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Research article

In vitro AND *in vivo* CHARACTERISTICS OF HEPATIC OVAL CELLS MODIFIED WITH HUMAN HEPATOCYTE GROWTH FACTOR

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Abstract: Hepatocyte growth factor (HGF) is a multifunctional growth factor that controls cell scattering. It has been suggested that it regulates the proliferation of hepatic oval cells (HOCs). Using a HOC line that stably expresses the human HGF gene (*hHGF*), we investigated the *in vitro* proliferation and differentiation characteristics of hHGF-modified HOCs and explored their potential capacity for intrahepatic transplantation. A modified 2-acetylaminofluorene and partial hepatectomy (2-AAF/PH) model was established to activate the proliferation of oval cells in the rat liver. HOCs were transfected with the pBLAST2-hHGF plasmid and hHGF-carrying HOCs were selected based on blasticidin resistance. The level of hHGF secretion was determined via ELISA. Cell proliferation was determined using the MTT assay. Differentiation was induced by growth factor withdrawal. A two-cuff technique was used for orthotopic liver transplantation, and HOCs or hHGF-modified HOCs were transplanted into the recipients. The levels of biochemical indicators of liver function were measured after transplantation. An HOC line stably

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Abbreviations used: 2-AAF – 2-acetylaminofluorene; ALB – albumin; ALP – alkaline phosphate; ALT – alanine aminotransferase; ChE – cholinesterase; DBil – direct bilirubin; EGF – epidermal growth factor; ELISA – enzyme-linked immunoassay; GGT – γ-glutamyltransferase; HGF – hepatocyte growth factor; hHGF – human hepatocyte growth factor; HOCs – hepatic oval cells; I/R – ischemia/reperfusion; LIF – leukemia inhibitory factor; MST – median survival time; PH – partial hepatectomy

expressing hHGF was established. The transfected line showed greater hHGF secretion than normal HOCs. The *hHGF* gene promoted the proliferation capability of HOCs by reducing the peak time *in vitro*. The hHGF-modified HOCs differentiated into hepatocytes and bile duct epithelial cells upon growth factor withdrawal *in vitro*. In addition, hHGF-modified HOC transplantation significantly prolonged the median survival time (MST) and improved the liver function of recipients compared to HOC transplant recipients and non-transplanted controls. Our results indicate that hHGF-modified HOCs may have valuable properties for therapeutic liver regeneration after orthotopic liver transplantation.

Key words: Hepatic oval cells, Hepatocyte growth factor, pBLAST2-hHGF, Plasmid transfection, Proliferation, Differentiation, Liver transplantation, Rats, *In vitro*, Liver regeneration

INTRODUCTION

Hepatocytes and hepatic oval cells (HOCs) are an important source of endogenous stem cells (progenitor cells) for liver regeneration [1, 2]. The proliferation of differentiated hepatocytes generally occurs in conjunction with liver regeneration after injury [3]. However, when hepatocyte proliferation is blocked or delayed, HOCs may also proliferate, invade the adjacent liver parenchyma, and differentiate into hepatocytes and biliary epithelial cells [2]. HOCs are considered the preferred source for hepatic transplantation after acute injury [4], but their inefficient proliferation and differentiation, which is possibly regulated by wingless-type MMTV integration site family member 1 (WNT1), may hinder their use in such applications [5].

Hepatocyte growth factor (HGF), also known as scatter factor, is a multifunctional growth factor that is responsible for activating a genetic program that includes cell detachment, repulsion, protection against apoptosis, invasiveness of extracellular matrices, and proliferation [6, 7]. It is currently recognized as the most potent mitogen for mature hepatocytes and it seems to act as a hepatotropic factor [8]. Recent studies indicated that HGF can accelerate the *in vitro* and *in vivo* proliferation of HOCs [9-11]. Although the mechanism of HGF-regulated HOC proliferation remains unclear, the v-akt murine thymoma viral oncogene homolog 1 (AKT) pathway might be involved in this process [11]. Nevertheless, the effect of HGF on HOC differentiation and HOC-based intra-hepatic cell transplantation is poorly understood.

In this study, we established a HOC line that stably expresses the human HGF (*hHGF*) gene. The *in vitro* proliferation and differentiation characteristics of these hHGF-modified HOCs were investigated and their potential for intra-hepatic transplantation was determined. Their *in vivo* influence on transplanted and ischemia/reperfusion (I/R) injured livers was examined.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Gibco (USA). Dulbecco's modified Eagle's medium/F12 (DMEM/F12) culture medium was obtained from Sigma-Aldrich (USA). HGF, epidermal growth factor (EGF), stem cell factor (SCF) and leukemia inhibitory factor (LIF) were purchased from Cytolay (USA). The pBLAST2-hHGF plasmid, blasticidin and Lipofectamine 2000 Transfection Reagent were purchased from Invitrogen (USA). The anti-β-actin antibody was obtained from Sigma-Aldrich (USA). An ELISA kit for the detection of hHGF was purchased from Shanghai ExCell Biology (Shanghai, China).

Animals

Male and female specific pathogen-free (SPF) Lewis rats weighing 200 ± 20 g (used for HOC isolation) or 250-300 g (used for transplantation) were obtained from Vital River Lab Animal Technology Co., Ltd. (Beijing, China). Female SPF Dark Agouti (DA) rats weighing 200-250 g (used as donors) were obtained from the Laboratory Animal Center of Harbin Medical University, Harbin, China. Adult Sprague Dawley rats (n = 8 per group) were used for the I/R injury experiment. All efforts were made to minimize animal suffering and to reduce the number of animals used. All animal procedures were approved by the Ethics Committee of Liaocheng People's Hospital.

Preparation of pBLAST2-hHGF plasmid

The *Escherichia coli* DH5α strain of competent cells was prepared using the calcium chloride method. To chemically transform competent cells, they were mixed with the pBLAST2-hHGF plasmid on ice and then briefly heat shocked at 37ºC. The cells were plated on LB/agar plates containing blasticidin (6 μg/ml) and antibiotic-resistant clones were isolated for small-scale preparation of the plasmid. Plasmid DNA was then extracted from cells using the Mini Plasmid Extraction Kit according to the manufacturer's instructions (Omega, USA).

Primary culture of HOC

The slightly modified 2-acetylaminofluorene/partial hepatectomy-induced injury (2-AAF/PH) Lewis male rat model was used to activate HOCs. In our modified model, a 1/3-1/2 partial hepatectomy was performed using a left lateral lobe excision instead of a 2/3 partial hepatectomy [12]. HOCs were isolated 10 days after the operation. The tissue samples were minced, washed with D-Hank's Solution, and digested with 0.1% collagenase IV and 0.025% EDTA at 37ºC. The cells were filtered through a 100 mesh filter and centrifuged at 500 RPM for 5 min at 4ºC. HOCs were separated by Percoll gradient centrifugation (Amersham Biosciences, USA). The cells were seeded onto gelatin-coated flasks and maintained in DMEM/F12 culture medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 ng/ml amphotericin B at

37ºC in a 5% CO_2 incubator. Three days after seeding, 20 ng/ml SCF, 10 ng/ml HGF, 10 ng/ml EGF and 10 ng/ml LIF were added to the culture medium. To induce differentiation, LIF was withdrawn from the culture medium until examination [13, 14]. After growth factor withdrawal, cells underwent differentiation depending on endogenous secretion of HGF.

Stable transfection

Transient transfection was carried out when HOCs grew to 90% confluence using Lipofectamine 2000 Transfection Reagent according to the manufacturer's instructions. Briefly, 15 µl DNA was combined with 15 µl Lipofectamine 2000 in 150 µl serum- and antibiotic-free DMEM/F12 culture medium for each 25-cm^2 flask of cells. After 10-15 min of incubation, the mixture was added to cells in the presence of 3 ml serum- and antibiotic-free DMEM/F12 culture medium. The cultures were incubated for 4 h in this transfection medium, and then the medium was replaced with complete culture medium. The cells expressing pBLAST2-hHGF were screened using blasticidin. Blasticidin-resistant cells were maintained in DMEM/F12 culture medium containing 15% FBS, 20 ng/ml SCF, 10 ng/ml HGF, 10 ng/ml EGF and 20 ng/ml LIF. Endogenously secreted and exogenously added HGF may increase the potential for cell differentiation, so the concentration of LIF was increased to suppress cell differentiation. For ELISA, passage 4 HOCs stably expressing hHGF were seeded into a new culture flask containing DMEM/F12 medium supplemented with 12% FBS without growth factors. Non-transfected passage 4 HOCs were cultured under same conditions and used as the control.

ELISA

The secretion of hHGF in culture medium was evaluated via ELISA according to the manufacturer's instructions for the kit. The culture medium was collected and the supernatant was removed after centrifuging at 3000 RPM for 3 min. The primary and secondary antibodies were used in a 1:250 dilution. A biotinylated antibody was added to the 96-well plate (100 µl per well) and incubated at 37ºC for 60 min. Then, 100 µl of enzyme solution was added to each well and the plate was incubated for an additional 30 min at 37ºC. After washing, 100 µl of substrate solution was added to each well and the plate was incubated at 37ºC for 15 min in the dark. Finally, the sealer was removed and the stop solution was added (100 µl for each well). The absorbance at 450 nm was recorded on a plate reader (BioTek, USA) immediately after the plates were shaken to ensure complete mixing.

Induction of hHGF-HOC differentiation

Passage 4 hHGF-HOCs were randomly assigned into three groups: 1) complete growth factor group; 2) LIF only group; and 3) growth factor withdrawal group. Due to the increased concentration of HGF in the culture medium, the dosage of LIF was also increased. In the complete growth factor group, cells were maintained with 20 ng/ml SCF, 10 ng/ml HGF, 10 ng/ml EGF and 20 ng/ml LIF.

In the LIF only group, cells were cultured in the presence of 10 ng/ml LIF. In the growth factor withdrawal group, the cells were grown in media without growth factor supplementation.

Electron microscope analysis

For transmission electron microscopy (TEM), cells were fixed in 2.5% glutaraldehyde for 2 h and then post-fixed in 1% OsO_4. Dehydration was done using a graded ethanol and acetone series, with 30% (v/v) ethanol for 15 min; 50% (v/v) ethanol for 15 min; 70% (v/v) ethanol for 15 min; 90% (v/v) ethanol for 15 min; 95% (v/v) ethanol for 15 min; 50% (v/v) acetone for 10-15 min; 70% (v/v) acetone for 10-15 min; and 90% (v/v) acetone for 10-15 min; followed by three washes with 100% (v/v) acetone (30 min). Then, the cell samples were embedded in Epon812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a HF-3300 electron microscope (Hitachi Science Systems, Ltd., Japan). For scanning electronic microscopy (SEM), cell samples were fixed in 2.5% glutaraldehyde for 12 h. Dehydration was done in an acetone gradient of 50% (v/v) acetone for 10-15 min; 70% (v/v) acetone for 10-15 min; and 90% (v/v) acetone for 10-15 min. Then, spray dried samples were analyzed with an S-3400-N electron microscope (Hitachi Science Systems, Ltd., Japan).

Cell proliferation assay

Cell proliferation was evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay technique. Cells were seeded onto 24-well plates at a density of 1×10^7 cells/well. At the indicated time points after cell seeding, 20 µl of MTT (5 mg/ml) was added to each well and the cells were incubated for an additional 4 h at 37ºC. The medium was removed and 150 µl dimethyl sulfoxide (DMSO) was added to each well to resuspend the MTT metabolic product. The absorbance of the dissolved formazan was measured at 570 nm with a scanning microplate spectrophotometer. The wells were measured in triplicate for each experimental group.

Cell cycle analysis

Cell cycle staging was determined by flow cytometry. Cell samples were processed with a DNA-PREP Reagents Kit (Beckman-Coulter, Fullerton, CA) according to the manufacturer's instructions and then transferred to an EPICS ALTRA flow cytometer (Beckman-Coulter, USA). The cytometry data were analyzed with MultiCycle software.

Cell transplantation

A two-cuff technique (portal vein and infrahepatic vena cava) was used to perform an orthotopic liver transplantation in rats as previously described [15]. Female DA rats were used as donors and female Lewis rats as recipients. Recipients were randomly divided into three groups of 60 rats each: the control group, the HOC transplantation group and the hHGF transplantation group. In the control group, animals underwent orthotopic liver transplantation. In the two

cell transplantation groups, 1 ml of 1×10^6 cells/ml HOCs or hHGF-modified HOCs (passage 4-10) were respectively injected into the portal vein or hepatic artery during the orthotopic liver transplantation. The recipients were given tacrolimus from one day before surgery until 13 days after surgery. Liver function indicators, including alanine aminotransferase (ALT), direct bilirubin (DBil), albumin (ALB), γ-glutamyltransferase (GGT), alkaline phosphate (ALP) and cholinesterase (ChE) were measured at the indicated times post-transplantation using a Beckman CX9 automatic biochemical analyzer (Beckman-Coulter, Germany).

I/R injury model and cell transplantation

In the control group (n = 8), I/R injury was induced by clamping the porta hepatis for 50 min, followed by reperfusion. In the cell transplantation groups, 1 ml of 1×10^6 cells/ml HOCs (n = 8) or passage 4 through 10 hHGF-modified HOCs (n = 8) were injected into the portal vein 40 min after the initiation of reperfusion.

Histology

The animals were killed 20 days after surgery or 5 days after I/R injury, and their liver tissues were removed, fixed in 10% buffered formaldehyde, and embedded in paraffin. The specimens were then cut into 4-μm sections, stained with hematoxylin and eosin (HE) using a standard procedure, and examined under a phase contrast microscope (Olympus, Japan).

Quantitative real time RT-PCR (qPCR)

Total RNA was extracted from HOCs, differentiated HOCs, hHGF-modified HOCs and differentiated hHGF-modified HOCs, and from liver and bile duct tissue using Trizol reagent (Sigma-Aldrich, USA). After purification, the integrity of the purified mRNA was confirmed by agarose gel electrophoresis. The cDNA was then transcribed with a Fermentas cDNA synthesis kit according to the manufacturer's instructions. The sequences of the specific primers used are: ALB, forward, 5'-TGTCACGGCGACCTGTTG-3', reverse, 5'-GGAGATAGTGGCCTGGTTCTCA-3'; CK19, forward, 5'-GACTTCC GGACCAAGTTTGAG-3', reverse, 5'-CGCAGGCCGTTGATGTC-3'; and β-actin, forward, 5'-ACCGAGCGCGGCTACAGC-3', reverse, 5'-CTCATT GCCAATGGTGAT-3'. PCR was carried out on a real-time fluorescence quantitative instrument (Roche Diagnostics, Switzerland). The primer concentration was 20 pM and the efficiency of the PCR of kit was 96.3%. The thermocycling conditions were: 60ºC for 2 min, 94ºC for 10 min, and then 40 cycles at 94ºC for 15 s and 60ºC for 60 s. The amplified products were analyzed with real-time fluorescence quantitative instrument software. Fold changes in target gene expression were the normalized with the following formula:

$$\text{Fold change} = 2^{-\Delta(\Delta Ct)}$$

The negative log was calculated and data were analyzed from 20 independent experiments.

Statistical analysis

Data were analyzed with Statistical Package for the Social Sciences (SPSS) version 16.0 (IBM, Inc., USA) and were expressed as means ± standard deviation (SD). A comparison of median survival time (MST) was performed via Kaplan-Meier analysis. Differences in measurement data were compared with analysis of variance (ANOVA) or independent sample *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Establishment of hHGF-expressing HOCs by stable transfection

HOCs were isolated from the liver tissues of rats that had received 2-AAF/PH. After adhesion, round, oval or short spindle-like cells multiplied rapidly in the culture medium supplemented with growth factors. The pBLAST2-hHGF plasmid lacked a reporter gene, such as GFP, but it did carry the DNA sequence of the blasticidin-resistance gene (*BSR*); therefore, blasticidin was used to select transfected cells carrying the inserted plasmid. As shown in Fig. 1, most of the cells died 6 days after receiving the antibiotic, whereas cell colonies carrying the pBLAST2-hHGF plasmid survived and proliferated rapidly starting 11 days after blasticidin administration. The stable expression of hHGF in hHGF-modified HOCs was confirmed via Western blot using a specific antibody against hHGF, as previously described [16]. No hHGF expression was detected in non-transfected HOCs, while a strong signal for hHGF was observed in HOCs stably transfected with the pBLAST2-hHGF plasmid [16]. The secretion of hHGF into the culture medium was significantly higher for the hHGF-modified HOCs than for normal HOCs ($p < 0.05$; Fig. 2). These results indicated that an HOC line stably expressing hHGF had successfully been established.

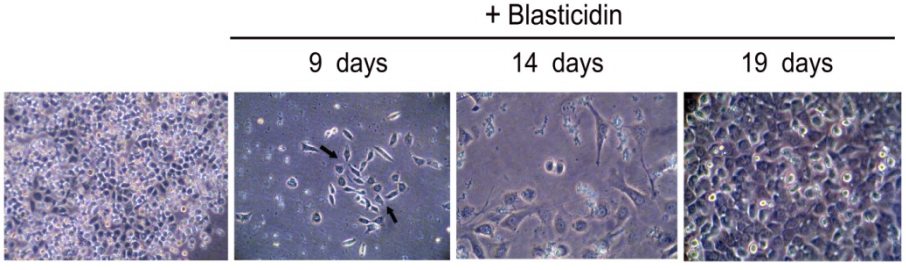

Fig. 1. Morphology of the HOC line stably expressing hHGF. Blasticidin screening was performed 3 days post-transfection. The morphology of pBLAST2-hHGF transfected HOCs prior to transfection or 9, 14, and 19 days post-transfection was observed under a phase contrast microscope. The blasticidin-resistant cell colonies are indicated by arrows. (Magnification: 200 ×).

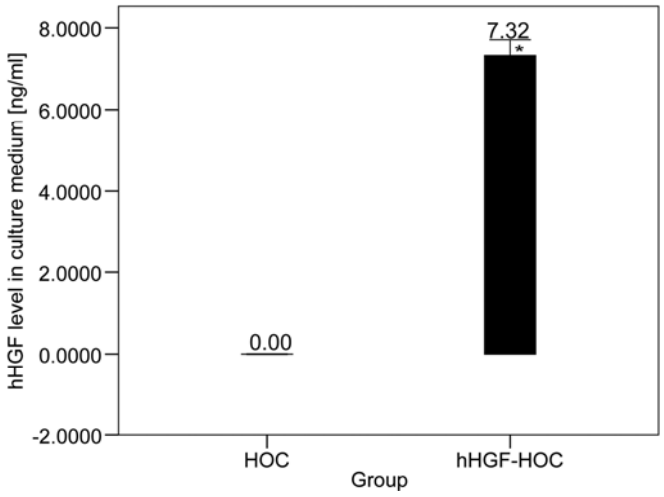

Fig. 2. Determination of hHGF expression in hHGF-modified HOCs. The production and secretion of hHGF into the culture medium by passage 4 HOCs or hHGF-modified HOCs was confirmed via ELISA. *$p < 0.05$ compared with HOCs.

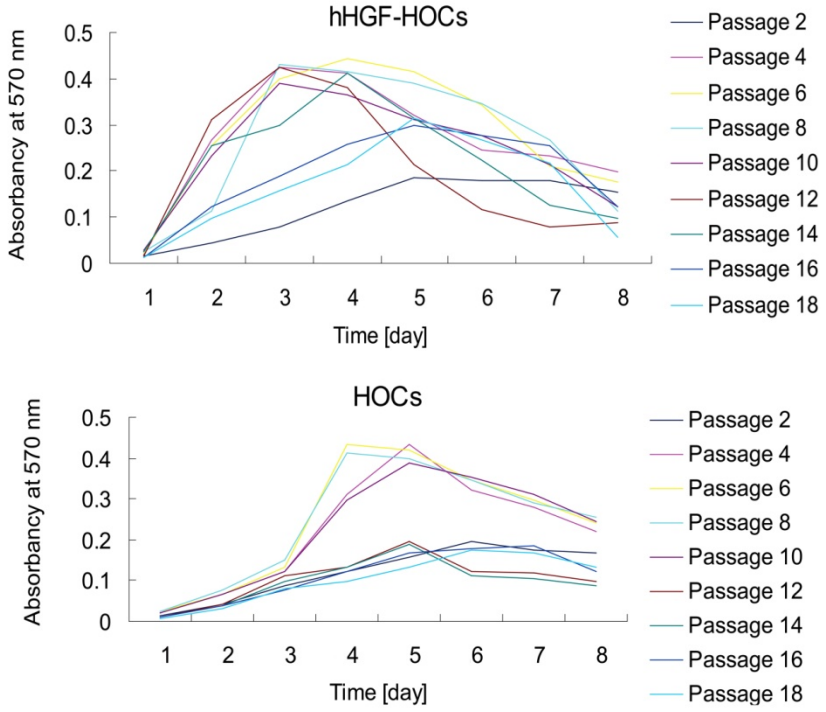

Fig. 3. Growth curves of hHGF-modified HOCs collected at different passages. The growth viability of passage 2 through 18 hHGF-modified HOCs was determined via MTT. The x-axis indicates the incubation time and the y-axis indicates the optical density (OD) value.

Proliferation of hHGF-modified HOCs

We next determined the proliferation capability of hHGF-modified HOCs using the MTT assay. As shown in Fig. 3, rapid proliferation was observed in passage 4 through 14 HOCs that possessed the *hHGF* gene. It should be noted that the peak time was greatly reduced in hHGF-modified HOCs of any passage compared to that of normal HOCs (unpublished data of normal HOC growth curve), suggesting that the *hHGF* gene may promote HOC proliferation. Flow cytometry analysis showed that the percentage of hHGF-modified HOCs in S and G2/M phase was markedly higher than that of non-modified HOCs (65.34 ± 1.67% vs. 51.52 ± 2.83%, respectively; $p < 0.05$; Table1), confirming the enhanced proliferation capability of hHGF-modified HOCs.

Table 1. Cell-cycle analysis.

	G0/G1	S and G2/M	sub G0/G1
HOCs	45.22 ± 1.20*	51.52 ± 2.83*	3.20 ± 0.14*
hHGF-modified HOCs	33.07 ± 2.19	65.34 ± 1.67	1.43 ± 0.89

*p < 0.05 compared with HOCs. Data are presented as means ± SEM %.

Table 2. Quantification of mRNA expression of ALB and CK19 by RT-PCR.

	ALB	CK19
Liver	−1.139 ± 0.588*#	−1.926 ± 0.653*#
Bile duct	−6.138 ± 0.521*#	−1.739 ± 0.704*
HOCs	−1.820 ± 0.614	−3.852 ± 0.974#
hHGF-modified HOCs	−1.813 ± 0.522	−3.676 ± 0.871#
Differentiated HOCs	−1.758 ± 0.543	−1.492 ± 0.634*^
Differentiated hHGF-modified HOCs	−2.134 ± 0.337	−1.107 ± 0.483*

Data were quantified from 20 independent experiments and normalized with β-actin. The negative log was calculated as described in the Materials and Methods section.
*$P < 0.05$ vs. HOCs and hHGF-modified HOCs; #$P < 0.05$ vs. differentiated HOCs and differentiated hHGF-modified HOCs; ^$P < 0.05$ vs. differentiated hHGF-modified HOCs.

***In vitro* differentiation capability of hHGF-HOCs**

In order to investigate the differentiation potential of hHGF-modified HOCs, cells were maintained in culture medium supplemented with LIF only or medium containing LIF, SCF, HGF and EGF (complete growth factor group). Cells without growth factor supplementation were used as a control. The HOCs grown in the presence of complete growth factor medium had a round, oval, or short spindle-like morphology as observed under a phase contrast microscope (Fig. 4A-C). Similar cell morphology was observed in hHGF-modified HOCs cultured in medium with LIF supplementation. However, the withdrawal of growth factors for 4-6 days induced the differentiation of HOCs, and large, round-shaped hepatocytes and long spindle-like bile duct epithelial cells were both observed (Fig. 4C, E). TEM analysis showed that some of the differentiated

cells were enriched with intracellular organelles and contained microvilli on their cell surfaces, consistent with typical hepatocyte ultrastructure (Fig. 4D).
RT-PCR analysis showed that the CK-19 mRNA levels were clearly elevated after differentiation in both hHGF-modified HOCs (−3.676 ± 0.871 vs. −1.107 ± 0.483, respectively; $p < 0.05$; Table 2) and HOCs (−3.852 ± 0.974 vs. −1.492 ± 0.634, respectively; $p < 0.05$; Table 2), suggesting that *in vitro* cultured hHGF-modified HOCs and HOCs can differentiate into hepatocytes and bile duct epithelial cells.

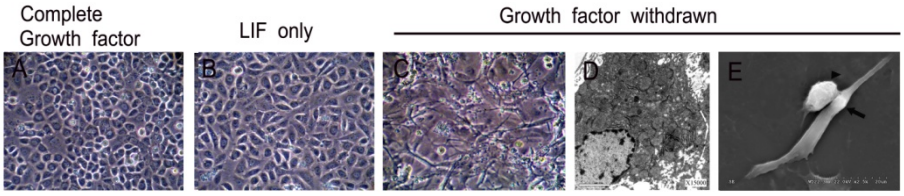

Fig. 4. Differentiation of HOCs. Cells in the complete growth factor group were maintained in 20 ng/ml SCF, 10 ng/ml HGF, 10 ng/ml EGF and 20 ng/ml LIF. Cells in the LIF only group were cultured in the presence of 10 ng/ml LIF. Cells in the growth factor withdrawal group were grown without growth factor supplementation. A to C – The morphology of cells was monitored under a phase contrast microscope 6 days after incubation. (Magnification: 200×). D – TEM revealed differentiated cells exhibiting a typical hepatocyte structure. Scale bar, 2 μm. E – SEM revealed round-shaped hepatocytes (arrowhead) and long spindle-like bile duct epithelial cells (arrow). Scale bar, 20 μm.

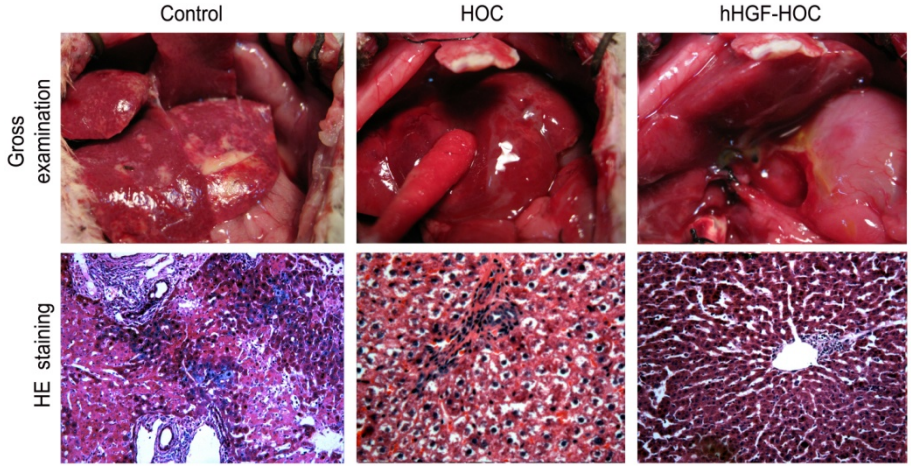

Fig. 5. Gross examination and histological analysis of livers 20 days after transplantation. In the control group, animals received orthotopic liver transplantation only. In the cell transplantation groups, 1 ml of 1×10^6 cells/ml HOCs or hHGF-modified HOCs (passage 4 through 10) was injected into the portal vein and hepatic artery respectively during orthotopic liver transplantation. Histological analysis was performed on HE-stained cells. (Magnification: 100×).

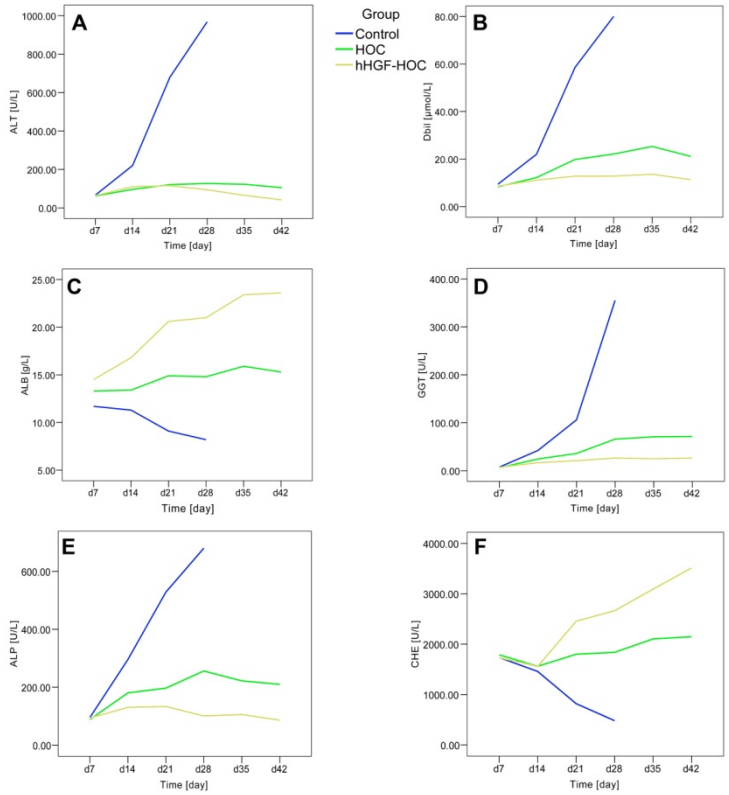

Fig. 6. The average levels of liver function indicators measured on the indicated day following transplantation. Orthotopic liver transplantation was carried out in conjunction with HOC (green) or hHGF-modified (yellow) HOC transplantation. In the control group (blue), animals received orthotopic liver transplantation only. Liver function indicators, including (A) alanine aminotransferase (ALT), (B) direct bilirubin (DBil), (C) albumin (ALB), (D) γ-glutamyltransferase (GGT), (E) alkaline phosphate (ALP) and (F) cholinesterase (ChE) were measured 1, 2, 3, 4, 5 and 6 weeks post-transplantation.

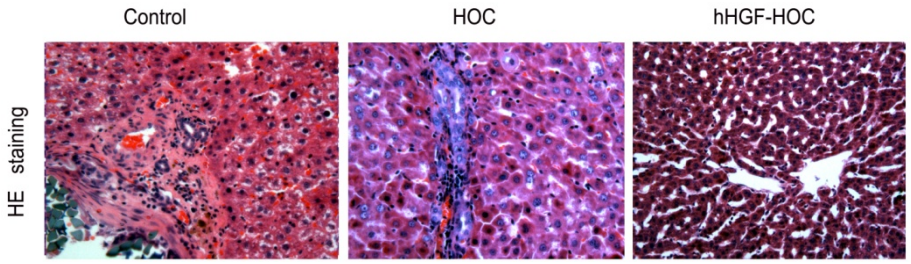

Fig. 7. Cell transplantation in I/R-injured livers. In the control group, animals suffered liver I/R injury only. In the cell transplantation groups, 1 ml of 1×10^6 cells/ml HOCs or hHGF-modified HOCs (passage 4 through 10) was injected into the portal vein following 50 min of ischemia. Histological analysis was performed in HE-stained specimens obtained 5 days post-injury. (Magnification: 100×).

The hHGF-modified HOCs showed a bias towards bile duct epithelial cell differentiation (−1.107 ± 0.483 vs. −1.492 ± 0.634, respectively; $p < 0.05$; Table 2). In addition, the majority of the differentiated HOCs were bile duct epithelial cells, indicating that hHGF-modified HOCs had a high efficiency for differentiating into bile duct epithelial cells *in vitro*.

Transplantation of hHGF-HOCs

To further examine the *in vivo* activity of hHGF-modified HOCs, cells were injected into the portal vein and hepatic artery of female Lewis rats in conjunction with orthotopic liver transplantation. Transplantation with HOCs (n = 33) and hHGF-modified HOCs (n = 20) remarkably prolonged the median survival time (MST) of recipients compared to the control (n = 47): 38 and 48 days vs. 21 days, respectively ($p < 0.05$). In addition, the MST of hHGF-HOC transplantation rats was significantly longer than that of HOC transplantation rats ($p < 0.05$). Examination revealed swelling accompanied by ischemia, congestion and hemorrhage in the livers of the control group (Fig. 5).

HE staining showed that severe acute rejection occurred in the control group livers. In the HOC transplantation group, local liver congestion was still observed, although no obvious necrosis was detected. Mild rejection responses were found in HOC transplanted livers. Importantly, the hHGF-HOC transplantation group livers had normal structures without the appearance of necrosis, ischemia, congestion, or rejection. The ALT, DBil, GGT and ALP levelswere all significantly higher for the rats of the control and HOC transplantation groups than those for the rats of the hHGF-modified transplantation group ($p < 0.05$; Fig. 6), suggesting that hHGF-modified HOC transplantation can improve liver function. Furthermore, hHGF-modified HOC transplantation also improved liver synthesis function, as evidenced by the lower ChE and ALB values observed in this group compared to the HOC and control groups. Therefore, HOC or hHGF-HOC transplantation dramatically improved liver function in recipients after the transplantation (Fig. 6), and hHGF-HOC transplantation provided a higher MST and a better outcome in liver function compared to HOC transplantation ($p < 0.05$).

We confirmed that HOC or hHGF-HOC transplantation reduced liver damage in rats with I/R injury-induced liver damage (Fig. 7). Histological examination of HE-stained liver specimens collected 5 days after I/R injury revealed that a better reduction of injury was obtained with hHGF-HOC transplantation than with non-transfected HOC transplantation.

DISCUSSION

HGF is a multifunctional growth factor that controls a variety of cellular biological activities [6]. The cellular responses to HGF are mediated by the c-Met tyrosine kinase receptor [7, 17]. It has been reported that HGF deficiency affects the embryonic liver of mice and leads to incomplete development and animal death *in utero* [18]. However, HGF is not expressed in hepatic parenchymal

cells. Non-parenchymal cells are the main sources of tumor necrosis factor (TNF), interleukin-6 (IL-6), HGF, and heparin binding-epidermal growth factor (HB-EGF), which are required for the replication of hepatocytes [2]. Importantly, hepatocytes and HOCs are the predominant cell source for liver regeneration after injury [1, 2, 4]. Nevertheless, the effects of HGF on the *in vitro* and *in vivo* proliferation and differentiation of HOCs still remain unclear.

Previous studies have indicated that administration of recombinant human HGF can accelerate the proliferation of HOCs and can possibly promote HOC differentiation in a 2-AAF/PH model in rats [9]. In addition, the combination of adenovirus-mediated HGF gene transfer with signal transduction inhibitors, including LY294002, rapamycin and U0126, can regulate the proliferation of oval cells [11]. In this study, an HOC line stably expressing hHGF was established through blasticidin selection. No obvious morphological difference was found between HOCs and hHGF-modified HOCs. Moreover, rapid proliferation was observed in passage 4 through 14 HOCs with hHGF modification, which was in agreement with the results for normal HOCs. Importantly, the peak time was greatly reduced in hHGF-modified HOCs of any passage compared to normal HOCs. These observations were consistent with previous studies and indicated that the *hHGF* gene may accelerate the *in vitro* proliferation capability of HOCs. In addition, this gene modification technique provides a novel approach that could benefit HOC-based cell transplantation.

It was previously reported that the *in vivo* transfer of the *HGF* gene into liver cells accelerates HOC proliferation in the Solt-Farber rat model [10]. Here, the *in vivo* activity of hHGF-modified HOCs was determined by transplanting cells into female Lewis rats in conjunction with orthotopic liver transplantation. A variety of biochemical markers were used to evaluate liver function. ALT was used to assess liver cell damage; DBil, GGT and ALP were used to assess bile duct damage; and ChE and ALB were applied to evaluate the protein synthesis function of the livers. Our results showed that combined hHGF-HOC transplantation downregulated ALT, DBil, GGT and ALP levels but upregulated ChE and ALB expression levels, suggesting that transplantation in combination with the administration of HOCs improved liver function, prevented bile duct damage, and protected against the development of chronic hepatocyte injury. Accumulating evidence suggests that the MET tyrosine kinase receptor (also known as the HGF receptor) promotes tissue remodeling and organ homeostasis in response to environmental stimuli or cell-autonomous perturbations [19]. Therefore, it is possible that hHGF-modified HOCs may improve liver function through the HGF-Met pathway. We will continue to investigate the detailed mechanism involved in this process.

The protective effect of hHGF-modified HOC transplantation was further confirmed in rats with I/R-injured livers. It has been suggested that alterations induced by oxidative stress during I/R can exceed the compensatory capacity of the liver, resulting in cell death [20]. HGF is an endogenous cardioprotective

factor against oxidative stress [21]. Therefore, it is possible that hHGF may prevent liver damage by suppressing oxidative stress.

In our previous study, we found that the combination of hHGF-modified HOCs with liver transplantation decreased host anti-graft immune responses, resulting in a reduction of allograft rejection rates and prolonging graft survival in recipient rats [16]. Collectively, these findings indicate that hHGF-modified HOCs may exhibit better proliferation and differentiation capability both *in vitro* and *in vivo* based on our establishment of an HOC line that stably expresses hHGF. These findings suggest that hHGF-modified HOCs could serve as a potential cell source for therapeutic liver regeneration after orthotopic liver transplantation. Additional studies are needed to explore the potential molecular mechanism of hHGF-mediated proliferation and differentiation of HOCs.

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