

## HNF-4 $\alpha$ determines hepatic differentiation of human mesenchymal stem cells from bone marrow

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### Abstract

**AIM:** To investigate the differentiation status and key factors to facilitate hepatic differentiation of human bone-marrow-derived mesenchymal stem cells (MSCs).

**METHODS:** Human MSCs derived from bone marrow were induced into hepatocyte-like cells following a previously published protocol. The differentiation status of the hepatocyte-like cells was compared with various human hepatoma cell lines. Overexpression of hepatocyte nuclear factor (HNF)-4 $\alpha$  was mediated by adenovirus infection of these hepatocyte-like cells. The expression of interesting genes was then examined by either re-

verse transcription-polymerase chain reaction (RT-PCR) or real-time RT-PCR methods.

**RESULTS:** Our results demonstrated that the differentiation status of hepatocyte-like cells induced from human MSCs was relatively similar to poorly differentiated human hepatoma cell lines. Interestingly, the HNF-4 isoform in induced MSCs and poorly differentiated human hepatoma cell lines was identified as HNF-4 $\gamma$  instead of HNF-4 $\alpha$ . Overexpression of HNF-4 $\alpha$  in induced MSCs significantly enhanced the expression level of hepatic-specific genes, liver-enriched transcription factors, and cytochrome P450 (P450) genes.

**CONCLUSION:** Overexpression of HNF-4 $\alpha$  improves the hepatic differentiation of human MSCs from bone marrow and is a simple way of providing better cell sources for clinical applications.

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**Key words:** Bone marrow; Cytochrome P450 genes; Differentiation of hepatocyte; Hepatocyte nuclear factor 4; Human mesenchymal stem cells

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## INTRODUCTION

The liver is an important organ and performs many biological functions, such as plasma protein synthesis, glucose and fatty acid metabolism, and detoxification. The standard treatment for irreversible liver failure has been focused on liver transplantation; however, the availability of donor tissues is limited and enables only 10% of candidate patients to receive transplants. Recently, hepatocyte transplantation or bioartificial livers have become promising alternatives for treatment<sup>[1,2]</sup>, but the development of cell-based therapies is hindered by the propagation of hepatocytes and the maintenance of their hepatic functions *in vitro*. Therefore, it would be of great benefit to develop *in vitro* models of hepatocyte differentiation to facilitate future clinical applications.

Recently, success in driving stem cells into hepatic lineage has provided great hope for overcoming the limitation of cell sources for hepatocyte transplantation<sup>[3-6]</sup>. Previous studies have demonstrated that reservoirs of stem cells may reside in several types of adult and fetal tissues, including liver stem cells as a hepatic source and embryonic stem cells, bone marrow cells, and umbilical cord blood cells as a non-hepatic source. Most of these cells can be induced toward hepatic differentiation and to exhibit hepatic functions in both *in vivo* and *in vitro* systems<sup>[7-9]</sup>. Among them, the study of hepatic differentiation of bone marrow cells is most attractive because the autologous stem cells can be easily isolated, expanded extensively, and induced into hepatic differentiation for transplantation back into the patient. It has been reported that mesenchymal stem cells (MSCs) derived from bone marrow have the potential to differentiate into cells of mesodermal lineage, such as osteoblasts, chondrocytes, and adipocytes, as well as into various types of cells of other lineages, including neural and liver cells<sup>[10]</sup>. Furthermore, the hepatic differentiation of MSCs *in vivo* has been established in rat and mouse models<sup>[11-13]</sup>. These observations bring new hope for the possible application of cell-based therapy in severe liver diseases. However, the hepatic differentiation status of hepatocyte-like cells derived from stem cells is not sufficient for clinical use because the relatively low expression levels of drug metabolizing enzymes and their metabolic activities are not fully induced<sup>[14]</sup>. Therefore, it is important to develop a simple strategy for the efficient induction of hepatic differentiation.

The coordinated expression of various liver-specific genes is required for hepatic differentiation and the biological functions of adult liver. Previous studies have demonstrated that most hepatic gene expression is regulated primarily through the combinational action of several liver-enriched transcription factors<sup>[15-17]</sup>. Among them, hepatocyte nuclear factor (HNF)-4 $\alpha$  plays a crucial role in the liver-specific phenotype through induction of various liver-specific functions<sup>[18]</sup>. Several studies have demonstrated that HNF-4 $\alpha$  may act as a master gene in a transcription factor cascade that could drive hepatic differentiation<sup>[19,20]</sup>. These studies suggest that high expression of HNF-4 $\alpha$

may be a simple strategy for the induction of hepatic differentiation and the functions of hepatocyte-like cells derived from stem cells in the cell culture system.

In this study, we demonstrated that human bone marrow MSCs can be differentiated into hepatocyte-like cells according to the procedure established by Lee *et al.*<sup>[3]</sup>. Furthermore, overexpression of a single liver-enriched transcription factor, HNF-4 $\alpha$ , could significantly improve the differentiation status of hepatocyte-like cells through activation of several target genes. Apparently, these more differentiated hepatocyte-like cells will provide a better cell source for future clinical applications and *in vitro* hepatotoxicity models for drug screening.

## MATERIALS AND METHODS

### Propagation of human bone marrow MSCs

Human bone marrow MSCs were isolated and characterized by Lee *et al.*<sup>[3]</sup>. Briefly, human bone marrow was collected from healthy donors with informed consent and approved by the institutional review board of the Taipei Veterans General Hospital. Mononuclear cells were obtained by negative immunodepletion of CD3, CD14, CD19, CD38, CD66B, and glycophorin-A positive cells using a commercially available kit (RosetteSep, StemCell Technologies) according to the manufacturer's instructions, followed by Ficoll-Paque density-gradient centrifugation (1.077 g/cm<sup>3</sup>), and plated in tissue culture plates in expansion medium. The expansion medium consisted of Iscove's modified Dulbecco's medium (IMDM) (Gibco) and 10% fetal bovine serum (Hyclone) supplemented with 10 ng/mL epidermal growth factor (EGF) (Becton Dickinson) and 10 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems). The adherent cells were amplified within the expansion medium until hepatic induction.

### Hepatic differentiation of human MSCs

The hepatic induction of human bone marrow MSCs was performed according to the protocol developed by Lee *et al.*<sup>[3]</sup>. In brief, 5th- to 13th-passage stem cells (at  $1.0$  to  $1.3 \times 10^4$ /cm<sup>2</sup>) were serum deprived for 2 d in IMDM supplemented with 20 ng/mL EGF and 10 ng/mL bFGF prior to induction using a two-step protocol. Differentiation was induced by treating MSCs with step-1 differentiation medium, consisting of IMDM supplemented with 20 ng/mL hepatocyte growth factor (R&D Systems), 10 ng/mL bFGF and 0.61 g/L nicotinamide for 7 d, followed by treatment with step-2 maturation medium, consisting of IMDM supplemented with 20 ng/mL oncostatin M (R&D Systems), 1  $\mu$ mol/L dexamethasone (Sigma), and 50 mg/mL ITS<sup>+</sup> premix (Becton Dickinson). Medium changes were performed twice weekly.

### Cell culture

Human hepatoma cell lines HepG2<sup>[21]</sup>, HA22T/VGH<sup>[22]</sup> and SK-Hep-1<sup>[23]</sup> were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal calf serum (Gibco) as described previously<sup>[24]</sup>.

Table 1 Primer sets used for reverse transcription-polymerase chain reaction analysis

Gene	Forward primer	Reverse primer	Product size (bp)
Alb	TGCTTGAATGTGCTGATGACAGGG	AAGGCAAGTCAGGGCATCTCATC	161
AFP	TGCAGCCAAAGTGAAGAGGGAAGA	CATAGCGAGCAGCCCAAAGAAGAA	216
TAT	TGAGCAGTCTGTCCACTGCCT	ATGTGAATGAGGAGGATCTGAG	358
G6P	GCTGGAGTCCTGTCAGGCATTGC	TAGAGCTGAGGCGGAATGGGAG	350
TO	ATACAGAGACTTCAGGGAGC	TGGTTGGGTTTCATCTTCGGTATC	299
HNF-1 $\alpha$	GTGTCTACAACCTGGTTTGCC	TGTAGACACTGTCACTAAGG	251
HNF-3 $\beta$	CACCACTACGCCTTCAACC	GGTAGTAGGAGGTATCTGCGG	235
HNF-4	CTGCTCGGAGCCACAAAGAGATCCATG	ATCATCTGCCACGTGATGCTCTGCA	371
C/EBP $\alpha$	CAAGAAAGTCGGTGGACAAGAAC	CCTCATCTTAGACGCACCAAGT	450
$\beta$ 2M	TGACGTGTGAACCATG	TGGAGACAGCACTCAAAG	242

The thermo-cycling parameters for  $\beta$ 2M: 94°C for 5 min/28 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s/72°C for 10 min; The thermo-cycling parameters for other genes: 94°C for 5 min/38 cycles of 94°C for 30 s, 56–58°C for 30 s, and 72°C for 30 s/72°C for 10 min. Alb: Albumin; AFP:  $\alpha$ -fetoprotein; TAT: Tyrosine-aminotransferase; TO: Tryptophan 2,3-dioxygenase; G6P: Glucose 6-phosphatase;  $\beta$ 2M:  $\beta$ -2-microglobulin.

Table 2 Primer sets for real-time reverse transcription-polymerase chain reaction analysis

Gene	Forward primer	Reverse primer	Probe
HNF-1 $\alpha$	TGAGTCCGGGCTTCACAC	GGCTGCTGGAGGACACTG	42
HNF-3 $\beta$	GCACCTGCAGATTCTGATTTT	GACTTCCCTGCAACAACAGC	25
HNF-4 $\alpha$	ATTGACAACCTGTGCGAGGA	CGTGGTCCCATATGTTCC	77
HNF-4 $\gamma$	CTTGGTGGAATGGGCTAAA	AGCTCTCAACAGTGCCACCT	8
HNF-6	CCTGGAGCAAACCTCAAATCC	TCTTTCTTTTGCATGCTG	88
C/EBP $\alpha$	CAACACTTGTATCTGGCCTCTG	CGAGCAAAACCAAAACAAAAC	3
PPAR $\alpha$	GTCCCCGCAGATTCTACATT	GAAGCTGTCTGGCTCAGAT	14
Alb	TGTTGCCAAGCTGCTGATA	CCTTCATCCCGAAGTTCATC	27
TAT	TGCTGAGCAGTCTATCCACT	CTGCTCAGAACTCCTGGAT	67
G6P	GCTGCTCATTTTCTCATCAA	TCTGTAAACAGCAATGCCTGA	67
CYP1A1	GACAGATCCCATCTGCCCTA	CAAACTTGTCTCTTGTGTGTC	80
CYP1A2	CAAGAAATGCTGTGTCTTCGTAA	AGAGGGGTCTCCACAG	59
CYP2A6	CAAAAAGGACACCAAGTTTCG	AGAGCCCAGCATAGGGTACA	69
CYP2B6	GAAGCTTTTATCCCTTCTCCT	GCCATGGAGAAGTTCTGGAG	35
CYP2C8	AAGAAAAGTGACTACTTCATGCCTTT	CAAGTCCTTCTCTGCACAA	18
CYP2C9	ATTGACCTTCTCCCCACCA	CAGGGAATTAATATGGTTGTGC	43
CYP2C19	GTCCAGAGATACATCGACCTCA	AGTGAGGGAAGTTAATATGGTTGTG	43
CYP2D6	AGGAGGAGTCGGGCTTCT	CGCTGGGATATGCAGGAG	56
CYP2E1	CAAGCCATTTTCCACAGGA	CAACAAAAGAAACAACCTCCATGC	67
HPRT	AGGTGTCAGCGAGGAAAAA	AGCCACCCTGGAAGAAACTT	59

The thermo-cycling parameters for real-time reverse transcription-polymerase chain reaction: 95°C for 10 min/50 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s/40°C for 30 s. Alb: Albumin; AFP:  $\alpha$ -fetoprotein; TAT: Tyrosine-aminotransferase; G6P: Glucose 6-phosphatase; PPAR $\alpha$ : Peroxisome proliferator-activated receptor  $\alpha$ ; HPRT: Hypoxanthine-guanine phosphoribosyltransferase.

### Immunofluorescence

To stain for albumin, cells were fixed with 4% paraformaldehyde at 4°C, and permeabilized with 0.1% Triton X-100 for 15 min. The slides were incubated with mouse monoclonal antibodies against human albumin (Santa Cruz) (1:50 dilution) for 1 h, followed by fluorescein-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) (1:100 dilution) for 1 h. Hoechst stain was used for labeling DNA. Between incubations, samples were washed with phosphate-buffered saline (PBS).

### Uptake of low-density lipoprotein

The Dil-Ac-low-density lipoprotein (LDL) staining kit was purchased from Biomedical Technologies and the assay was performed following the manufacturer's instructions.

### Reverse transcription-polymerase chain reaction and real-time reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from induced MSCs using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA templates were obtained by reverse transcription of total RNA with oligo(dT) 18-mers and Superscript II reverse transcriptase (Invitrogen). The products were then subjected to either reverse transcription-polymerase chain reaction (RT-PCR) analysis with the specific primer pairs and conditions<sup>[3]</sup> listed in Table 1 or quantitative real-time RT-PCR analysis with the specific primer pairs and Taqman probes listed in Table 2 (according to the instruction of the Assay Design Center, Universal ProbeLibrary System, Roche Applied Science).

### Recombinant adenoviruses

The HNF-4 $\alpha$  expression vector (Ad/HNF4 $\alpha$ -IRES-EGFP) contained the rat HNF-4 $\alpha$  cDNA fragment (kindly provided by Dr. Ou JH)<sup>[25]</sup> in the parental adenovirus vector (Ad-IRES-EGFP) which was constructed based on the Adeno-X expression system (Clontech) containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus and EGFP gene. The adenoviruses were packaged and amplified in 293 cells and then purified by banding on CsCl gradients. The titer of recombinant adenoviruses was estimated according to the amount of viral genome. The 8w-induced MSCs were infected with purified adenoviruses at a 1500 multiplicity of infection (m.o.i.) for 1 h in serum-free medium. The overexpression of rHNF-4 $\alpha$  was confirmed by real-time RT-PCR (forward primer: CAAGAGGTCCATGGTGTTC; reverse primer: CCGAGGGACGATGTAGTCA; Taqman probe: #68). For the induction of P450 genes, 50  $\mu$ mol/L of B-naphthoflavone or rifampicin (Sigma) was added to the culture medium for 2 d.

### Statistical analysis

Statistical significance for the induction effect was determined by *t*-test. Differences were considered significant if the *P* value was less than 0.05.

## RESULTS

### The differentiation status of hepatocyte-like cells induced from human bone marrow MSCs

Human MSCs were isolated from bone marrow, expanded in growth medium, and differentiated into hepatic lineage according to the protocols previously described by Lee *et al.*<sup>[3]</sup> Under hepatic induction conditions, the fibroblastic morphology of MSCs (Figure 1A, 0 wk and 1 wk) gradually proceeded toward the polygonal shape of hepatocytes with the appearance of abundant granules in the cytoplasm (Figure 1A, 4 wk, 7 wk, and 10 wk). Interestingly, compared with human hepatoma cell lines of different differentiation status-HepG2, HA22T/VGH, and SK-Hep-1 cells; the morphology of induced MSCs at an early stage was similar to that of spindle-shaped SK-Hep-1 and HA22T/VGH cells. After 7 wk of induction, the morphology of MSCs gradually approached that of HepG2 cells (Figure 1B). These observations suggest that the induction of the differentiation process of human MSCs may have some similarities to human hepatoma cell lines with different stages of differentiation.

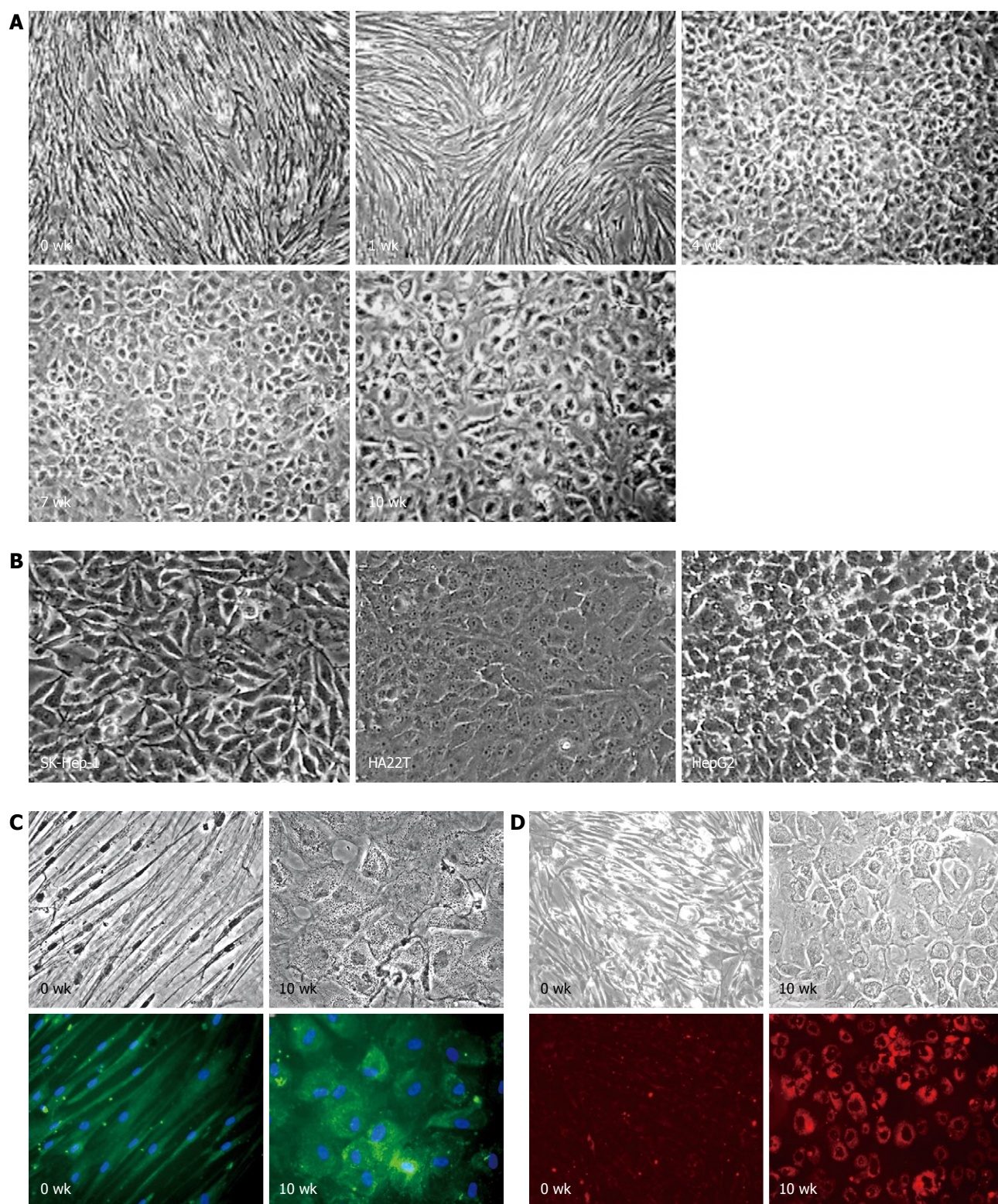
Beside the morphological differences, we also evaluated biological properties to characterize the hepatic maturation of induced MSCs including uptake of low-density lipoprotein (LDL) and albumin expression. After 10 wk of induction, most of the induced MSCs exhibited positive signals for albumin expression (Figure 1C) and LDL uptake (Figure 1D). These results also demonstrated that human MSCs have the potential to differentiate into hepatocyte-like cells as previously described<sup>[3]</sup>.

Furthermore, to compare the differentiation status

of human MSCs at a different period of induction with human hepatoma cell lines, the expression pattern of several hepatic genes was investigated using RT-PCR in human MSCs induced at 0, 1, 4, 7, and 10 wk together with HepG2, HA22T/VGH, and SK-Hep-1 cells (Figure 2). These hepatic genes included (1) an indicator for the early hepatic gene, albumin (Alb); (2) indicators for the middle hepatic gene, tyrosine-aminotransferase (TAT), and glucose 6-phosphatase (G6P); (3) indicators for the late hepatic gene, tryptophan 2,3-dioxygenase (TO)<sup>[26]</sup>; and (4) liver-enriched transcription factors including HNF-1 $\alpha$ , HNF-3 $\beta$ , HNF-4, and C/EBP $\alpha$ . Our data indicated that most of the detected genes, including Alb, TAT, TO, HNF-3 $\beta$ , HNF-4, and C/EBP $\alpha$ , were expressed in MSCs 10 wk after induction, whereas the immature marker  $\alpha$ -fetoprotein was not expressed during the entire period of induction (Figure 2A). Among them, the Alb gene was expressed as early as serum starvation treatment and maintained its expression during the whole induction period. The TAT gene was induced to be expressed 1 wk post-induction and the TO gene was activated 4 wk post-induction. This unique pattern is similar to the sequential expression of hepatic genes during hepatocyte maturation<sup>[26]</sup>. However, the expression of G6P was not detectable, even 10 wk after induction. The expression of liver-enriched transcription factors HNF-4 and C/EBP $\alpha$  was also activated 4 wk after induction. In contrast, HNF-3 $\beta$  was only weakly expressed until 7 wk post-induction and HNF-1 $\alpha$  was not expressed 10 wk after induction. In the human hepatoma cells (Figure 2B), all genes except for TO were well expressed in HepG2 cells; Alb, TO, and HNF-4 genes were expressed in HA22T/VGH cells; and TAT, TO, HNF-3 $\beta$ , HNF-4, and C/EBP $\alpha$  genes were expressed in SK-Hep-1 cells. In general, the expression level of investigated hepatic genes was much lower in the hepatocyte-like cells derived from human MSCs than those in HepG2 cells, the well-differentiated hepatoma cell line, and relatively better than those in poorly differentiated hepatoma cell lines HA22T/VGH and SK-Hep-1. Taken together, human bone marrow MSCs can be specifically induced toward hepatic lineage and expressed certain hepatic-specific genes and liver-enriched transcription factors. However, the expression potency of hepatic genes was weak compared with well-differentiated hepatoma cells. Our results suggested that the differentiation status of induced MSCs seems to lie between the well-differentiated and poorly differentiated cell types of human hepatoma cell lines.

To confirm the specificity of these RT-PCR analyses, we performed a sequence analysis of the PCR fragments followed by nucleotide BLAST analysis. Interestingly, we found that the sequence of the PCR fragments from induced MSCs, HA22T/VGH, and SK-Hep-1 cells corresponded to the HNF-4 $\gamma$  gene, which is not expressed in adult liver<sup>[27]</sup>, and that from HepG2 cells corresponded to the HNF-4 $\alpha$  gene (data not shown). These results revealed that HNF-4 $\alpha$  was only expressed in HepG2 cells, whereas HNF-4 $\gamma$  was expressed in the induced MSCs, HA22T/VGH and SK-Hep-1 cells. To further study the

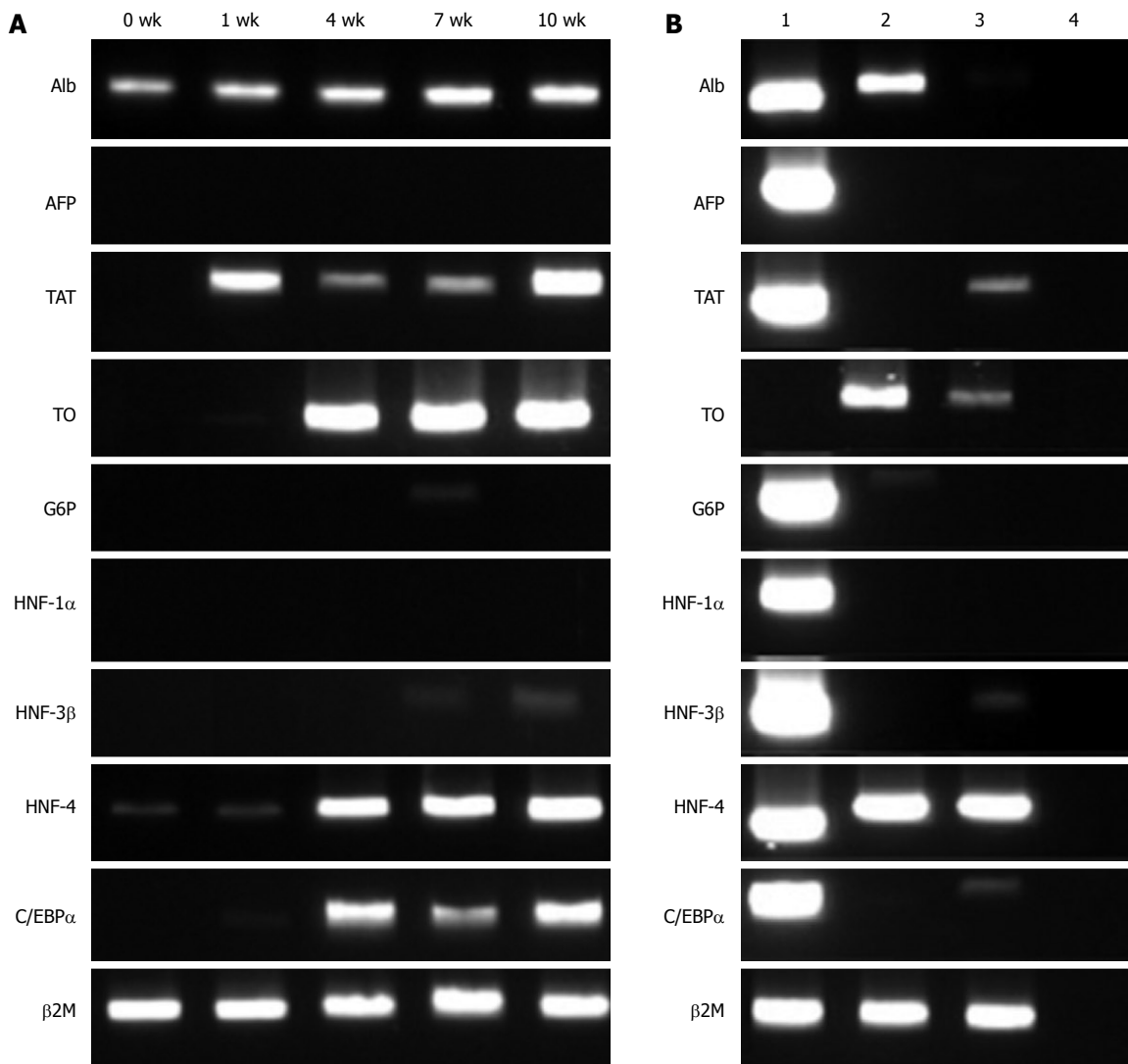




**Figure 1** Hepatic differentiation of human bone marrow-derived mesenchymal stem cells. A: Morphological characterization of differentiated mesenchymal stem cells (MSCs) under hepatic induction. 0 wk: Undifferentiated MSCs; 1 wk: 1 wk post-induction; 4 wk: 4 wk post-induction; 7 wk: 7 wk post-induction; and 10 wk: 10 wk post-induction (original magnification,  $\times 50$ ); B: Morphology of human hepatoma cell lines: SK-Hep-1, HA22T/VGH, and HepG2 (original magnification,  $\times 100$ ); C: Production of albumin (green color) in differentiated MSCs after 10 wk induction (10 wk) and counterstained with Hoechst (blue color). The undifferentiated MSCs (0 wk) were used as negative control cells (original magnification,  $\times 200$ ); D: Uptake of low-density lipoprotein (red color) in differentiated MSCs after 10 wk induction (10 wk). The undifferentiated MSCs (0 wk) were used as negative control cells (original magnification,  $\times 100$ ).

kinetic expression of HNF-4, we use real-time RT-PCR analysis with specific isoform primers (Table 2) to esti-

mate the expression level of HNF-4 $\alpha$  or HNF-4 $\gamma$  in these samples. Similarly, expression of the HNF-4 $\gamma$  gene was



**Figure 2** Detection of liver-specific genes and liver-enriched transcription factors in induced mesenchymal stem cells by reverse transcription-polymerase chain reaction. A: Induced mesenchymal stem cells at the indicated time point: 0, 1, 4, 7 and 10 wk post-induction; B: Human hepatoma cell lines: 1, HepG2; 2, HA22T/VGH; 3, SK-Hep-1; and 4, negative control. Expression of  $\beta$ 2M was used as the control for adjustment of sample amount. Alb: Albumin; AFP:  $\alpha$ -fetoprotein; TAT: Tyrosine-aminotransferase; TO: Tryptophan 2,3-dioxygenase; G6P: Glucose 6-phosphatase;  $\beta$ 2M:  $\beta$ -2-microglobulin.

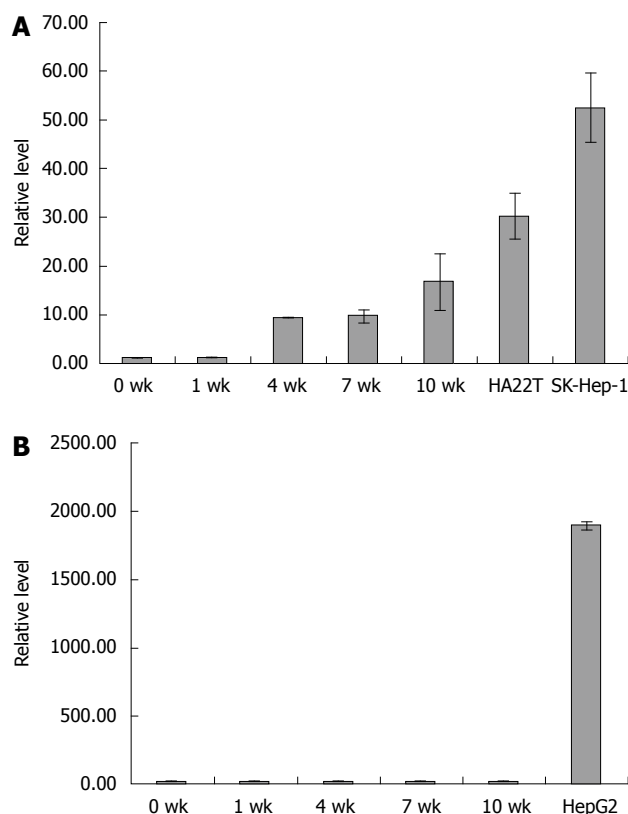
gradually increased during the hepatic induction of MSCs, and HA22T/VGH as well as SK-Hep-1 cells (Figure 3A). In contrast, the HNF-4 $\alpha$  gene was not expressed during the hepatic induction of MSCs, whereas it was significantly expressed in HepG2 cells (Figure 3B). These results suggested that the hepatic induction of human MSCs in this study was not efficient because of the failed expression of HNF-4 $\alpha$  during the induction process.

#### Enhancement of hepatic differentiation by overexpression of HNF-4 $\alpha$

Several studies have demonstrated that hepatic gene expression is regulated by the combinational action of liver-enriched transcription factors. In this study, we found that the expression of HNF-3 $\beta$  and C/EBP $\alpha$  was significantly weaker than that of well-differentiated hepatocytes in induced MSCs for 10 wk. Furthermore, the expression of HNF-1 $\alpha$  and HNF-4 $\alpha$  was undetectable in these cells. Our results clearly indicated that the induced MSCs were

not well differentiated because of the low expression of these liver-enriched transcription factors. Among them, HNF-4 $\alpha$  is crucial for hepatic differentiation because it exhibits a central determinant of hepatic gene expression including liver-enriched transcription factors<sup>[18]</sup>. Therefore, we investigated whether the transcription efficiency of hepatic genes could be simply activated by HNF-4 $\alpha$  overexpression in induced MSCs and further improve their differentiation status. Overexpression of the rat HNF-4 $\alpha$  gene was introduced into 8w-induced MSCs by adenovirus-mediated gene transfer (Ad/HNF4 $\alpha$ -IRES-EGFP). Most of the cells (more than 80%) were successfully infected at a 1500 m.o.i. as monitored by the coexpressed EGFP gene, and the overexpression of HNF-4 $\alpha$  was confirmed by real-time PCR analysis (Figure 4A). The expression level of other liver-enriched transcription factors in the HNF-4 $\alpha$ -infected cells was examined by real-time RT-PCR analysis and compared with that of mock control cells infected with control adenoviruses (Ad-IRES-EGFP) (Figure 4B).



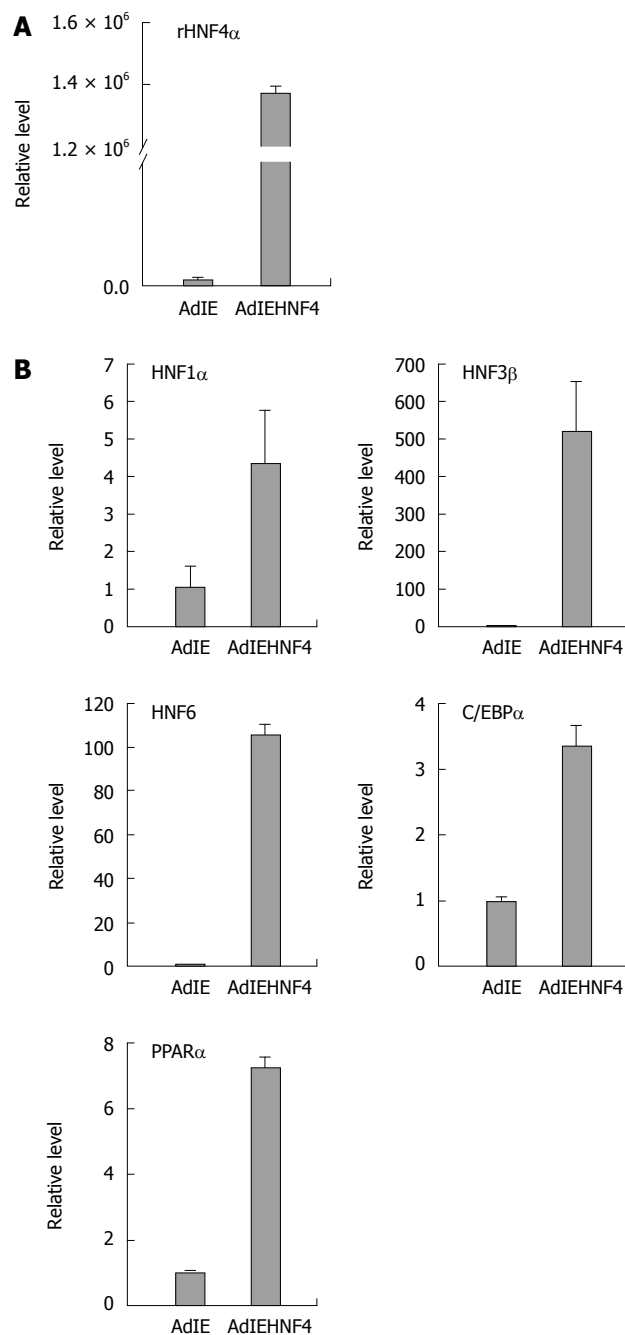


**Figure 3** Detection of hepatocyte nuclear factor-4 isoforms in induced mesenchymal stem cells and human hepatoma cell lines by real-time reverse transcription-polymerase chain reaction. A: The relative level of hepatocyte nuclear factor (HNF)-4 $\gamma$  in induced mesenchymal stem cells (MSCs) at the indicated time point (0, 1, 4, 7 and 10 wk post-induction), HA22T/VGH and SK-Hep-1 cells; B: The relative level of HNF-4 $\alpha$  in induced MSCs at the indicated time point (0, 1, 4, 7 and 10 wk post-induction) and HepG2 cells. The relative level at 0 wk was set to 1. The amount of input RNA was normalized using the housekeeping gene  $\beta$ 2M. Each column represents the mean  $\pm$  SD of three independent experiments.

The expression of HNF-1 $\alpha$ , C/EBP $\alpha$ , and peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) was induced 3- to 7-fold in the HNF-4 $\alpha$ -infected hepatocyte-like cells. Furthermore, the expression of HNF-3 $\beta$  and HNF-6 was dramatically induced by more than 100-fold (500-fold and 100-fold, respectively) in the HNF-4 $\alpha$ -infected hepatocyte-like cells. The effect of HNF-4 $\alpha$  overexpression on the induction of hepatic-specific genes was also evaluated by the same method. Among them, expression of Alb, TAT, and G6P genes was markedly induced in HNF-4 $\alpha$ -infected cells, compared with mock control cells, by 12-, 40-, and 2000-fold, respectively (Figure 5).

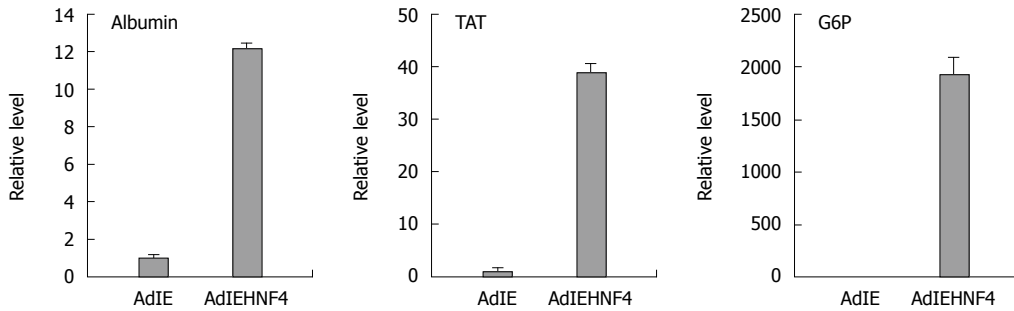
#### Induction of the P450 gene family by HNF-4 $\alpha$ overexpression

The drug-metabolizing enzymes, cytochrome P450 enzymes, are a superfamily of monooxygenases that play an important role in the detoxification of xenobiotics and the metabolic activation of chemical carcinogens in mature hepatocytes. Therefore, the expression of P450 genes is also designated as an indicator for the late hepatic genes. In this study, the expression patterns of P450 genes, including CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and

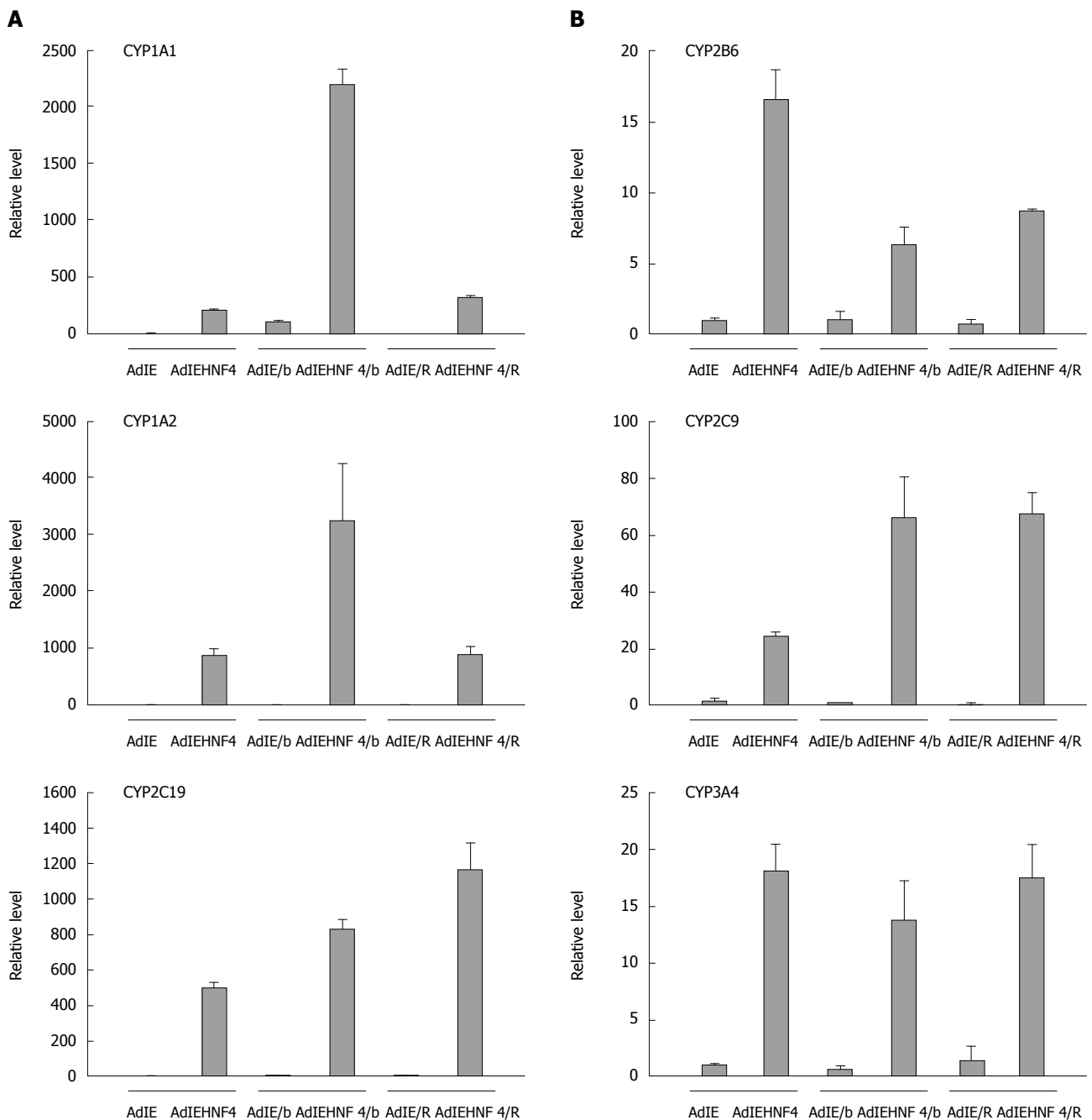


**Figure 4** Detection of liver-enriched transcription factors in hepatocyte nuclear factor-4 $\alpha$  overexpressing induced mesenchymal stem cells by real-time reverse transcription-polymerase chain reaction. The induced mesenchymal stem cells (8 wk post-induction) were infected with adenovirus containing rHNF-4 $\alpha$  gene (Ad/HNF4 $\alpha$ -IRES-EGFP) (indicated as AdIEHNF4) or parental control adenovirus (Ad-IRES-EGFP) (indicated as AdIE). The relative level in the control group was set to 1. A: The overexpression of rHNF4 $\alpha$  was demonstrated in the Ad/HNF4 $\alpha$ -IRES-EGFP infected cells; B: The induction effect of HNF-4 $\alpha$  on the expression of liver-enriched transcription factors was represented as HNF1 $\alpha$ , HNF3 $\beta$ , HNF6, C/EBP $\alpha$  and peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), respectively. The amount of input RNA was normalized using the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase. Each column represents the mean  $\pm$  SD of three independent experiments and the induction fold for each gene was significant ( $P < 0.05$ ). HNF: Hepatocyte nuclear factor.

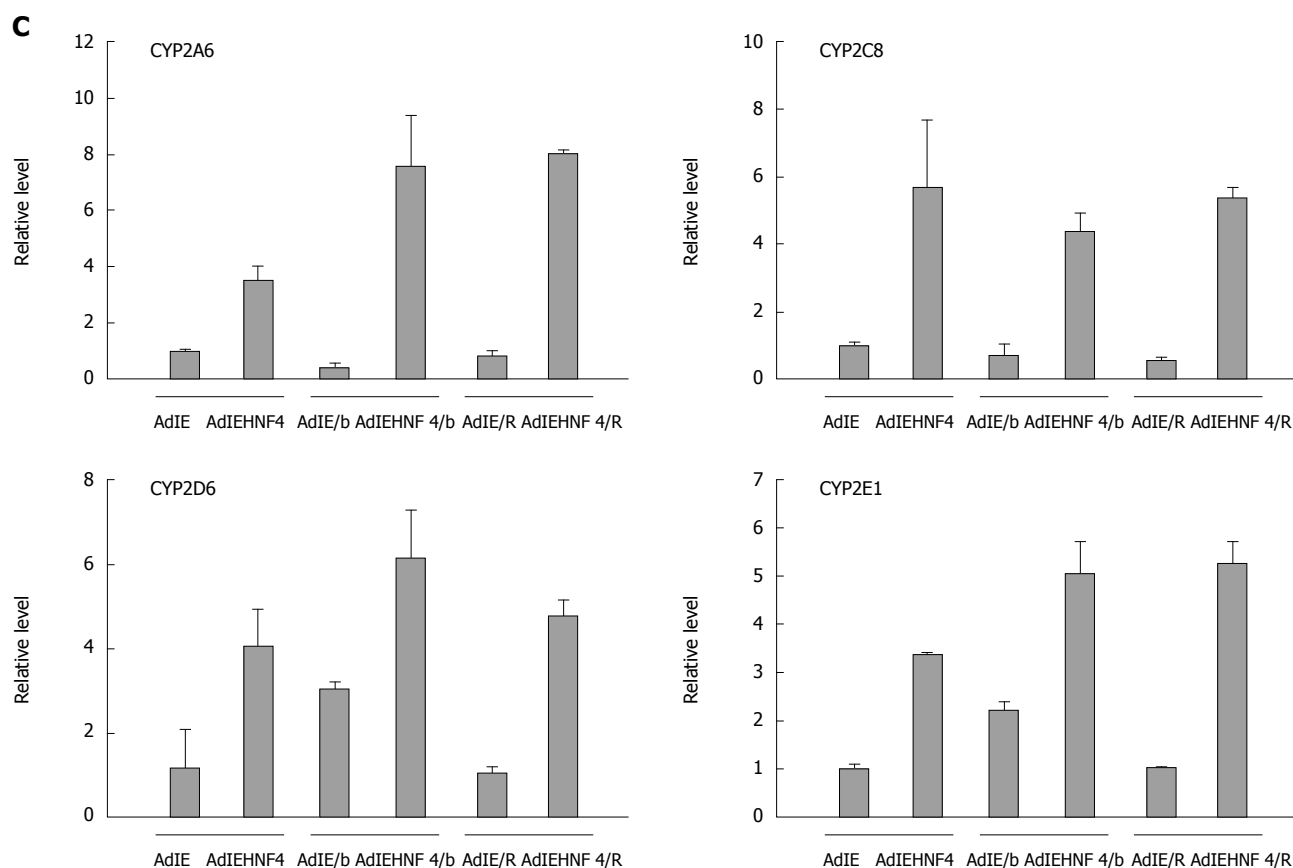
3A4, were examined in induced MSCs, which were induced for 8 wk and then infected with Ad/HNF4 $\alpha$ -IRES-EGFP for 1 wk, and compared with that in the mock control cells



**Figure 5** Detection of liver-specific genes in hepatocyte nuclear factor-4 $\alpha$  overexpressing induced mesenchymal stem cells by real-time reverse transcription-polymerase chain reaction. The induced mesenchymal stem cells (8 wk post-induction) were infected with adenovirus containing rHNF-4 $\alpha$  gene (indicated as AdIEHNF4) or parental control adenovirus (indicated as AdIE). The relative level of each gene in the control group was set to 1. The induction effect of HNF-4 $\alpha$  on the expression of liver-specific genes was represented as Albumin, tyrosine-aminotransferase (TAT), and glucose 6-phosphatase (G6P), respectively. The amount of input RNA was normalized using the hypoxanthine-guanine phosphoribosyltransferase gene. Each column represents the mean  $\pm$  SD of three independent experiments and the induction fold for each gene was significant ( $P < 0.05$ ). HNF: Hepatocyte nuclear factor.







**Figure 6** Detection of P450 genes in hepatocyte nuclear factor-4 $\alpha$  overexpressing induced mesenchymal stem cells by real-time reverse transcription-polymerase chain reaction. The induced mesenchymal stem cells (8 wk post-induction) were infected with adenovirus containing rHNF-4 $\alpha$  gene (indicated as AdIEHNF4) or parental control adenovirus (indicated as AdIE). The infected cells were further treated with inducer B-naphthoflavone (indicated as AdIE/b or AdIEHNF4/b) or rifampicin (indicated as AdIE/R or AdIEHNF4/R) for 2 d. The relative level of each gene in the control group was set to 1. The induction effect of HNF-4 $\alpha$  and/or inducer on the expression of P450 genes is represented as (A) CYP1A1, CYP1A2, and CYP2C19; (B) CYP2B6, CYP2C9, and CYP3A4; (C) CYP2A6, CYP2C8, CYP2D6, and CYP2E1, respectively. The amount of input RNA was normalized using the HPRT gene. Each column represents the mean  $\pm$  SD of three independent experiments and the induction fold by HNF-4 $\alpha$  overexpression for each gene was significant ( $P < 0.05$ ). HNF: Hepatocyte nuclear factor.

infected with Ad-IRES-EGFP. As shown in Figure 6, most of the detected genes were not expressed in the induced MSCs (indicated as AdIE). However, all of the detected genes were significantly activated by the overexpression of HNF-4 $\alpha$  (indicated as AdIE/HNF4). Among them, CYP1A1, CYP1A2, and CYP2C19 genes were markedly activated by more than 100-fold (Figure 6A); CYP2B6, CYP2C9, and CYP3A4 genes were markedly activated by more than 10-fold (Figure 6B); and CYP2A6, CYP2C8, CYP2D6, and CYP2E1 genes were moderately activated by 3- to 6-fold (Figure 6C).

It is well known that expression of P450 genes can be activated by a range of chemicals<sup>[28]</sup>. Therefore, the induction potential of P450 gene expression was also investigated after cells were incubated for 48 h with either B-naphthoflavone (BNF) or rifampicin (RIF), which are reported to be inducers of selective P450 genes<sup>[29]</sup>. As shown in Figure 6, the inducibility of P450 genes was observed in the presence of BNF or RIF both in induced MSCs (indicated as AdIE/b or AdIE/R) and in HNF-4 $\alpha$ -overexpressing cells (indicated as AdIEHNF4/b or AdIEHNF4/R). Among them, CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2D6, and CYP2E1 genes were sig-

nificantly activated by BNF, whereas CYP2A6, CYP2C9, CYP2C19, and CYP2E1 genes were significantly induced by RIF ( $P < 0.05$ ). However, the expression of CYP2C8 and CYP3A4 was not induced and that of CYP2B6 was reduced by either BNF or RIF. Taken together, in the presence of a high amount of HNF-4 $\alpha$ , the MSCs-derived hepatocyte-like cells can be further induced toward a more differentiated hepatocytic status. The expression of liver-specific genes, liver-enriched transcription factors, and P450 genes could be significantly activated by HNF-4 $\alpha$  overexpression and move toward that of well-differentiated cell types.

## DISCUSSION

Recently, several sources of stem cells have been successfully driven to hepatic differentiation and display several hepatic functions such as LDL uptake, glycogen storage and albumin expression<sup>[3,5]</sup>. In this study, we induced the hepatic differentiation of human bone marrow MSCs according to the protocol previously described by Lee *et al.*<sup>[3]</sup>. The expression pattern of hepatic genes in induced human MSCs seems to be correlated with the developmental pro-

cess of the liver *in vivo*. The differentiated cells expressed early hepatic genes as early as serum starvation treatment, middle hepatic genes at 1 wk post-induction, and late hepatic genes at 4 wk post-induction. However, their differentiation status was much less than that of HepG2 cells as evaluated by their potency of expression of hepatic-specific genes, liver-enriched transcription factors, and the P450 gene family. It is likely that the differentiation status of induced human MSCs lies between HepG2 cells and poorly differentiated hepatoma cells (HA22T/VGH and SK-Hep-1 cells).

It is well known that several liver-enriched transcription factors can coordinately regulate the expression of hepatic genes that are involved in liver-specific functions<sup>[15-17]</sup>. Among them, HNF-4 $\alpha$  may act as a master gene in the transcriptional cascade that regulates constitutive expression of target genes<sup>[18-20]</sup>. Interestingly, we found that one of the HNF-4 isoforms, HNF-4 $\gamma$  was expressed in the middle stage of induced MSCs and poorly differentiated hepatoma cell lines. The human HNF-4 $\gamma$  gene encodes for a 408 amino acid protein with 70% overall identity to human HNF-4 $\alpha$ . A previous study revealed that HNF-4 $\gamma$  is expressed in the kidney, pancreas, small intestine, and colon but not in the liver, while HNF-4 $\alpha$  is significantly expressed in the liver<sup>[27]</sup>. Studies also demonstrated that HNF-4 $\gamma$  is able to activate transcription through the same binding sites as HNF-4 $\alpha$ , however, the transactivation potential of HNF-4 $\gamma$  is significantly less active than HNF-4 $\alpha$ <sup>[30]</sup>. These results suggest that the poorly differentiated status of hepatocyte-like cells induced from human MSCs is likely due to the expression of HNF-4 $\gamma$  instead of HNF-4 $\alpha$ . Moreover, we demonstrated that the differentiation status of hepatocyte-like cells derived from human MSCs could be further progressed toward that of well-differentiated HepG2 cells by adenovirus-mediated HNF-4 $\alpha$  overexpression. Most of the detected hepatic genes, liver-enriched transcription factors, and P450 genes were significantly activated by HNF-4 $\alpha$  overexpression. These findings indicate that HNF-4 $\alpha$  plays a key role in facilitating hepatic differentiation of human MSCs derived from bone marrow and are consistent with previous studies in rodents<sup>[31,32]</sup>.

Metabolism by cytochrome P450 is a major route of detoxification for a large number of xenobiotics. The induction of P450 gene expression is a common cellular defensive mechanism of hepatocytes against the toxicity of foreign compounds. Hepatic expression of P450 genes can be activated following exposure to various classes of inducers including B-naphthoflavone (BNF), rifampicin (RIF), and phenobarbital (PB)<sup>[29]</sup>. Among them, expression of CYP1A1 and 1A2 can be significantly induced by exposure to BNF, but weakly induced by RIF and PB<sup>[33-35]</sup>. Expression of CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 can be increased by RIF and PB, whereas CYP2D6 was not obviously induced by RIF or PB<sup>[34-36]</sup>. In this study, the inducibility of P450 genes by either BNF or RIF was also demonstrated in the hepatocyte-like cells and HNF-4 $\alpha$ -overexpressing cells

(Figure 6). In addition, we found that CYP2A6, CYP2C9, CYP2D6, and CYP2E1 genes can be activated by BNF, which had not been examined in previous studies. Relatively little or no induction of CYP2C8 and CYP3A4 or a reduction in CYP2B6 was shown in the presence of RIF. The reason for this observation is unknown at the present time and remains to be further investigated. Taken together, hepatic differentiation of stem cells *in vitro* can be improved by the ectopic expression of a single liver-enriched transcription factor such as HNF-4 $\alpha$ .

In conclusion, HNF-4 $\alpha$  is a key factor in determining the differentiation status of hepatocyte-like cells derived from human MSCs. Overexpression of HNF-4 $\alpha$  can activate various hepatic-specific genes and enhance the differentiation status in differentiated MSCs. This may provide a simple and convenient way to obtain better cell sources for clinical applications and drug screening *in vitro*. Adenoviral vectors are efficient systems for gene delivery both *in vitro* and *in vivo*. However, several limitations including innate immune responses by host cells, genomic integration into target cells, and cytotoxicity for target cells have impeded their clinical utility and studies regarding the determination of certain P450 detoxification. Therefore, delivery of HNF-4 $\alpha$  genes by other systems should be considered for future studies. Recently, adeno-associated virus, which has the capacity to deliver genes to both dividing and non-dividing cells in numerous tissues, has shown significant promise in clinical trials because of its safety and delivery efficiency<sup>[37,38]</sup>. It will be a suitable strategy for the future application of HNF-4 $\alpha$  overexpression.

## COMMENTS

### Background

Mesenchymal stem cells (MSCs) derived from bone marrow have the potential to differentiate into hepatocyte-like cells both *in vitro* and *in vivo*. These observations bring new hope for the possible application of cell-based therapy in severe liver diseases. However, the differentiation status of induced MSCs is poor and not sufficient for future clinical applications.

### Research frontiers

The combinational action of several liver-enriched transcription factors regulates hepatic gene expression. Among them, hepatocyte nuclear factor (HNF)-4 $\alpha$  plays a crucial role. However, the expression of HNF-4 isoforms in human hepatoma cell lines and induced MSCs has not been studied. In this study, the authors demonstrated that the overexpression of HNF-4 $\alpha$  could enhance the hepatic differentiation of human MSCs.

### Innovations and breakthroughs

This is the first report to show that the differentiation status of induced human MSCs is similar to poorly differentiated human hepatoma cell lines, all of which expressed HNF-4 $\gamma$  instead of HNF-4 $\alpha$ . Overexpression of HNF-4 $\alpha$  can significantly activate the expression of hepatic-specific genes, liver-enriched transcription factors and P450 genes and, therefore, enhance hepatic differentiation.

### Applications

Overexpression of HNF-4 $\alpha$  is a simple and convenient way to facilitate hepatic differentiation of human MSCs, and provide better cell sources for clinical application such as *in vitro* hepatotoxicity models for drug screening.

### Peer review

This is a well-written paper that characterized human mesenchymal stem cells differentiating into hepatocyte-like cells. They found these cells express low levels of HNF-4 $\alpha$  and high levels of HNF-4 $\gamma$ . Overexpression of HNF-4 $\alpha$  induces more hepatocyte-like gene expression including p450-related genes.

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