

## Basic Study

## Hepatic differentiation of rat induced pluripotent stem cells *in vitro*

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**Supported by** National Natural Science Foundation of China, No. 81000173, No. 81070344 and No. 81100297.

**Institutional review board statement:** The study did not involve human or animal subjects.

**Institutional animal care and use committee statement:** The study did not involve human or animal subjects.

**Conflict-of-interest statement:** The authors declare no financial conflict of interest.

**Data sharing statement:** No additional data are available.

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Received: January 22, 2015

Peer-review started: January 24, 2015

First decision: April 14, 2015

Revised: April 23, 2015

Accepted: August 25, 2015

Article in press: August 25, 2015

Published online: October 21, 2015

### Abstract

**AIM:** To show the efficient generation of hepatocyte-like cells (HLCs) differentiated from the induced pluripotent stem cells (iPSCs) of rats.

**METHODS:** Hepatic differentiation was achieved using a three-step protocol with several growth factors. First, rat iPSCs were differentiated into definitive endoderm cells using Activin A and Wnt3a treatment. Then fibroblast growth factor 4 and bone morphogenetic protein 2 were added to the culture medium and used to induce hepatic differentiation. Finally, hepatocyte growth factor, Oncostatin M and dexamethasone were used for hepatic maturation. The liver-related markers and functions of HLCs were assessed at the gene and protein levels.

**RESULTS:** After endodermal induction, the differentiated cells expressed endodermal markers forkhead box protein A2 and SRY-box containing gene 17 at the mRNA and protein levels. After 20 d of culture, the iPSCs were differentiated into HLCs. These differentiated cells expressed hepatic markers including  $\alpha$ -fetoprotein, albumin CK8, CK18, CK19, and transcription factor HNF-4 $\alpha$ . In addition, the cells expressed functional proteins such as  $\alpha$ 1-antitrypsin, cytochrome P450 1A2 and CYP 3A4. They acted like healthy hepatic cells, storing glycogen and taking up indocyanine green and low-density lipoproteins. Also, the rates of urea synthesis (20 d  $1.202 \pm 0.080$  mg/dL vs 0 d  $0.317 \pm 0.021$  mg/dL,  $P < 0.01$ ) and albumin

secretion (20 d  $1.601 \pm 0.102$  mg/dL vs 0 d  $0.313 \pm 0.015$  mg/dL,  $P < 0.01$ ) increased significantly as differentiation progressed.

**CONCLUSION:** Rat iPSCs can differentiate into HLCs rapidly and efficiently. These differentiated cells may be an attractive resource for treatment of end-stage liver disease.

**Key words:** Hepatic differentiation; Induced pluripotent stem cells; Hepatocyte-like cells; Rat

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**Core tip:** Induced pluripotent stem cells (iPSCs) can differentiate into many kinds of cells, including hepatocytes. However, the most important animal model for studying human diseases, especially liver diseases, is the rat model. This study is the first to show the efficient generation of hepatocyte-like cells (HLCs) differentiated from the iPSCs of rats. Hepatic differentiation was achieved using a three-step protocol. Rat iPSCs were differentiated into HLCs after 20 d of culture. These differentiated cells expressed hepatic markers and exhibited functional hepatic characteristics. These differentiated cells may be an attractive resource for treatment of liver disease.

Sun C, Hu JJ, Pan Q, Cao Y, Fan JG, Li GM. Hepatic differentiation of rat induced pluripotent stem cells *in vitro*. *World J Gastroenterol* 2015; 21(39): 11118-11126 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i39/11118.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i39.11118>

## INTRODUCTION

So far, there has been no ideal treatment for end-stage liver disease attributable to chronic hepatitis or cirrhosis. Recent studies have suggested that differentiated hepatocytes derived from transplanted stem cells may be a suitable form of therapy<sup>[1,2]</sup>. Embryonic stem cells (ESCs) and fetal liver epithelial cells can form mature hepatocytes *in vivo*. This might reduce the severity of hepatic fibrosis<sup>[3,4]</sup>. However, the collection of these cells from human embryos involves certain ethical and political issues, which limit their use in clinical settings. According to a previous study, bone marrow-derived liver stem cells may suppress hepatic fibrosis and ameliorate liver function<sup>[5]</sup>. However, their ability to proliferate is much poorer than that of ESCs, leaving them unable to produce a large number of cells<sup>[6]</sup>. Bone marrow contains several different components. This low purity produces a non-homogeneous crop of differentiated cells. This affects the treatment after transplantation<sup>[7]</sup>.

Induced pluripotent stem cells (iPSCs) sidestep most of the ethical issues surrounding ESCs. They

can produce functional hepatocytes and provide new seed cells for the treatment of hepatic diseases<sup>[8,9]</sup>. Studies have shown that iPSCs can differentiate into neural cells<sup>[10,11]</sup>, osteogenic cells<sup>[12,13]</sup>, cardiac cells<sup>[14,15]</sup>, vascular cells<sup>[16,17]</sup> and pancreatic cells<sup>[18,19]</sup>. The differentiation of iPSCs into hepatic lineages has been observed in humans and mice using similar protocols<sup>[20-22]</sup>. However, the laboratory rat was the first mammalian species domesticated for scientific research, and the rat model is commonly used in research involving hepatic diseases. So far, no reports have been published on the differentiation of rat iPSCs into hepatocyte-like cells (HLCs). In order to develop such technologies in rat models, a method suitable for the differentiation of rat iPSCs into HLCs is here presented and the necessary conditions are given.

In the present study, rat iPSCs were differentiated into HLCs using several growth factors. The characteristics and functions of the HLCs were evaluated. These cells might be suitable for use as a cell resource for transplantation into model animals with liver diseases.

## MATERIALS AND METHODS

### Culture of rat iPSCs

Rat iPSC line M13 was provided by the research group led by Professor Lei Xiao of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences<sup>[23]</sup>. Rat iPSCs were cultured with a feeder layer of irradiated mouse embryonic fibroblasts (MEF) in iPSC medium (DMEM/F12 containing 20% knockout serum replacement, 2 mmol/L L-glutamine, 1% nonessential amino acids, 0.1 mmol/L β-mercaptoethanol (all from Invitrogen/Gibco, Grand Island, NY, United States) in a humid atmosphere containing 5% CO<sub>2</sub>. Before differentiation, the cells were cultured on gelatin-coated tissue culture dishes with MEF-conditioned medium.

### Hepatic differentiation of rat iPSCs

During the first 5 d, rat iPSCs were cultured in the Roswell Park Memorial Institute 1640 medium (Invitrogen/Gibco) with 25 ng/mL Wnt3a and 100 ng/mL Activin A (both from R and D Systems, Minneapolis, MN, United States) for endodermal induction. Next, the inductive factors were replaced with 30 ng/mL fibroblast growth factor 4 (FGF4, R and D Systems) and 20 ng/mL bone morphogenetic protein 2 (BMP2, R and D Systems) for 5 d. Finally, during the maturation step, the differentiated cells were incubated in the same basal medium containing 10 ng/mL Oncostatin M (OSM, R and D Systems), 20 ng/mL hepatocyte growth factor (HGF, R and D Systems), and 0.1 μmol/L dexamethasone (Dex, Sigma-Aldrich, St. Louis, MO, United States) for another 10 d.

### Immunofluorescence staining

On day 5 after endodermal induction, differentiated cells were tested for SRY-box containing gene 17 (SOX17)

**Table 1 Primers, GenBank accession numbers, and amplicon size of genes for reverse transcription-polymerase chain reaction**

Gene	Primer	Accession Number	Amplicon size (bp)
FOXA2	TGTCAGGAGCACAAGCGAGG (F) GGGTGGTTGAAGGCGTAATGGTG (R)	NM-012743.1	194 bp
SOX17	GGCACGGAACCCAACCAGC (F) CAGTCGTGTCCTGGTAGGGAAGAC (R)	NM-001107902.1	119 bp
AFP	TCTGAAACGCCATCGAAATGCC (F) AATGTAAAATGTCGGCCAGTCCCT (R)	NM-012493.2	285 bp
ALB	GGCACAGTGCTTCGAGAATTCAG (F) CACAGACGGTTCAGGATGGCAG (R)	NM-134326.2	272 bp
CK8	TGAAGGATGCCAATGCCAAGCTG (F) AACTCAGTCCCTCGTAGCC (R)	NM-199390.1	237 bp
CK18	ACGATTCAGTCTCAACGACGCC (F) TCCCTTCTCTGAGCCTTAGTGCC (R)	NM-053976.1	147 bp
CK19	GCTCAGCATGAAAGCTGCCCT (F) CGCACTGGTAGCAAGGTAGGAG (R)	NM-199498.1	299 bp
HNF4 $\alpha$	GATGTGCTGCTCTAGCAATGAC (F) CTGCCGGTCGTGATGTAATCCTC (R)	NM-001270931.1	261 bp
GADPH	CCITCATTGACCTCAACTAC (F) GGAAGGCCATGCCAGTGAGC (R)	NM-017008.4	594 bp

and forkhead box protein A2 (FOXA2). On day 20, they were tested for the other markers. Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% TritonX-100, and blocked with 10% goat serum (Sigma-Aldrich) for 1 h at room temperature. Then the cells were incubated overnight with primary antibodies at 4 °C. The primary antibodies against rat FOXA2 (1:200, Chemicon/Millipore, Billerica, MA, United States), SOX17 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, United States), albumin (ALB, 1:500),  $\alpha$ -fetoprotein (AFP, 1:200),  $\alpha$ 1-antitrypsin (AAT, 1:200), cytokeratin 18 (CK18, 1:200, all from Santa Cruz Biotechnology), cytochrome P450 1A2 (CYP1A2, 1:200, Abcam, La Jolla, CA, United States) and cytochrome P450 3A4 (CYP3A4, 1:200, Abcam) were obtained. Then the cells were incubated with TRITC-conjugated secondary antibody (Invitrogen, Carlsbad, CA, United States) for 1 h at room temperature. The nuclei were then counterstained with 4,6-diamidino-2-phenylindole (DAPI, Roche, Mannheim, Germany). The cells were analyzed using a fluorescence microscope (IX71, Olympus, Japan).

#### Reverse-transcription PCR analysis of gene expression

To assess endodermal and hepatic markers, differentiated cells were tested for FOXA2 and SOX17 on day 5 and for other markers on day 20. Trizol reagent was used to extract total RNA from differentiated iPSCs (Invitrogen). Reverse transcription-PCR was used to produce cDNA. GenBank accession numbers, primer sequences, and amplicon size are listed in Table 1 for each gene. The PCR procedure was as follows: cDNA denaturation at 94 °C for 5 min followed by 28 cycles (ALB, 40 cycles) of denaturation at 94 °C for 15 s, annealing at 66 °C for 10 s, and extension at 72 °C for 20 s. The final extension took place at 72 °C and lasted 7 min. PCR products were separated by electrophoresis on agarose gels. The GAPDH housekeeping gene was

used as an internal control.

#### Urea assay

The urea production test was performed using a colorimetric QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA, United States). Undifferentiated cells and differentiated cells on days 5, 10, 15, and 20 were trypsinized and counted with a hemocytometer. The assay was performed in accordance with the manufacturer's instructions. The sample supernatants were stored at -20 °C. Absorbance was measured using a microplate reader. Urea production was normalized to the total number of cells.

#### Albumin secretion

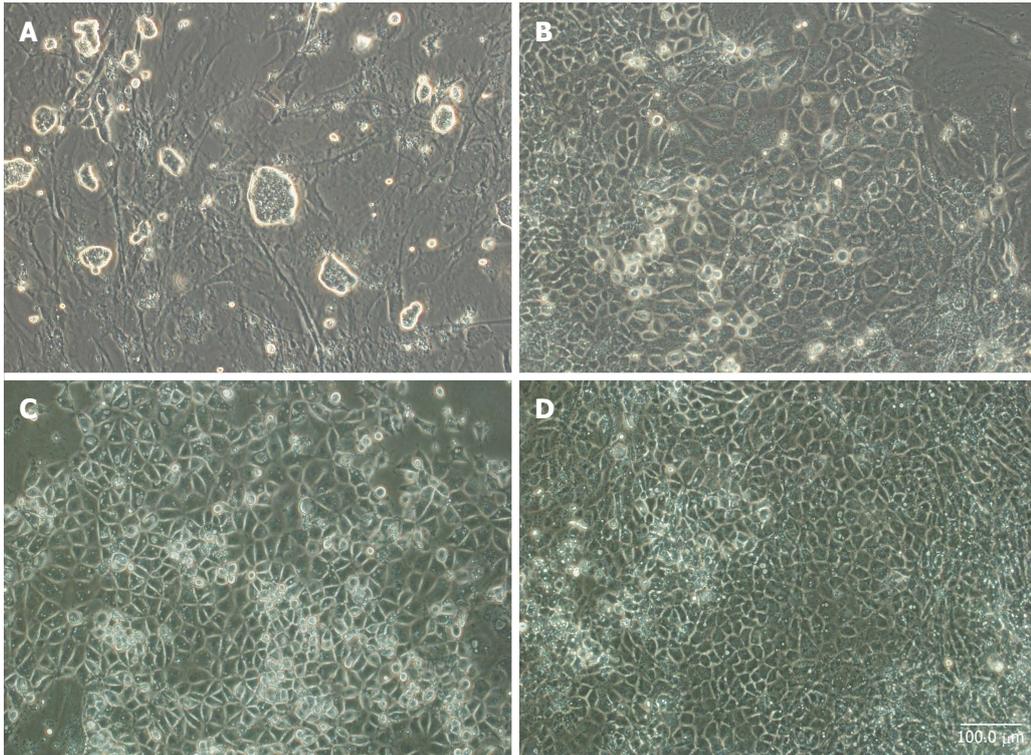
The concentration of rat albumin in the supernatant was determined using a Rat Albumin ELISA Quantitation Kit (ICL, Portland, OR, United States) in accordance with the manufacturer's instructions. Undifferentiated iPSCs and differentiated cells were trypsinized and counted on days 5, 10, 15, and 20 using a hemocytometer. Albumin secretion was normalized to the total number of cells.

#### Periodic acid-Schiff staining

On day 20, differentiated iPSCs were stained using a Periodic acid-Schiff (PAS) staining system (Sigma-Aldrich). The cells were first fixed in 4% paraformaldehyde for 20 min and oxidized with 1% periodic acid solution for 5 min. Then they were washed. The cells were treated with Schiff's reagent for 15 min at room temperature. The cells were washed with PBS and then stained with hematoxylin for 90 s. They were then examined under a light microscope (IX50, Olympus, Japan).

#### Uptake of indocyanine green and low-density lipoprotein

After differentiation, cells were incubated for 1 h with indocyanine green (ICG, Sigma-Aldrich) in basal



**Figure 1** Morphological changes of rat induced pluripotent stem cells at different stages of differentiation. A: Undifferentiated induced pluripotent stem cells; B: Differentiated cells on day 5; C: Differentiated cells on day 15; D: Differentiated cells on day 20. Scale bar = 100  $\mu$ m.

medium at 37 °C. Uptake of ICG was detected under a light microscope. The ability of these cells to take up low-density lipoprotein was determined using an LDL Uptake Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI, United States) according to the manufacturer's instructions. The cells were visualized under a fluorescent microscope.

### Statistical analysis

Results are shown as mean  $\pm$  SD. One-way ANOVA was used for statistical analysis.  $P < 0.05$  was considered significant.

## RESULTS

### Morphological and phenotypic hepatic markers

A simple three-stage differentiation method for iPSCs was developed. During the first five days, iPSCs were treated in a medium with Activin A and Wnt3a for endodermal induction. The cells gradually took on flatter and spikier shapes (Figure 1B). Then, the cells were cultured under feeder-free conditions with high concentrations of FGF4 and BMP2 for the next five days. Cells in these colonies had compact, polygonal shapes like those of differentiated hepatocytes (Figure 1C). The medium was replaced with mature medium supplemented with HGF, OSM, and Dex for an additional 10 d. The cells exhibited the morphology of mature hepatocytes with large amounts of cytoplasm and distinct, round nuclei (Figure 1D).

Cell characteristics were determined at different

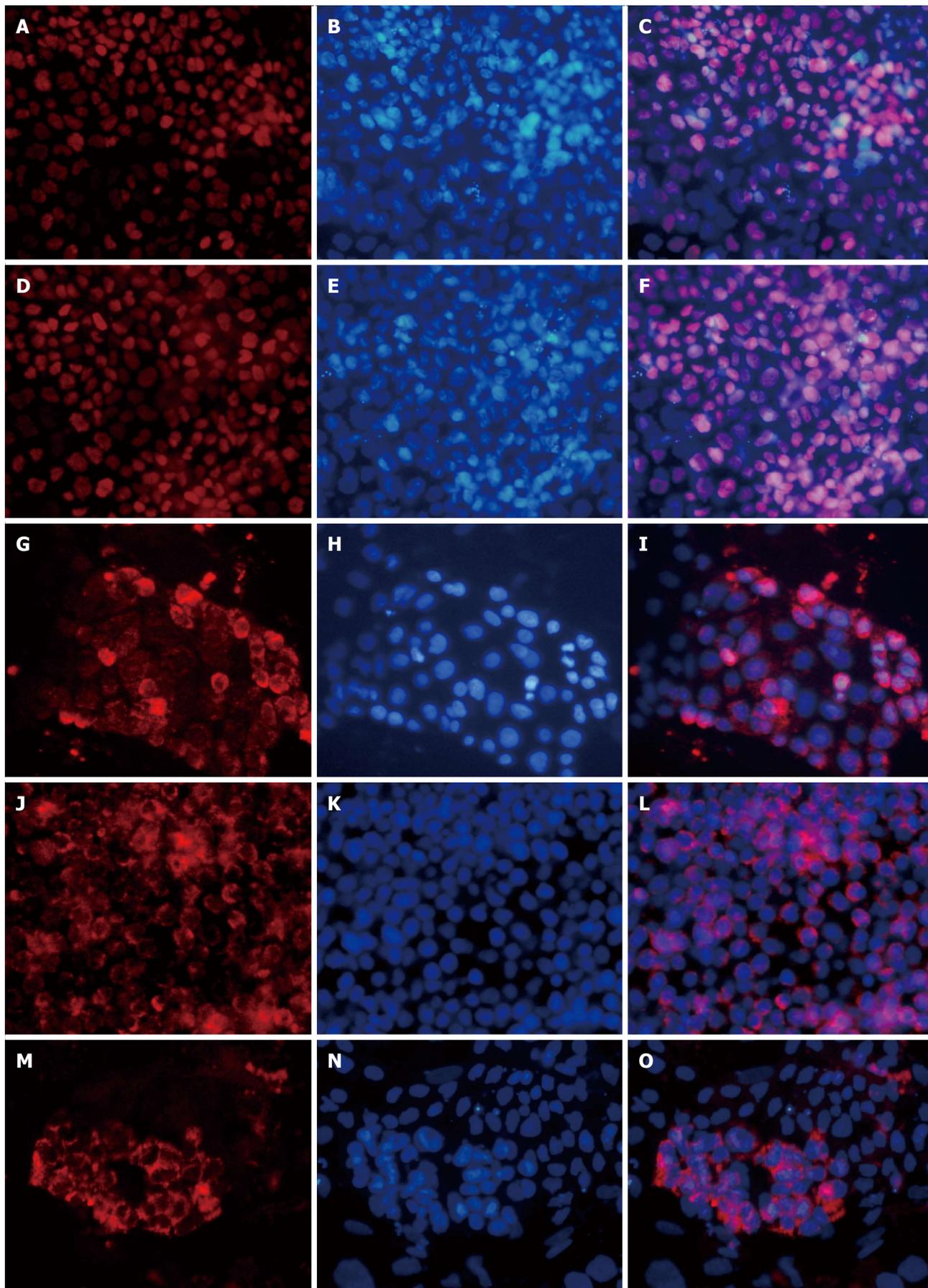
stages of differentiation using immunofluorescence staining. After endodermal induction, approximately 70%-80% of the differentiated cells expressed endodermal markers FOXA2 and SOX17 at the protein level (Figure 2). These results indicate that rat iPSCs could form endoderm efficiently if exposed to Activin A and Wnt3a. After hepatic maturation at stage 3, immunostaining analysis showed that 50%-60% of the cells expressed hepatic markers, including AFP, ALB, CK18, and functional proteins such as AAT, CYP1A2 and CYP 3A4 (Figure 2).

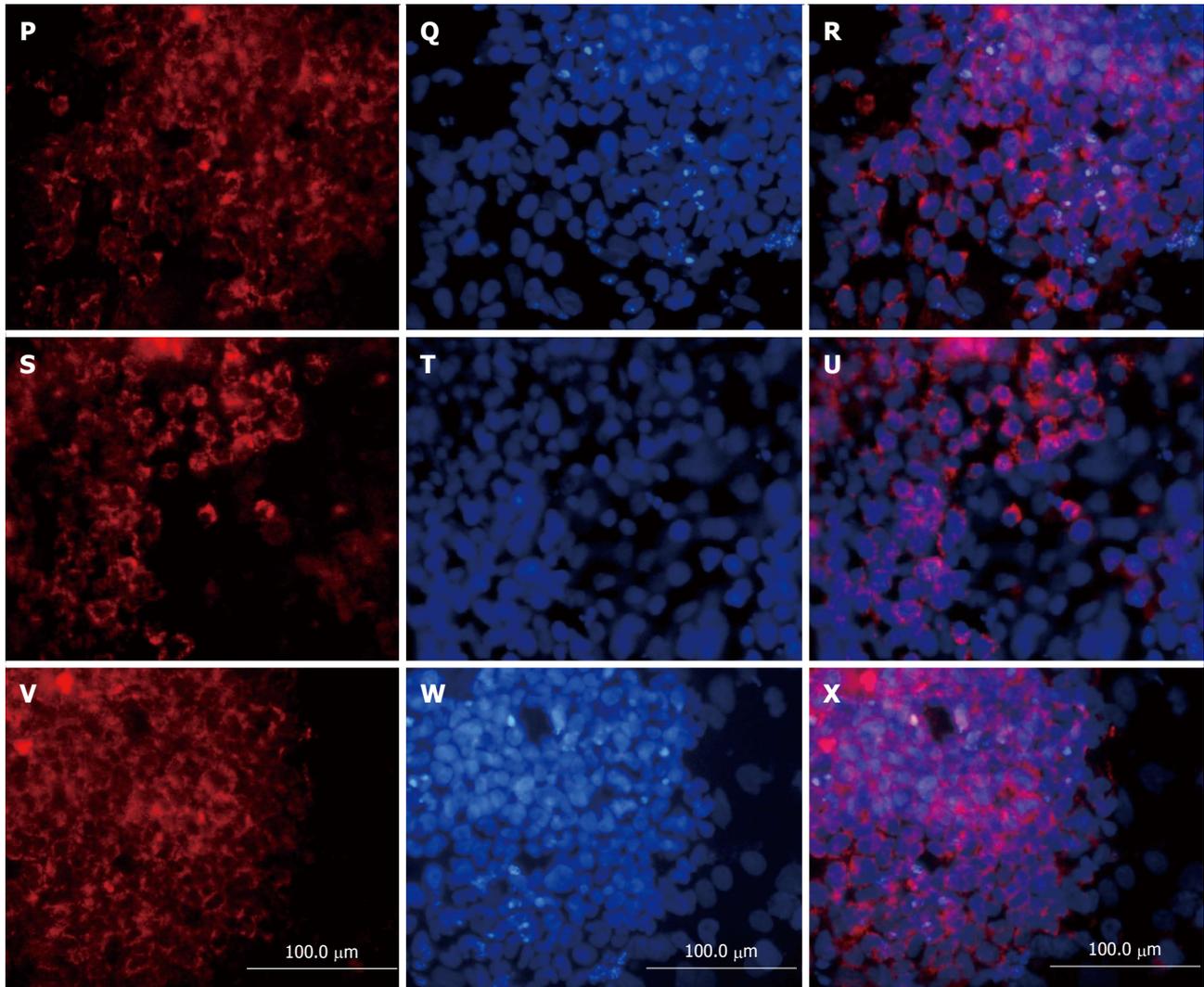
### Gene expression profiles of HLCs

To further analyze the properties of iPSCs that developed into endodermal cells and HLCs, RT-PCR analysis was performed in the cells at various stages of differentiation. After induction of endodermal differentiation, endoderm markers FOXA2 and SOX17 were detected in the cells at stage 1 (Figure 3). This was consistent with the results of the immunostaining assay. After the cells differentiated into HLCs at stage 3, RT-PCR analysis indicated the expression of liver associated genes such as AFP, ALB, CK8, CK18, CK19, and transcription factor HNF-4 $\alpha$  (Figure 3). These results collectively indicate that the cells had changed from endodermal to hepatic.

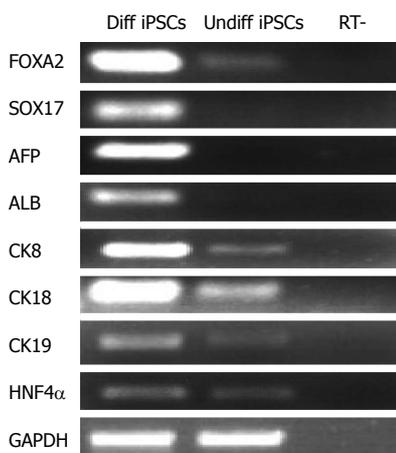
### Functional characteristics of rat iPSC-derived HLCs

In order to evaluate the liver-specific function of iPSC-derived differentiated cells, the functional properties of HLCs of urea synthesis and albumin secretion were





**Figure 2** Protein expression in differentiated induced pluripotent stem cells on days 5 and 20 as indicated by immunofluorescence staining. A, B, C: FOXA2; D, E, F: SOX17; G, H, I: AFP; J, K, L: ALB; M, N, O: CYP1A2; P, Q, R: CYP3A4; S, T, U: AAT; V, W, X: CK18. Nuclei were stained blue with DAPI. Scale bar = 100  $\mu$ m. FOXA2: Forkhead box protein A2; SOX17: SRY-box containing gene 17; AFP:  $\alpha$ -fetoprotein; ALB: Albumin; CYP1A2: Cytochrome P450 1A2; AAT:  $\alpha$ 1-antitrypsin.



**Figure 3** RT-PCR analysis of differentiated induced pluripotent stem cells on days 5 and 20. Differentiated cells expressed endodermal markers FOXA2 and SOX17 after 5 d of endoderm induction. Liver associated genes AFP, ALB, CK8, CK18, CK19, and HNF-4 $\alpha$  mRNA were expressed on day 20. H2O served as a negative control. FOXA2: Forkhead box protein A2; SOX17: SRY-box containing gene 17; AFP:  $\alpha$ -fetoprotein; ALB: Albumin; iPSCs: Induced pluripotent stem cells.

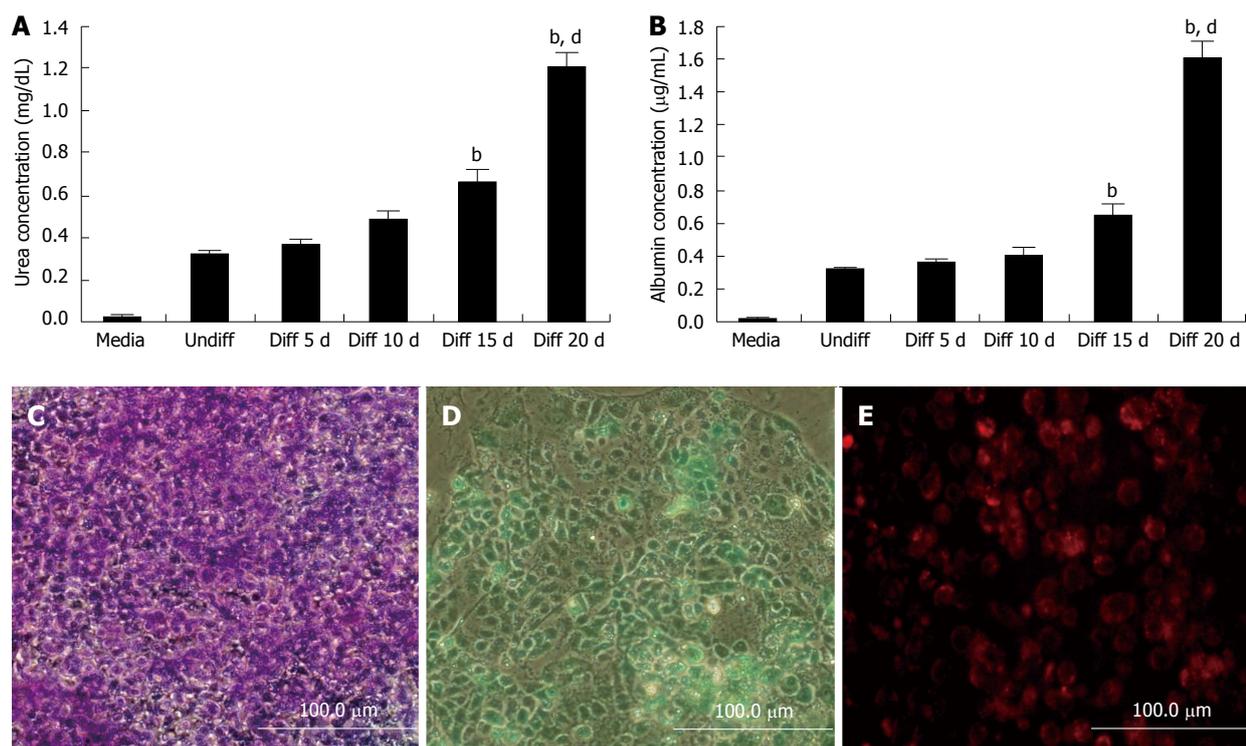
analyzed. The rates of urea synthesis (20 d  $1.202 \pm 0.080$  mg/dL vs 0 d  $0.317 \pm 0.021$  mg/dL,  $P < 0.01$ ; Figure 4A) and albumin secretion (20 d  $1.601 \pm 0.102$  mg/dL vs 0 d  $0.313 \pm 0.015$  mg/dL,  $P < 0.01$ ; Figure 4B) increased significantly as differentiation progressed, indicating hepatic maturation.

The ability of the cells to store glycogen was tested. PAS staining was used to confirm the presence of stored glycogen. Differentiated cells showed glycogen staining, indicating glycogen accumulation (Figure 4C).

The iPSCs-HLCs were also found to take up ICG and LDL (Figure 4D and E). These results showed that the iPSC-HLCs had the same function as hepatocytes *in vitro*.

## DISCUSSION

iPSCs have been used to investigate hepatocyte differentiation and possible stem cell therapies. Studies have shown that human and mouse iPSCs can be



**Figure 4** Functional analysis of differentiated induced pluripotent stem cells. A: Urea production of differentiated induced pluripotent stem cells (iPSCs); B: Albumin secretion of differentiated iPSCs; C: Glycogen synthesis of differentiated iPSCs as indicated by PAS staining on day 20; D: ICG uptake test of differentiated iPSCs; E: LDL uptake test of differentiated iPSCs. Scale bar = 100 µm. <sup>b</sup>*P* < 0.01 vs undifferentiated cells; <sup>d</sup>*P* < 0.01 vs differentiated cells on day 15.

differentiated into functional hepatocytes<sup>[20,21]</sup>. Currently, some of the studies of liver disease and possible therapeutic strategies involve animal models. Most models of liver diseases are based on rodents, especially rats. The present work is the first to demonstrate the efficient generation of HLCs differentiated from iPSCs of rat species. These differentiated cells were found to express the hepatic markers AFP, ALB, CK8, CK18, and CK19 and transcription factor HNF-4 $\alpha$ . They were also able to store glycogen, secrete urea and albumin, and take up LDL and ICG. Results showed that these differentiated cells had a gene profile similar to that of HLCs derived from human and mouse iPSCs, as reported previously<sup>[20,24]</sup>.

In the present study, a three-step protocol designed to generate HLCs from rat iPSCs was developed. The process is very simple and highly efficient. It takes two to three weeks and requires only a few inductive factors. The microenvironment in which the hepatocytes develop involves several biological events. Cell growth and differentiation are tightly regulated by a series of intra- and intercellular signals. All the inductive factors used in the process are extracellular signals<sup>[24]</sup>. As in previous studies, nodal signaling through the transforming growth factor- $\beta$  pathway was found to play a crucial role in the development of the endoderm. Activin A initiated this nodal pathway, causing the phosphorylation of Smad2 and Smad3. It also interacted with Smad4 and then activated gene-specific transcription factors in the nucleus<sup>[25]</sup>. Several

studies have demonstrated that Activin A can be used during the formation of definitive endoderm. Hay and colleagues were able to enhance the endodermal differentiation of ESCs using Wnt3a in combination with Activin A. They postulated that Activin A plus Wnt3a would induce the rapid formation of a relatively consistent hepatic endoderm and cells that are more capable of acting like hepatocytes than other combinations of inductive factors<sup>[26]</sup>. In the present differentiation system, the iPSCs quickly became spiky in shape, and the formation of endoderm was induced using Activin A combined with Wnt3a, as previously reported<sup>[22,27]</sup>.

FGFs and BMPs were found to play significant roles in liver specification. FGFs from the cardiac mesoderm and BMPs from mesenchymal cells of the septum transversum have been shown to be essential for the induction of foregut endoderm cells into hepatocytes<sup>[28]</sup>. Cai *et al.*<sup>[29]</sup> found that both FGF4 alone and BMP2 alone in the culture medium had little effect on definitive endoderm cells into liver cells, but both together led to a pronounced increase in the number of cells expressing ALB *in vitro*. They also demonstrated that the induction of definitive endoderm is essential for the effectiveness of FGF4 and BMP2 in hepatic induction<sup>[29]</sup>. Another study showed that FGF4 plus BMP2 can generate hepatic cells from human iPSCs<sup>[20]</sup>. These factors were used in the present protocol at stage 2 of differentiation. Cells were driven toward commitment to hepatic cells.

To generate mature liver cells, the iPSCs were subjected to a process meant to foster *in vitro* maturation. At this stage, cells are generally grown in media that had previously been optimized for the culture of hepatocytes with the addition of factors thought to promote the maturation or proliferation of hepatocytes during liver development. OSM has been reported to facilitate the differentiation of liver progenitor cells into hepatocytes<sup>[30]</sup>. HGF enhances hepatic maturation and proliferation<sup>[27]</sup>. Dex is a synthetic glucocorticoid hormone. It is active in induction of enzymes involved in gluconeogenesis<sup>[24]</sup>. In the present work, during the part of maturation that involves these factors, differentiated cells presented a cuboidal morphology and showed the properties of hepatic cells.

The enzymes CYP1A2 and CYP3A4 are critical to drug metabolism. The present study showed that rat iPSC-derived HLCs expressed the hepatocyte function-specific proteins CYP1A2 and CYP3A4. This suggested that these cells gain liver-specific properties and may be used in drug metabolism tests and toxicity screens.

In summary, the data collected here suggest that rat iPSCs can differentiate into HLCs in a rapid and efficient manner through a three-stage process involving the application of inductive factors. Differentiated cells may be an attractive resource for treatment of end-stage liver disease.

## COMMENTS

### Background

Recent studies have suggested that differentiated hepatocytes derived from transplanted stem cells may be a suitable form of therapy. Embryonic stem cells (ESCs) and bone marrow-derived liver stem cells can form mature hepatocytes and reduce the severity of hepatic fibrosis. However, the ethical issues of ESCs and the low purity of bone marrow stem cells limit their use in clinical settings. Induced pluripotent stem cells (iPSCs) sidestep most of the ethical issues surrounding ESCs. The differentiation of iPSCs into hepatic lineages has been observed in humans and mice. So far, no reports have been published on the differentiation of rat iPSCs into hepatocyte-like cells (HLCs).

### Research frontiers

The laboratory rat was the first mammalian species domesticated for scientific research, and the rat model is commonly used in research involving hepatic diseases. This study is the first to show the efficient generation of HLCs differentiated from the iPSCs of rats.

### Innovations and breakthroughs

Hepatic differentiation was achieved using a three-step protocol. Rat iPSCs were differentiated into HLCs after 20 d of culture. These differentiated cells expressed hepatic markers and exhibited functional hepatic characteristics.

### Applications

This study suggests that rat iPSCs can differentiate into HLCs in a rapid and efficient manner through a three-stage process involving the application of inductive factors. Differentiated cells may be an attractive resource for treatment of end-stage liver disease.

### Terminology

Induced pluripotent stem cells are a type of pluripotent stem cell that can be

generated directly from adult cells. Pluripotent stem cells hold great promise in the field of regenerative medicine. Because they can propagate indefinitely, as well as give rise to every other cell type in the body, they represent a single source of cells that could be used to replace those lost to damage or disease.

### Peer-review

The manuscript is the first to demonstrate the efficient generation of HLCs differentiated from iPSCs of rat species.

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**P- Reviewer:** Liu L   **S- Editor:** Ji FF   **L- Editor:** Wang TQ  
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ISSN 1007-9327

