESC DIFFERENTIATION

Three different induction plans were designed, each of which employed culture medium free of serum and feeder layers. In the first induction plan, after trypsinizing the cells cultured in the medium free of serum and feeder layers, the cells were subjected to suspension culture to obtain embryoid bodies (EBs) and were cultured in elementary induction medium. Then, the EBs was observed on the 5th and 10th days. For the second induction plan, the cells were adherently cultured and induced. During the first phase, they were induced for 5 consecutive days in elementary induction medium supplied with sodium butyrate (1 mM) and Activin A (100 ng/ml). During the second phase, they were induced in the elementary induction medium supplied with DMSO, insulin and dexamethasone for 5 days. Samples on the 5th and 8th days of culture were obtained and studied.

SYBR GREEN REAL-TIME QUANTITATIVE PCR

Total RNA was extracted from ESCs. Total RNA (1 μg) was reverse transcribed using the ReverTraAce-α (Toyobo) according to the manufacturer's recommended protocol. Real-time RT-PCR primers are listed in Table I. The cDNAs were used for PCR using SYBR Green Real-Time PCR Master Mix-Plus (Toyobo) in duplicate. Optimization of the real-time PCR reactions was performed according to the manufacturer's instructions (iCycle, Bio-Rad). All calculations were normalized to an endogenous control, β-actin. Results are expressed as relative quantitative gene expression data that were presnt using the comparative CT method [Schmittgen and Livak, 2008].

IMMUNOFLUORESCENCE

The location and expression of hepatic markers, such as Hnf4a, Foxa2, ALB, CYP7A1, and CK18 were examined by immunofluorescence. Cells on slides were fixed in 4% paraformaldehyde and washed three times with PBS, then incubated with PBS containing 0.1% Triton X-100 (Sigma–Aldrich) and 1% normal serum for 30 min at room temperature. The primary and secondary antibodies were diluted as follows: rabbit anti-Hnf4a and rabbit anti-Foxa2 (1:100) and goat anti-ALB, rabbit anti-CYP7A1 (1:500), goat anti-CK18 (1:500), FITC-conjugated goat anti-rabbit IgG, goat anti-rabbit IgG R, and Cy5-conjugated donkey anti-goat IgG (1:400). Nuclear DNA was dyed with DAPI. For all immunochemistry experiments, negative staining controls were carried out by omitting the primary antibody. Each staining batch included slides with unmodified CCE cells or hepatocytes derived from mouse liver to serve as negative- and positive-staining controls, respectively. Images were captured using the Leica TCSNT confocal microscopy system (Leica TCSNT, Germany, http://www.leica-microsystems.com).

WESTERN BLOT ANALYSIS

Genetically modified mouse ESCs and control cells (mouse ESCs and mouse primary hepatocytes) were lyzed in RIPA strong lysis buffer (Beyotime Institute of Biotechnology, China) according to the manufacturer's recommended protocol and were centrifuged at 800*g* at 4°C for 10 min. Protein concentrations were measured, and aliquots of 20–40 μg of total lysates were fractionated on SDS–PAGE and electroblotted onto PVDF membranes. Antibody incubation and chemiluminescence detection were performed according to the manufacturer's instructions. Primary antibodies and secondary antibodies were used at the following working concentrations: rabbit anti-Foxa2 polyclonal antibody (1:200), rabbit anti-Hnf4a polyclonal

TABLE I. Primers for SYBR Green Real-Time Quantitative PCR

Gene	Primer sequence (forward/reverse)	GenBank accession	Annealing temperature (°C)	Product size (bp)
β-actin	5′-tgttaccaactgggacgaca-3′ 5′-ggggtgttgaaggtctcaaa-3′	NM_007393	55	165
AFP	5′-agcaaagctgcgctctctac-3′ 5′-gagttcacagggcttgcttc-3′	NM_007423	55	247
ALB	5′-gacaaggaaagctgcctgac-3′ 5′-ttctgcaaagtcagcattgg-3′	NM_009654	59.7	174
CK18	5′-cgaggcactcaaggaagaac-3′ 5′-cttggtggtgacaactgtgg-3′	NM_010664	55	246
AAT	5′-gctgaaaatgactccctcca-3′ 5′-ccgatgtttgtgtgaggttg-3′	NM_009244	55	326

antibody (1:200), mouse anti-β-actin (1:2,000), anti-mouse IgG-HRP (1:2,500) and anti-rabbit-HRP (1:2,500).

FACS ANALYSIS

For intracellular staining of albumin and α1-antitrypsin (AAT), 10^6 cells were harvested and fixed with 4% paraformaldehyde for 30 min, and then permeabilized in staining buffer (PBS with 10% FBS and 0.5% saponin) for 15 min. Cells were then incubated with primary antibody (rabbit anti-albumin, goat anti-AAT) for 45 min in staining buffer, followed with secondary antibody (PE-conjugated donkey anti-rabbit IgG, FITC-conjugated donkey anti-goat IgG) incubation for 30 min. Cells were analyzed by the Calibur flow cytometer (Becton Dickinson).

PERIODIC ACID–SCHIFF (PAS) STAINING

Cells were fixed with 10% formaldehyde oxidized in 1% periodic acid (Sigma–Aldrich) for 10 min and rinsed twice with water. Afterwards, cells were treated with Schiff's reagent for 10 min and rinsed with water.

STATISTICAL ANALYSIS

Statistical analyses were performed using PRISM 5.01 (GraphPad Software Inc). The data are presented as the mean values ± SD of n determinations, as indicated in the figure legends. Student's *t*-test analysis or one-way ANOVA with Tukey post-hoc testing for multiple comparisons was employed to determine the levels of significance of the differences observed between the examined treatment groups. A P value < 0.05 considered statistically significant.

RESULTS

CONSTITUTIVE EXPRESSION OF Foxa2 AND Hnf4a IN MOUSE ESCs

Three different gene groups were transduced into ESCs. Cells expressing Hnf4a, Foxa2, or Foxa2/Hnf4a were obtained, with four–five clones for each type of cells. These clones were then preliminarily examined by PCR to specifically amplify the resistance gene segments to ensure the existence of the target sequence (data not shown). Cell clones were labeled as CCE-Foxa2, CCE-Hnf4a, and CCE-Foxa2/Hnf4a, which represent the stable constitutive expression of Foxa2, Hnf4a, and the Foxa2/Hnf4a, respectively, in mouse ESCs. These cells were further examined by western blot and immunofluorescence. Immunofluorescence results indicate that the target proteins were located in the nucleus and could be expressed in the corresponding cells (Fig. 1A and B). The western blot results confirmed the expression of the corresponding target proteins and showed that low expression of Hnf4a or Foxa2 in the ESCs could be mutually facilitated by forced expression of Foxa2 or Hnf4a (Fig. 1C and D).

THE EFFECTS OF THE CONSTITUTIVE EXPRESSION OF Foxa2 OR Hnf4a ON THE GROWTH CHARACTERISTICS OF ESCs AND TERATOMA FORMATION CAPABILITY IN VIVO

Constitutive expression of Foxa2 or Hnf4a did not affect the growth and proliferation rates of ESCs, which still maintained the typical growing patterns of aggregation and cloning in the ESC culture media. After transplanting the four types of cells (CCE, CCE-Foxa2, CCE-Hnf4a, and CCE-Foxa2/Hnf4a) into mice, 4 weeks of continuous observation was carried out to study the effects of the constitutive Foxa2 or Hnf4a expression on the ability to form in vivo teratomas and autonomous differentiation. After 1 month, the final results showed that obvious teratomas formed in all mouse groups (Fig. 2). The sizes of the teratomas among the groups demonstrated no significant differences in their abilities to form teratomas. HE staining of tissue sections indicated that all the four of the ESC types generated teratomas containing cells of the three embryonic layers, and there were no significant differences among the different types. Furthermore, the genetically modified ESCs did not tend to differentiate into a specific tissue. Three repeated teratoma formation experiments confirmed these results. These results indicate that the Constitutive Expression of Foxa2 or Hnf4a has no significant effects on the ESC proliferation capacity and teratoma formation.

EFFECTS OF CONSTITUTIVE EXPRESSION OF Foxa2 OR Hnf4a ON AUTONOMOUS HEPATIC DIFFERENTIATION OF ESCs

To analyze the effects of constitutive expression of Foxa2 or Hnf4a on autonomous hepatic differentiation of ESCs, external inducers were not added to the elementary induction medium. Though many lamp or lantern-like EBs was formed on the 10th day in the CCE-Foxa2 and CCE-Foxa2/Hnf4a groups (Fig. 3 A1,A2), but none formed in the CCE and CCE-Hnf4a groups. As determined by fluorescent quantitative PCR, no significant differences were demonstrated between the ESCs modified to express Foxa2 or Hnf4a and the unmodified ESCs with regard to the expression levels of the four liver-specific genes ALB, AFP, CK18, and AAT (Fig. 3B–E).

After the culture of EBs in plates, which had been prepared by the above-mentioned methods, a small number of hepatocyte-like cells were found on the clone edges of all cells. However, there were no significant differences in the amounts of these cells among the different groups. Later, after the extension of culture time (10 days of adherent culture), a mixture of cells appeared in the middle, including spindle cells, amoeboid cells, column-shaped cells, and some beating cell groups (Fig. 3A3,A4). The results suggest that the constitutive expression of Foxa2 or Hnf4a has no significant effects on autonomous hepatic differentiation of ESCs.

THE EFFECTS OF THE CONSTITUTIVE EXPRESSION OF Foxa2 OR Hnf4a ON HEPATIC DIFFERENTIATION OF ESCs

To analyze the effects of constitutive expression of Foxa2 or Hnf4a on hepatic differentiation of ESCs, an adherent culture model free of serum and feeder layers was adopted. The first phase of induction involved the inducers sodium butyrate and Activin A, whereas the second phase employed the inducers DMSO, insulin and dexamethasone instead. Under these induction conditions, there were some differences in the rate of morphological changes in the four types of cells and in their developing forms. At the end of the first phase, all cells changed from their aggregating and cloning patterns to an extension pattern of short-sided polygons. There seems be significantly more uniform and short-sided polygon-like cells formed by CCE-Foxa2-expressing cells (Fig. 4A).

Quantitative fluorescent PCR proved the following three points: (1) Foxa2 significantly facilitated the expressions of ALB, AFP, CK18 and AAT; (2) the constitutive expression of Foxa2/Hnf4a, although

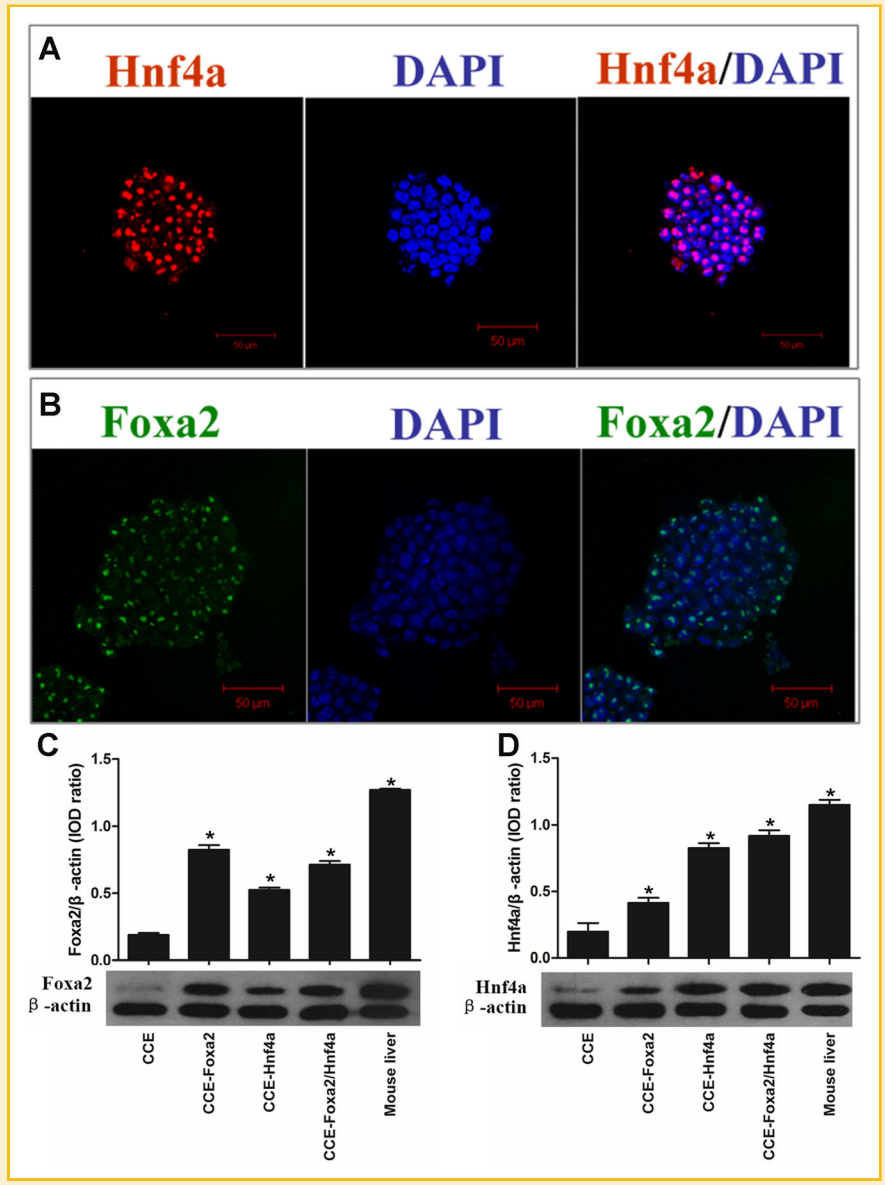

Fig. 1. The expressions of Foxa2 or Hnf4a in undifferentiated genetically modified ESCs were detected by Western blot and immunofluorescence. A and B: Immunofluorescent detection of Hnf4a in CCE-Foxa2/Hnf4a cells (A) or Foxa2 in CCE-Foxa2/Hnf4a cells (B). Scale bar = 50 μm; C and D: Western blot detection of the protein expression levels of Foxa2 (C) and Hnf4a (D). The Foxa2-, Hnf4a- or β-actin-specific bands were quantified as the relative integrated optical density (IOD) values of the ratio of Foxa2/β-actin or Hnf4a/β-actin for each treatment, mean ± SD. n = 5. $^*P < 0.05$, versus CCE cells (one-way ANOVA).

incomparable with Foxa2, could also increase the expressions of ALB, AFP, CK18 and AAT; and (3) Hnf4a alone did not significantly facilitate the expression of these specific liver genes (Fig. 4B–E).

Immunofluorescence detection after 10 days sequential differentiation indicated that the percentage of ALB-positive cells in CCE-Foxa2 cells was obvious higher than that in CCE cells (Fig. 5A and B). Using dual-color flow cytometry, we showed that 89.52% CCE-Foxa2 cells, in contrast to 32.43% CCE cells, expressed both ALB and AAT (Fig. 5C and D). In order to further verify whether CCE-Foxa2 cells express more mature and metabolism related indicators than CCE cells. CYP7A1 and CK-18 immunofluorescence detections were also done after 10 days sequential differentiation (Fig. 6A–D). The results indicated that percentage of CYP7A1-positive (Fig. 6B) or CK-18-postive cells (Fig. 6D) in CCE-Foxa2 cells was obvious higher than that in CCE cells (Fig. 6A and C). Moreover, glycogen production of liver-specific functions in vitro was assessed by PAS staining after inducing hepatic differentiation. Differentiated CCE-Foxa2 cells further demonstrated more obvious in vitro functions characteristic of glycogen storage than differentiated CCE cells (Fig. 6E and F). These results demonstrate that the constitutive expression of Foxa2 can increase the differentiation of ESCs into hepatocytes, with the assistance of inducers.

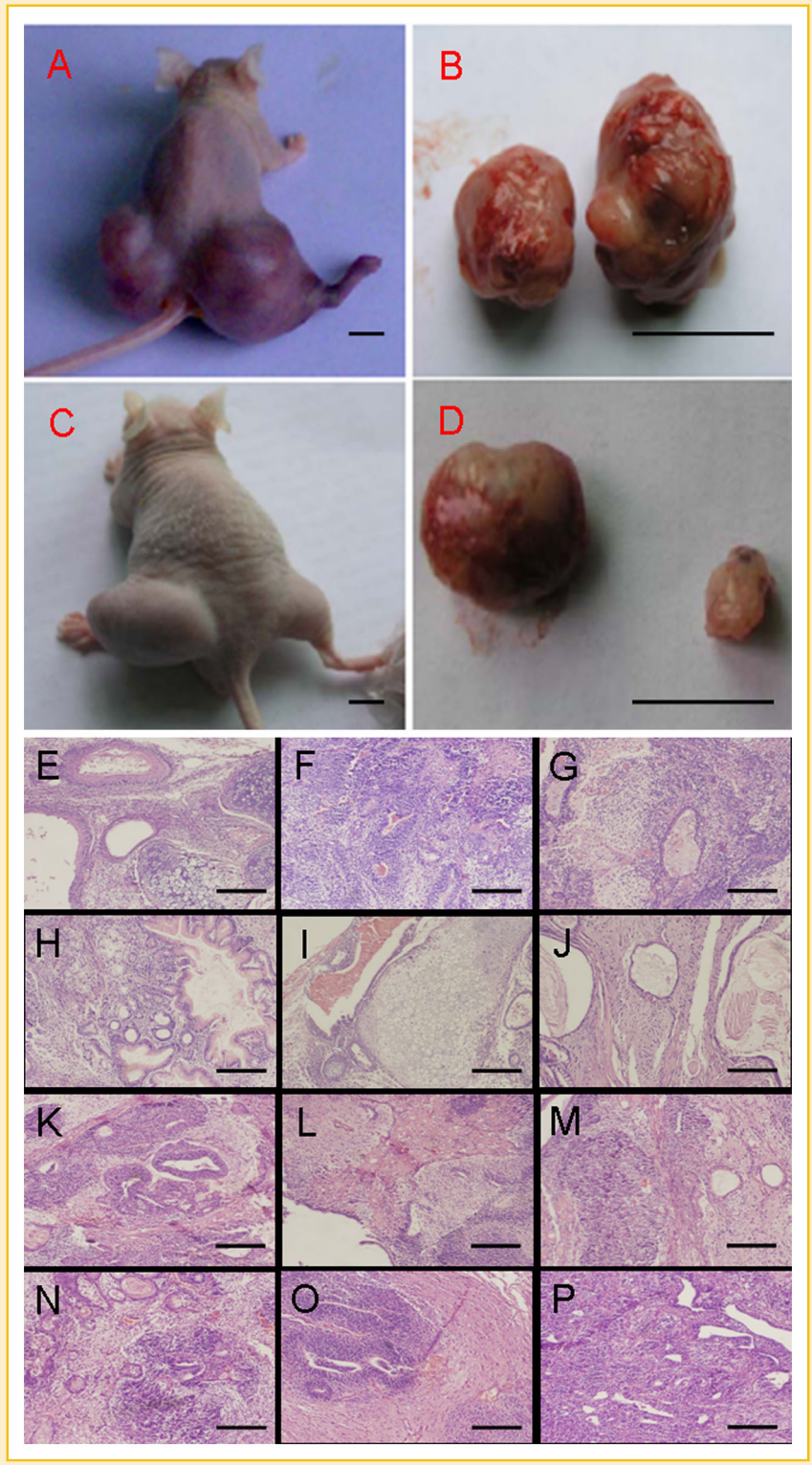

Fig. 2. Teratoma formation in vivo and H&E staining of the modified CCE cells. Left panel: Teratoma formation. A: CCE-Foxa2 cells (left leg) and CCE-Hnf4a cells (right leg); B: CCE-Foxa2 cells (left) and CCE-Hnf4a cells (right); C: CCE cells (left leg) and CCE-Foxa2/Hnf4a cells (right leg); D: CCE cells (left) and CCE-Foxa2/Hnf4a cells (right). Right panel ($\times$100): Eosin staining. E–G: CCE-Foxa2 cells; H–J: CCE-Hnf4a cells; K–M: CCE-Foxa2/Hnf4a cells; N–P: CCE cells. A–D: Scale bar = 1 cm; E–P: Scale bar = 200 μm.

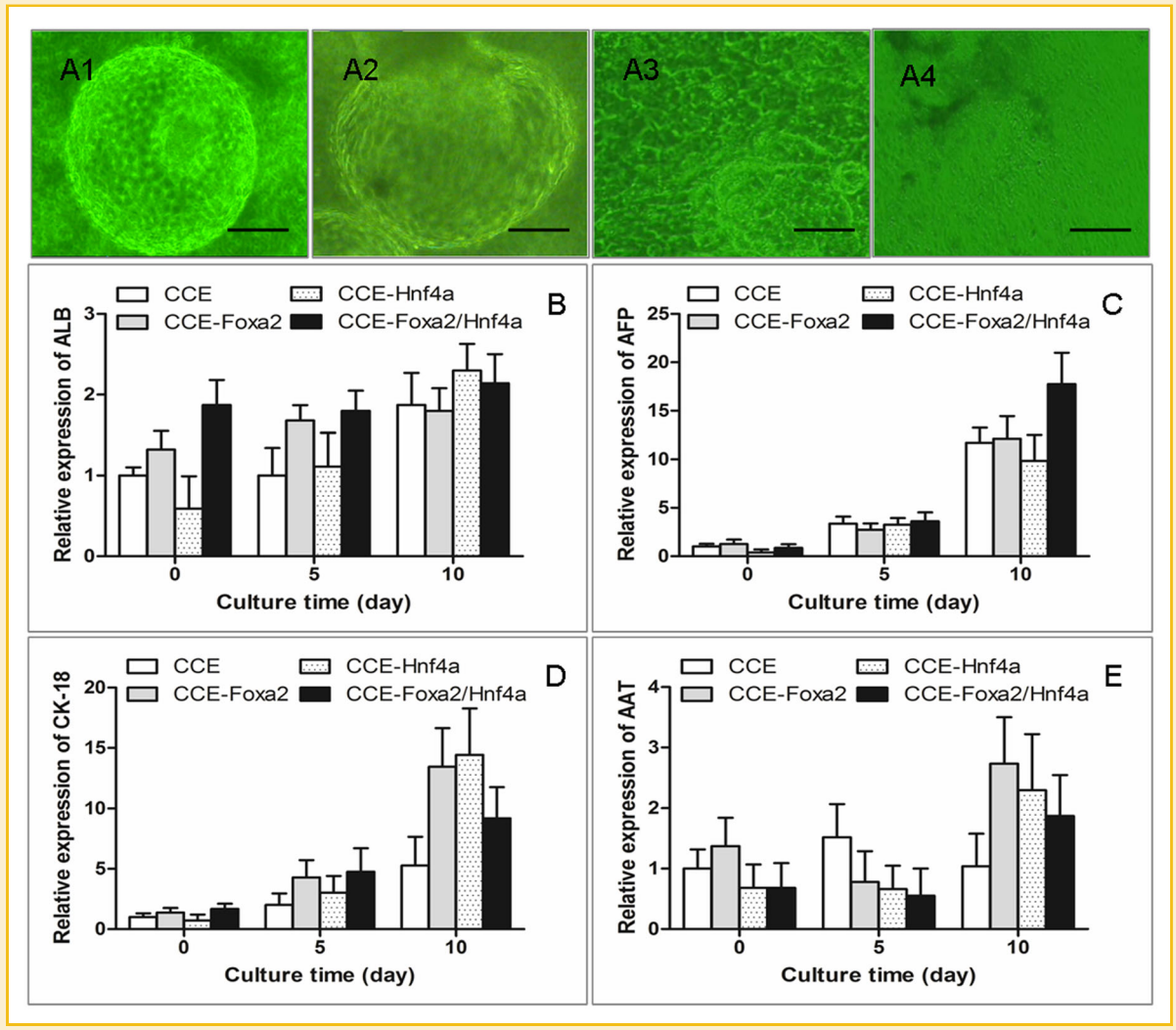

Fig. 3. The effects of constitutive expression of Foxa2 or Hnf4a on autonomous hepatic differentiation of ESCs. A1 and A2: Many lamp or lantern-like EBs on the 10th day was formed by the CCE-Foxa2 (A1) and CCE-Foxa2/Hnf4a (A2) groups; A3 and A4: Movies capture of beating cell groups. Scale bar = 50 μm; B–E: Relative expression levels of hepatic-specific genes including ALB (B), AFP (C), CK-18 (D), and AAT (E) in EB autonomous hepatic differentiation. Results are expressed as the relative expression levels compared with the undifferentiated CCE ESCs. Each value is the mean of duplicate qRT-PCR assays using thee independent samples.

DISCUSSION

With the recent research achievements in celluar reprogramming, it has been acknowledged that cell status in any phase can be subjected to change [Hanna et al., 2011]. Under the coordinating effects of extracellular and intracellular millieu, cells display the dynamic processes of differentiation, trans-differentiation and de-differentiation, which are accompanied by changes in the transcription factor network. Recent publications have indicated that forced expression of specific transcription factors can directly generate phenotypic changes of somatic cells [Takahashi and Yamanaka, 2006; Vierbuchen et al., 2010; Murry and Pu, 2011]. Some studies have argued that overexpression of specific transcription factors in adult cells can increase their ability to trans-differentiate [Murry and Pu, 2011; Tursun et al., 2011] or de-differentiate into certain specific types of stem cells [Cobaleda et al., 2007; Hanna et al., 2008; Liu et al., 2011] and that overexpression of specific transcription factors in stem cells can increase their differentiation into certain specific types of cells [Ishii et al., 2008; Liew et al., 2008; Unger et al., 2008]. All these findings support the significant role of critical transcription factors in resolving cell fate [Tuch et al., 2008]. During the process of liver development, hundreds of transcription factors are involved, and they constitute a complex control network that precisely and accurately regulate hepatic growth [Costa et al., 2003; Si-Tayeb et al., 2010]. Among these transcription factors, Foxa2, and Hnf4a were selected for research to explore the effects of their constitutive expressions on the hepatic-committed differentiation of ESCs.

Many publications have confirmed that when ESCs are maintained and cultured in standard ESC culture media, those that overexpress a foreign gene can maintain their vigorous proliferation rates and pluripotent differentiation capacities. It has also been theoretically assumed that if gene-modified ESCs are separated from their

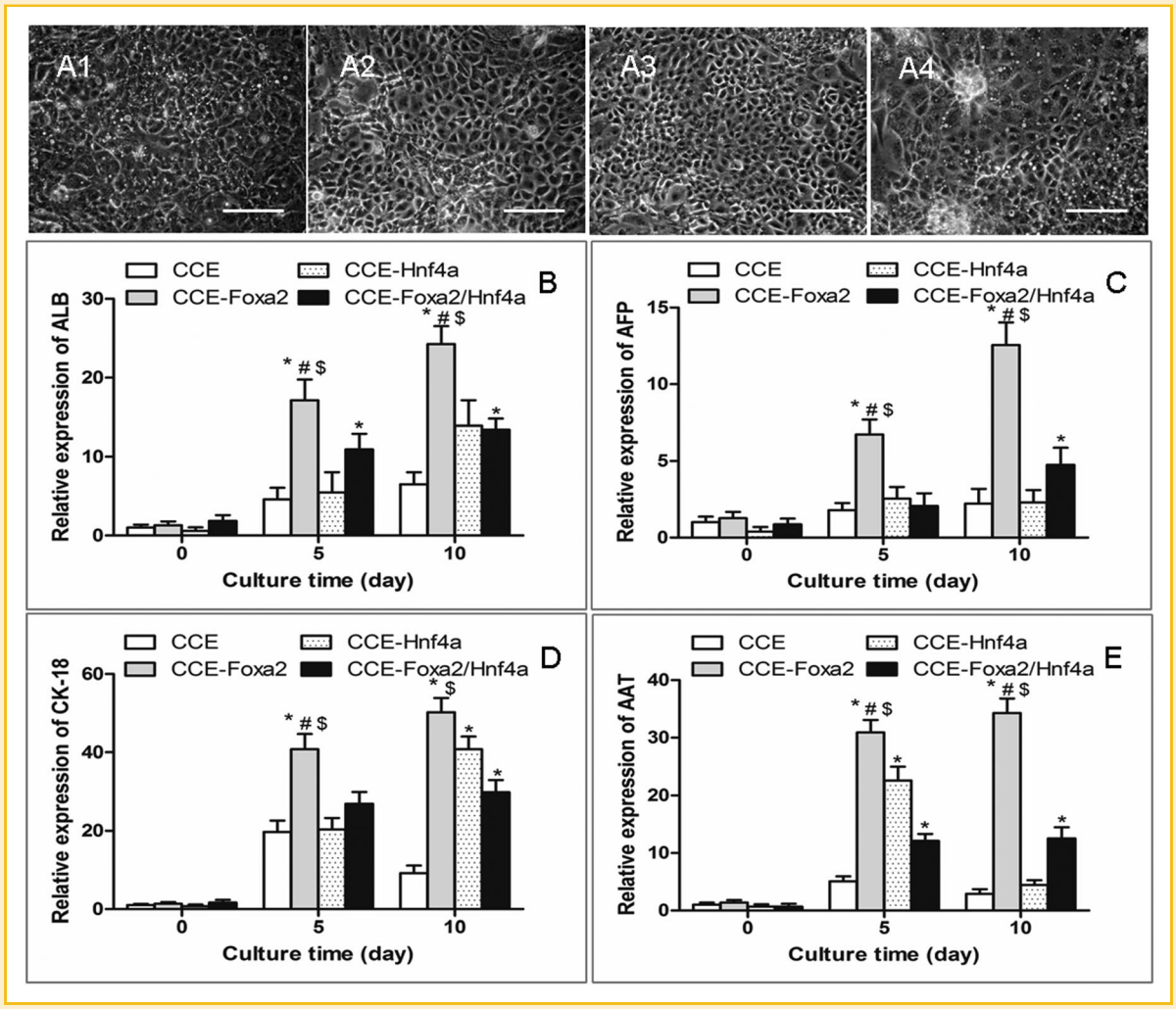

Fig. 4. The effects of constitutive expression of Foxa2 or Hnf4a on hepatic differentiation of ESCs after another 5 days of adherent culture (culture medium containing DMSO, etc.) following 5 days adherent culture (culture medium containing sodium butyrate and Activin A). A: Cell morphology of CCE (A1), CCE-Foxa2 (A2), CCE-Hnf4a (A3), and CCE-Foxa2/Hnf4a (A4) cells after 10 days of hepatic differentiation. Scale bar = 50 μm; B–E: Relative expression of hepatocyte-specific genes on the 5th and 10th day. Results are expressed as relative expression levels compared with the undifferentiated CCE ESCs. Each value is the mean of duplicate qRT-PCR assays using three independent samples. $^{*}P < 0.05$, versus CCE cells. $^{\#}P < 0.05$, versus CCE-Hnf4a cells. $^{\$}P < 0.05$, versus CCE-Foxa2/Hnf4a cells. (one-way ANOVA).

maintenance environment during teratoma development experiments, teratoma formation will be affected or the cells might differentiate into a particular embryonic layer or cell. However, such an effect or liability was not observed in our three repeated teratoma formation experiments. This may be attributed to the properties of the overexpressed genes or their strength of expression because some recent reports [Kawada et al., 2012; Kinney and McDevitt, 2012] have pointed out that cell differentiation has a certain dependence on space, time and quantity. Different levels of intracellular gene expression can occur in vivo, possibly resulting in differing amounts of growth and differentiation [Unger et al., 2008]. Induced factors in the extracellular milieu can also regulate the fate of ESCs in a concentration-dependent manner [Ameri et al., 2010]. Similarly, in this study, our results showed that the modified ESCs exercised no significant influence on teratoma formation, which may have been related to gene expression strength or a lack of beneficial external factors.

In culture media without inducing factors (sodium butyrate, Activin A, DMSO, insulin or dexamethasone), LIF, serum and a feeder layer, there were no significant differences between the gene-modified ESCs and unmodified ESCs. Expression of Foxa2 or Hnf4a independently or collectively did not increase the differentiation of ESCs into hepatocyte-like cells. Consistent with previous reports [Ishizaka et al., 2002; Kanda et al., 2003], when cells were cultured in induction media containing some inducing factors, Foxa2 expression significantly increased differentiation into hepatocyte-like cells. Although the stable constitutive expression of Foxa2 and Hnf4a increased differentiation to a less significant level compared with that of Foxa2 alone, Hnf4a expression alone could not increase differentiation, a result that is inconsistent with a previous report

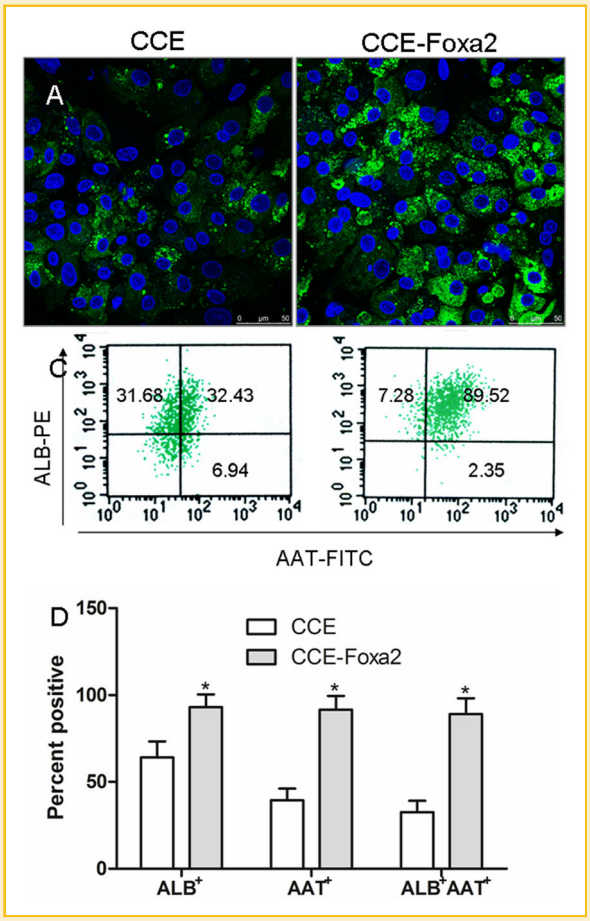

Fig. 5. Albumin (ALB) expression of CCE-Foxa2 cells as confirmed by immunofluorescence and FACS analysis after 10 days differentiation. A and B: Immunofluorescence staining indicated that the percentage of ALB-positive cells in CCE-Foxa2 cells (B) was obvious higher than that in CCE cells (B) after 10 days differentiaton. Scale bar = 50 μm; C: Dual-color FACS analysis of ALB and alpha antitrypsin (AAT) positive cells after 10 days differentiation. D: The percentage of both ALB and AAT positive cells in CCE-Foxa2 cells were higher than that in CCE cells after hepatic differentiation. $^{*}P < 0.05$, versus CCE cells (Student's *t*-test).

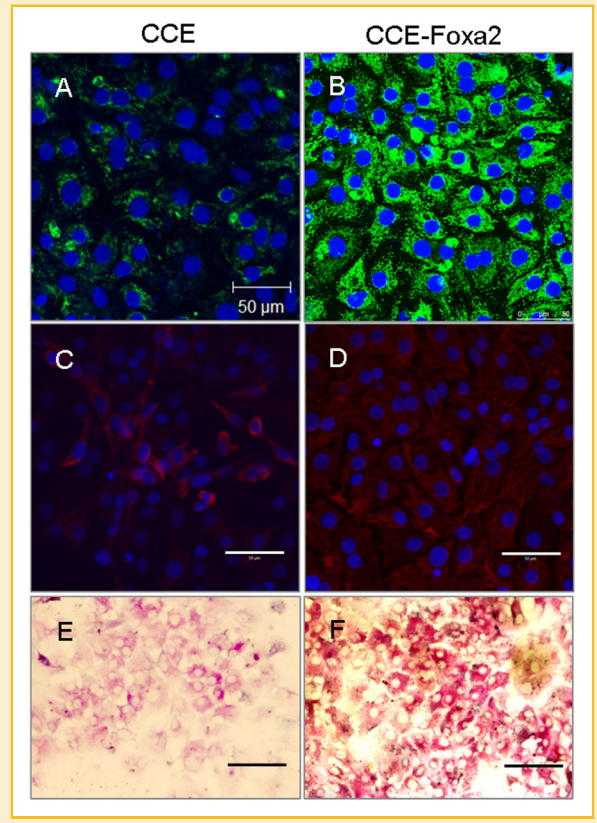

Fig. 6. Several liver-specific proteins and functions of differentiated CCE-Foxa2 cells. A and B: CYP7A1 expression in the differentiated CCE (A) and CCE-Foxa2 (B) cells. C and D: CK-18 expression in the differentiated CCE (C) and CCE-Foxa2 (D) cells. E and F: Periodic acid-schiff staining indicated that differentiated CCE-Foxa2 (F) cells demonstrated more obvious in vitro functions characteristic of glycogen storage than differentiated CCE cells (E). A–F: Scale bar = 50 μm.

[Kuai et al., 2006]. Our results were confirmed by quantitative PCR, immunofluorescence, FACS analysis and PAS staining.

According to our results, we can conclude that Foxa2, when combined with certain inducers in the culture media, can increase the differentiation of ESCs into hepatocyte-like cells. Other studies have shown that DMSO can increase the differentiation of definitive endoderm cells into hepatocyte-like cells [Hay et al., 2008] and that Foxa2 can facilitate the differentiation of ESCs into definitive endoderm cells [Zaret, 1999]. These studies may probably explain why DMSO and Foxa2 have synergistic effects. In almost all the latest effective protocols [Roelandt et al., 2010; Liu et al., 2011] for differentiation of ESCs into hepatocyte-like cells, one must always first induce ESCs into definitive endoderm cells, then induce definitive endoderm cells into hepatic progenitor cells, and finally differentiate these progenitor cells into mature hepatocyte-like cells. Any induction plan that excludes the step of differentiation into definitive endoderm cells will be largely devalued in effectiveness. The reason why Foxa2 could coordinate with some inducers to increase the differentiation of ESCs into hepatocyte-like cells is probably because the overexpression of Foxa2 first directly stimulates the differentiation of ESCs into definitive endoderm cells, and then accelerates the differentiation into hepatic progenitor cells with the assistance of various factors. Dexamethasone may also further induce hepatocyte maturation through promoting the expression of essential transcription factors for hepatocyte differentiation [Michalopoulos et al., 2003]. Therefore, further experiments must be performed to determine which factor in the media, insulin or dexamethasone has a synergistic effect with DMSO to facilitate Foxa2′s ability to induce the differentiation of ESCs into hepatocyte-like cells. In this study, without external induction factors, Foxa2 alone was unable to perform the above-mentioned functions. Likewise, Hnf4a failed to speed up the differentiation of ESCs into hepatocyte-like cells, although the Western blot results suggest that it could facilitate some Foxa2 expression. The overexpression of Foxa2 and Hnf4a did

facilitate the differentiation of ESCs into hepatocyte-like cells but to a less significant degree than Foxa2 alone.

Of note, hepatic special functional analysis such as urea secretion, albumin secretion, ammonia clearance, and so on, are also needed to be done to confirm these results. The lack of effect caused by Hnf4a expression in this study might be related to its ability to regulate hepatocyte maturation. Hnf4a acts during the middle and late phases [Naiki et al., 2005], whereas all of the induction strategies in our research were relatively short and focused on differentiation during the early and middle phases. Obviously, there is not a universal theory to explain all these phenomena, and further investigation is warranted.

During the differentiation of ESCs into progeny cells, ESCs are at the beginning or peak of developmental differentiation. Stem cells are at the intersection of these differentiation routes, whereas adult somatic cells are at the end. Traditionally, differentiation induction strategies attempt to copy normal internal differentiation signals with the use of inducers, cell matrices, and culture media. However, the high plasticity of cells and the critical roles of transcription factors in altering cellular phenotype have suggested the possibility of a new differentiation induction strategy, which utilizes a few critical transcription factors that then transcend regular differentiation routes or shorten directed differentiation [Young, 2011]. Although liver development and maturation is accomplished by hundreds of transcription factors in coordinating and sequencing work, this study focused on the effects of only Foxa2 and Hnf4a, two key transcription factors in liver development, on the differentiation of ESCs into hepatocyte-like cells. However, Foxa2 and Hnf4a have not synergistic effects in our study. Whether Foxa2 and Hnf4a have regulatory roles may probably be related to their expression levels and duration of function. The ideal research strategy may direct the differentiation of ESCs through controlling the expressions of key transcription factors in spatiotemporal sequences at different levels [Xia et al., 2008]. Then, the proper key transcription factors can be expressed at the correct time, with the right strength and in the right order so that they may cooperate better to promote the specific differentiation of ESCs.

In conclusion, this study investigated the effects of constitutive expression of Foxa2 or Hnf4a on the differentiation of ESCs into hepatocyte-like cells. From our data, we concluded the following three aspects: (1) Foxa2 or Hnf4a expression induced no significant effects on the ESC proliferation capacity and teratoma formation; (2) the constitutive expression of Foxa2 increased the differentiation of ESCs into hepatocyte-like cells, with the assistance of inducers; (3) the constitutive expression of Foxa2 and Hnf4a could also increase differentiation but to a less significant deree compared with that of Foxa2 alone. Therefore, our method employing the transduction of Foxa2 would be a valuable tool for the efficient generation of functional hepatocytes derived from ESCs.

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