Overexpression of transcription factor *Foxa2* and *Hnf1 α* induced rat bone mesenchymal stem cells into hepatocytes

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Abstract Hepatocytes differentiated from induced pluripotent stem cells and adult stem cells could be utilized as a tool for the study of liver diseases, screening for drug metabolism and hepatotoxicity. Thus further investigation of the method to efficiently generate hepatocytes is in great need. Bone Mesenchymal Stem Cells (BMSCs) were collected from rat femurs and tibias. *FOXA2* and *HNF1 α* genes were constructed into a lentiviral vector and introduced into BMSCs by a lentivirus-mediated overexpression system. Three weeks after the induction, the expressions of *FOXA2* and *HNF1 α* , and liver specific genes were analyzed, and hepatocyte-function related assays were performed. Overexpression of both *FOXA2* and *HNF1 α* induced the BMSCs to differentiate into

hepatocyte-like cells (HLCs). Hepatocyte-specific gene and protein were detected by RT-PCR, Western Blot and Immunofluorescence. These HLCs also exerted some typical hepatocyte functions such as glycogen storage, indocyanine green absorption and lipid accumulation. The combination of *FOXA2* and *HNF1 α* can effectively induce BMSCs to differentiate into HLCs. This is a novel and efficient method to prepare HLCs within a short timeline.

Keywords Bone marrow mesenchymal stem cells · Hepatocyte-like cells · *FOXA2* · *HNF1 α* · Lentivirus

Introduction

Supply of healthy hepatocytes is critical for study and treatment of liver-related diseases. At present, there are 600 million people suffering from liver diseases all over the world. Furthermore, the number of patients living with end-stage liver diseases is increasing continually and over 1 million people die from liver diseases every year (Gonzalez and Keefe 2011). Currently, orthotopic liver transplantation is the only effective treatment, but very limited clinical applications due to the number of donors as well as medical complications (Perera et al. 2009; Skvorak et al. 2009; Starzl and Fung 2010; Starzl et al. 1968; Yu et al. 2010). On the other hand, hepatocyte transplantation has been attracting great attentions worldwide due to

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its potential to relieve or overcome some of the essential problems. In vitro, primary hepatocytes cannot proliferate indefinitely and express liver-specific genes stably, which impede their usage as seed cells or target cells for drug metabolism and hepatotoxicity screening. Delightedly, the development of stem cell technology may bring a new hope to solve the shortage of liver cell source. Many researchers have discovered that stem cells can be induced into hepatocyte-like cells (HLCs) under the influence of different cytokines (Dong et al. 2010; Ghodsizadeh et al. 2010; Pauwelyn et al. 2011). As we know now that the differentiation of stem cells requires certain conditions. One of the current challenges is to define the culture conditions that can promote stem cells to differentiate into functional hepatocytes within a reasonable time period. Several transfection factors have been shown with potential to convert other type cells into hepatocytes. Huang's group used the combination of *GATA4*, *HNF4 α* and *FOXA3* to convert human fibroblasts into functional HLCs (Huang et al. 2011), and Sekiya and Suzuki confirmed the result by overexpression of *HNF4 α* and *FOXA1* or *FOXA2* or *FOXA3* (Sekiya and Suzuki 2011).

In recent years, researchers have found that mesenchymal stem cells (MSCs) can be induced to a variety of type of cells crossing three germ layers (Minguell et al. 2001). This finding has attracted great attention because it may offer an innovative approach to treat many kinds of liver diseases as well as for drug screening (Banas et al. 2007; Meirelles Lda et al. 2009; Wu et al. 2009). Of all kinds of MSCs, bone marrow mesenchymal stem cells (BMSCs) are particularly attractive because they can be isolated and cultured easily. In addition to the characteristics of stem cells for self-renewal, BMSCs can differentiate into a variety of types of cells under different culture conditions (Caplan 2007; Crisan et al. 2008; Ohishi and Schipani 2010).

FOXA2 and *HNF1 α* are vital members of the hepatocyte nuclear factor (HNF) family. They are also expressed abundantly in the liver and play an important role in cell differentiation (Kymizi et al. 2006). Based on these studies, we hypothesized that *FOXA2* and *HNF1 α* can directly induce BMSCs into functional hepatocytes. To test this hypothesis, we constructed lentiviral vector containing *FOXA2* and *HNF1 α* genes, over-expressed both *FOXA2* and

HNF1 α in rat BMSC by the method of lentivirus transduction, and obtained functional HLCs. Our results showed that the combination of *FOXA2* and *HNF1 α* could sufficiently induce rat BMSCs into functional HLCs. These HLCs were morphologically and functionally similar to rat hepatocytes, expressing the markers of hepatocytes and displaying numerous hallmark functions of hepatocytes (Willenbring 2011). Moreover, these cells were stable and expandable in culture. Thus, our finding will provide a novel and effective way to generate functional HLCs from BMSCs.

Materials and methodology

Isolation and culture BMSCs

BMSCs were isolation according to Friedenstein's method (Friedenstein et al. 1987). To harvest bone marrow, the femurs were aseptically dissected from rats (6–8 weeks). Briefly, the femurs were washed with PBS solutions and the ends of the femurs were cut open. The whole bone marrow was extruded with 10 ml Dulbecco's modified Eagle's medium–low glucose (DMEM-LG, Gibco BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (GIBCO BRL) and 1 % antibioticpenicillin/streptomycin (Hyclone, Logan, UT, USA) solution. The collected bone marrow cells were incubated at 37°C with 5 % CO₂. The medium was replaced twice a week. When the adherent cells reached approximately 70–80 % of the culture plate, they were detached with 0.125 % trypsin–EDTA (Gibco, NY, USA) and the cells were subcultured at 1:3 under the same culture conditions.

Plasmid constructs and lentivirus production

Coding sequences of rat *FOXA2* (NCBI RefSeq: NM_012743.1) and *HNF1 α* (NCBI RefSeq: NM_012669.1) were synthesized and inserted into the multiple cloning site (MCS) of the lentiviral vector pLVX-IRES-mCherry by Sangon Biotech (Shanghai, China). Vector particles were produced in HEK293T cells by transient cotransfection involving a three-plasmid expression system. Briefly, plasmid DNA was transfected into HEK293T cells together with helper plasmid pCMV Δ 8.91 and pVSV-G via the method of

calcium phosphate transfection (Kingston et al. 2003). After 12 h of incubation, the medium was replaced, virus particles were collected after 48 h, passed through a 0.45 µm filter, and concentrated by centrifugation at 25,000 rpm (15 °C) for 2 h (Bowles et al. 1996). The concentrated virus particles were suspended in PBS and stored at −80 °C.

Transduction of BMSCs

Transduction was performed in 24-well plates. BMSCs were seeded at 1×10^5 cells per well. One day later, the cells were transduced with 2×10^5 TU virus particles of both *FOXA2* and *HNF1α* for 8 h and the viral infection was serially repeated 2–3 times. Three days after the last round of transduction, the efficiency was measured by detecting the mCherry fluorescent protein using Fluorescence microscope. After 1 or 2 weeks, transduced cells in clusters were partially digested and seeded into new dishes to continue their culture.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RNA was extracted from the transduced cells using Total RNA Kit (Omega Bio-Tek, Doraville, GA, USA) according to the manufacturer's protocol. cDNA was synthesized from total RNA as described in the instructions of Reverse Transcription System (Promega, Madison, WI, USA). cDNA was amplified by recombinant Taq DNA polymerase (TaKaRa, Dalian, China) with the following specific primers: *HNF4a*, *FOXA3*, *ALB*, *AFP*, *G6P*, *TAT* and *TTR*, which were chose as the marker genes of hepatocytes. PCR was performed under the following conditions: denaturing for 3 min at 95 °C, followed by 32 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and a final extension for 5 min at 72 °C. The primer for *β-actin* gene was included as an internal control. At the same time, BRL-3A line derived from buffalo rat liver was obtained from cell bank of the School of Basic Medicine of Peking Union Medical College (Beijing, China), and was used as control (Table 1).

Western blot analysis

Western blot analysis was performed according to Towbin's method (Towbin et al. 1979). Cellular

protein was separated by SDS-PAGE and transferred to immobilon-P membrane. After the transfer, the membranes were blocked with 5 % non-fat milk in 0.1 % Tween 20 at room temperature for 1 h and incubated with the indicated specific primary antibodies, respectively: mouse anti-β-Actin (1:1000 dilution, Sigma, St. Louis, Mo., USA), goat anti-ALB (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-CK18 (1:1000 dilution, Santa Cruz Biotechnology), goat anti-G6P (1:1000 dilution, Santa Cruz Biotechnology), rabbit anti-TTR (1:1000 dilution, Bioss, Beijing, China), mouse anti-TAT (1:1000 dilution, Santa Cruz, Calif, USA), rabbit anti-AFP (1:1000 dilution, Bioss, Beijing, China) at 4 °C overnight. After washing with TBS-T for 30 min at room temperature, the membrane was further incubated with ALP-conjugated secondary antibodies (1:2000 dilution) including HRP-conjugated goat anti-mouse IgG (Zhongshan Biotechnology, Beijing, China), HRP-conjugated rabbit anti-goat IgG (Zhongshan Biotechnology, Beijing, China), HRP-conjugated goat anti-rabbit IgG (Zhongshan Biotechnology, Beijing, China),

Immunocytochemistry

Cells were cultured on the cover slips until confluence, fixed with 4 % paraformaldehyde for 1 h, and incubated with 0.1 % Triton for 15 min. Endogenous peroxidase was quenched with 0.3 % hydrogen peroxide solution. After being blocked with 3 % BSA in PBS at room temperature for 1 h, the slips were incubated with primary antibodies: rabbit anti-CD44 (1:100 dilution, Boster, Wuhan, China), rabbit anti-CD105 (1:100 dilution, Boster, Wuhan, China), goat anti-ALB (1:100 dilution, Santa Cruz Biotechnology), mouse anti-CK18 (1:100 dilution, Santa Cruz Biotechnology), goat anti-G6P (1:100 dilution, Santa Cruz Biotechnology), rabbit anti-TTR (1:100 dilution, Bioss, Beijing, China), mouse anti-TAT (1:100 dilution, Santa Cruz Biotechnology), rabbit anti-AFP (1:100 dilution, Bioss, Beijing, China) at 4 °C overnight, and then incubated with secondary antibody including FITC-conjugated goat anti-mouse IgG (1:200 dilution, Boster, Wuhan, China), FITC-conjugated rabbit anti-goat IgG (1:200 dilution, Boster, Wuhan, China), FITC-conjugated goat anti-rabbit IgG (1:200 dilution, Boster, Wuhan, China) and CY3-conjugated goat anti-rabbit IgG (1:200 dilution,

Table 1 Primers used in this study

	Primers primer sequences	Length
<i>CD44</i>		
Forward	5'-CAAGTGGGAATCAAGACAGTGG-3'	127 bp
Reverse	5'-GCAATGCAGACGGCAAGAATCAG-3'	
<i>CD105</i>		
Forward	5'-TCGGTTGTGATCTACAGCGTG-3'	274 bp
Reverse	5'-ACCAAGTGCAGTGGGATTTCT-3'	
<i>β-actin</i>		
Forward	5'-ACATCCGTAAAGACCTCTATGCCAACA-3'	109 bp
Reverse	5'-GTG CTA GGA GCC AGG GCA GTA ATC T-3'	
<i>Ex-hnf1α</i>		
Forward	5'-TAGAGGATCTATTTCCGGTGAATTC-3'	320 bp
Reverse	5'-AAGTGACTCCACCACGGCTTTC-3'	
<i>Ex-foxa2</i>		
Forward	5'-TAGAGGATCTATTTCCGGTGAATTC-3'	400 bp
Reverse	5'-CTCATGGAGTTCATATTGGCGTA-3'	
<i>Hnf4α</i>		
Forward	5'-CAGTGCGTGGTAGACAAAGATAAG-3'	94 bp
Reverse	5'-TTGGACGGCTTCTTTCTTCATG-3'	
<i>Foxa3</i>		
Forward	5'-GCTGGGCTCAGTGAAGATGG-3'	144 bp
Reverse	5'-GGAGAGCTAAGAGGGTTCAAGG-3'	
<i>ALB</i>		
Forward	5'-TCTGCACACTCCCAGACAAG-3'	114 bp
Reverse	5'-AGTCACCCATCACCGTCTTC-3'	
<i>G6P</i>		
Forward	5'-GATTCCGGTGCTTGAATGTC-3'	201 bp
Reverse	5'-AGGTGATGAGACAGTACCTC-3'	
<i>CK18</i>		
Forward	5'-ACAAGTACTGGTCTCAGCAG-3'	405 bp
Reverse	5'-GCATGGAGTTGCTGGAGTC-3'	
<i>TAT</i>		
Forward	5'-TGTCATGAGGCTCCTCTGGAA-3'	105 bp
Reverse	5'-GATGTTTTGTCCAGGATTGGC-3'	
<i>TTR</i>		
Forward	5'-GTAGTCACCACCAAGTCTG-3'	126 bp
Reverse	5'-GTTCTCCAAGTTGATGTTCTG-3'	
<i>AFP</i>		
Forward	5'-ACCTGACAGGGAAGATGGTG-3'	225 bp
Reverse	5'-GCAGTGTTGATACCGGAGT-3'	
<i>CYP1a1</i>		
Forward	5'-CCAAACGAGTTCCGGCCT-3'	91 bp
Reverse	5'-TGCCCAAACCAAAGAGAATGA-3'	
<i>CYP2b1</i>		
Forward	5'-GTCAGGGGACACCCAAAGTC-3'	123 bp
Reverse	5'-TTCTCGAAGCTGCATGAAGGAA-3'	

Boster, Wuhan, China) at room temperature for 45 min, and stained with DAPI (Sigma Chemical Co., St. Louis, MO, USA).

Periodic acid schiff (PAS) staining

In order to determine glycogen synthesis and storage capacity, cells were stained by periodic acid-Schiff (PAS, Senbeijia, Nanjing, China). Cells cultured in dishes were fixed in 4 % formaldehyde for 1 h, oxidized with 1 % periodic acid at room temperature for 40 min followed by 3 rinses with distilled water, then treated with Schiff's reagent for 15 min, and rinsed in distilled water for 5 min. Finally, the samples were counterstained with Mayer's hematoxylin and examined under light microscope

Indocyanine green (ICG) cellular uptake

In order to examine the drug absorption capacity of the transduced cells, the cells were cultured in the medium containing 1 mg/ml of ICG (Jiake, shanghai, China) for 1 h at 37 °C with 5 % CO₂, washed with PBS, and assessed under light microscope.

Oil red O staining

To test the lipid accumulation ability of the transduced cells, confluent cells were fixed with 4 % formaldehyde at room temperature for 1 h, then stained with oil red-O (Sigma Chemical Co., St. Louis, MO, USA) for 10 min, and washed with PBS. The result was recorded under light microscopic.

Results

Characterization of BMSCs

The initial isolated MSCs from rat bone marrow were heterogeneous cells and contained many hematopoietic cells, round or fibroblastic cells. In the third week of culture, we could observe the cells exhibiting a spindle-shape morphology with a unique vortex arrangement at 90 % confluence (Fig. 1a, b), and their nuclei were regular oval, large and obvious (Fig. 1c). Compared with the isolation method of FACS, this method has the advantage of reduced cell damage and cost, and the purity of the isolated cells

also meets the requirements, consistent with the results of Poliseti et al. (2010). RT-PCR analysis revealed that these cells presented strong positivity for CD44 and CD105 compared to the cells of primary culture, it indicating that BMSCs were purified, consistent with the studies of Kern et al. (2006). Besides, the results of immunofluorescence assays showed that the BMSCs purity was more than 95 % (Fig. 1d).

Effect of FOXA2 and HNF1 α on BMSCs morphology

After infection of the lentivirus carrying *ex-FOXA2* and *ex-HNF1 α* for 48 h, the BMSCs showed red fluorescent protein (Fig. 2a, b). Afterwards, due to the damage and toxicity of lentivirus to target cells, most cells were dead and the morphology of mCherry fluorescent positive cells was converted to epithelial-like cells (Fig. 2c, d). These transduced cells were selected by partial digesting and transferred to new dishes for subculture. The morphology of infection cells became triangular, polygonal or irregular epithelioid shape, and the size was smaller compared to parental BMSCs (Fig. 2e, f). In addition, we observed that some cells had 1–3 that some cells had 1–3 nucleoli. Those changes were similar with the observation by Sekiya and Suzuki (2011). The positive cells were isolated by partially digestion and seeded on 96-well plates, we tried to ensure that only one cell per well was seeded. Within 2 weeks after reseeding, we try to ensure that only one cell per well. Within 2 weeks after reseeding, we chose the clone which successfully maintained the mCherry fluorescent protein and had a high proliferation rate to detect the exogenous genes by RT-PCR. The results confirmed that those cells expressed the exogenous genes *FOXA2* and *HNF1 α* (Fig. 2g).

Confirmation of HLCs by hepatocyte markers

RT-PCR, Western blotting and immunofluorescence were used to determine whether the induced BMSCs expressed the markers of hepatocytes. Two weeks after induction, the results of RT-PCR revealed that these converted epithelial-like cells had the same characteristics as hepatocytes (Fig. 3a). Meanwhile, we found the expression of HNF4a, FOXA3, ALB, AFP, G6P, TAT and TTR as indicated by Western blotting (Fig. 3b), and the corresponding expression at

Fig. 1 Characterization of isolated BMSCs. **a** Cell morphology of the isolated BMSCs before passages. **b**, **c** Cell morphology of the isolated BMSCs on the third passage. **d** Expression of MSC related antigens CD44 and CD105 was confirmed by immunofluorescent staining. Red fluorescence stained with CY3-conjugated secondary antibody and nuclei were stained blue by DAPI. **e** Detection of *Cd44* and *Cd105* in BMSCs. (Color figure online)

the transcriptional level had been in accordance with the above results. Furthermore, we tested the hepatic proteins including ALB, G6P, TAT, TTR, CK18 and AFP by immunofluorescence, which indicated that more than 95 % the transduced cells expressed those hepatic proteins (Fig. 3c).

Functional analysis of HLCs

To assess the functional capacity of the induced cells, we used the Periodic Acid-Schiff (PAS) stain to detect the capacity of carbohydrate storage, and the result showed that, after 20 days of induction, red droplets was found deposited in the cytoplasm (Fig. 4b), indicating that these cells were able to produce and store glycogen. ICG stain was used to examine the drug absorption capacity of the induced cells (Fig. 4d), which showed that the most of cells had the ability of ICG uptake at 37 °C after an incubation

for 1 h in the medium with 1 mg/ml ICG. The oil red-O staining was utilized to detect lipids, and the results revealed that lipids were accumulated in some induced cells (Fig. 4f). On the other hand, non-induced BMSCs had no signs of glycogen storage, ICG uptake and lipid accumulation in the cytoplasm.

Discussion

A method to differentiate MSCs into HLCs plays a vital role for studying liver diseases, drug screening, and ultimately clinical application. Although a variety of methods have been reported to generate HLCs, they have own limitations, such as a great amount of time for inducing and immaturity. Nevertheless, trans-differentiating somatic cell directly into target cells was considered as the most simple and effective method. In 2014, Huang's group converted human fibroblasts into functional induced hepatocyte-like

Fig. 2 Morphological characterizations and exogenous genes detection of transduced BMSCs. **a, b** BMSCs transduced by the lentivirus. **c, d** BMSCs converted to epithelial cells. **e,**

f Transduced cells were partially digested and subcultured. **g** Expression of exogenous genes was analyzed by RT-PCR. The cells with red fluorescence were $Foxa2^{+}$ and $Hnf1\alpha^{+}$ positive

(iHep) cells by the combination of *FOXA3*, *HNF1 α* and *HNF4 α* (Huang et al. 2014). However, these iHep could not proliferation well, and SV40 large T antigen must be transferred into these cells to enhance their proliferation capacity, which reminded us to induce stem cells to functional iHep cells directly. Moreover,

the adult stem cell BMSCs had the characteristics of widely available high proliferation rate, and avoided the ethical issue compared with embryonic stem cells (ESCs).

Many researches have shown that *HNF4 α* , *FOXA1*, *FOXA2* and *FOXA3* are powerful factors which can

Fig. 3 Analysis of proliferative transduced BMSCs (marked as T-BMSC for short). **a** Detection of hepatic genes expression in the transduced cells, BRL-3A and BMSCs by RT-PCR. **b** Detection of hepatic proteins in T-BMSC, BRL-3A and BMSCs by Western blotting. **c** The expressions of ALB, G6P, CK18, TAT, TTR, KRT19 and AFP in transduced BMSCs by immunofluorescent staining

convert other types of cells into iHep cells (Takayama et al. 2012a). Although *SOX17*, *HEX*, *HNF4 α* can convert other types of cells into HLCs (Takayama et al. 2012b), the further differentiation and maturation of these HLCs still need other growth factors, such as Activin A, FGF, HGF, DEX, OSM and so on. It has been shown that *FOXA2* transduction was the most efficient way to promote dedifferentiation (Gragnoli et al. 1997). Although in the DE stage *HNF4 α* had a stronger effect than *HNF1 α* , *HNF1 α* was one of the

target genes of *HNF4 α* , which has been shown to have an obvious effect on the hepatic maturation. Thus, hepatic commitment and maturation are strongly promoted by the combination of *FOXA2* and *HNF1 α* transduction in the stage of hepatic commitment, expansion and maturation (Ang et al. 1993). Furthermore, *FOXA2* and *HNF1 α* were initially detected in DE differentiation during embryonic development, and their expressions are elevated during hepatic maturation (Klocke et al. 2002). Therefore, to mimic

Fig. 4 Functional characterization of transduced BMSCs (marked as T-BMSC for short). **a**, **b** Detection of Glycogen storage by PAS staining. **c**, **d** Detection of ICG uptake (green staining). **e**, **f** Detection of neutral triglycerides and lipids by oil red-O staining. BMSCs were used as negative control. (Color figure online)

the condition similar to embryonic liver development, we used the combination of *FOXA2* and *HNF1 α* to induce BMSCs into HLCs.

Interestingly, we found that the combination of *FOXA2* and *HNF1 α* transduction could convert BMSCs into hepatocytes efficiently. In addition, the T-BMSC not only expressed the specific marker genes of hepatocytes such as *ALB*, *AFP*, *G6P*, *CK18*, *TAT*, and *TTR*, but also had the capacity of glycogen storage, absorption of ICG and lipid accumulation. These results are consistent with the studies of Yamada and Mehlem (Mehlem et al. 2013; Yamada et al. 2002). Furthermore, we found that the T-BMSC by the combination of *FOXA2* and *HNF1 α* transduction had an elevated expression level of CYP enzymes in comparison to BRL-3A. This indicated that

T-BMSC might play a role in/be involved in drug metabolism. At the same time, the T-BMSC had a strong capacity of proliferation, and they were still alive after more than 20 passages. This was similar with immortalized hepatocytes as reported by Klocke et al. (2002). However, the specific molecular mechanism of this conversion was still unclear and needed further investigation.

In summary, the combination of *FOXA2* and *HNF1 α* transfection was sufficient to convert cultured BMSCs into functional HLCs. Compared with the published reports, our method has greatly simplified the preparation of HLCs with a high efficiency and short timeline. Furthermore, we provided a new source of liver seed cells that might be used in future basic and clinical studies.

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